STUDIES ON SURAMIN RESISTANCE IN KENYAN STOCKS OF *TRYPANOSOMA EVANSI* (Steel, 1885 Balbiani, 1888)

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A thesis submited for the Degree of Doctor of Philosophy
University of Edinburgh
1993

DECLARATION

The work described in this thesis is mine unless where acknowledged otherwise.

Marion W. Mutugi

ACKNOWLEDGEMENTS

I would first want to acknowledge that I would not have been able to do this work without the Almighty God who has not only given me life but also health, strength and opportunity.

I wish to express my gratitude to the public service commision of Kenya and the Kenya Trypanosomiasis Research Institute (KETRI) for allowing me time off my normal duties to take a study leave. I am grately indebted to the British Council for their grant which made it financially possible to study in the U.K. I would also like to acknowledge the financial assistance given to me by KETRI during the part of my work which took place in Kenya.

My thanks go to all three of my supervisors; Dr Peter Stevenson for providing the day to day assistance as needed at KETRI, Dr. A.G. Luckins for all his assistance and support especially with the writing, and last but not least, Dr. R. Boid for everything especially going through my numerous thesis drafts with a fine-toothed comb.

I wish to thank Prof. J. Boothroyd (Stanford University, U.S.A.) and Dr. M. Ouellette (Centre Hospitalier de L'Universite Laval, Quebec, Canada) who provided the ribosomal and P-glycoprotein probes respectively as well as Dr. G.R. Scott for his ready assistance in de-mystifying statistics and bringing it down to my level.

I would like to recognise the support given to me by the staff at KETRI. I would first want to thank the Director, who persevered my many visits to his office with requests every time. I am grateful to all the staff in the veterinary/epidemiology division for all their assistance. My special gratitude go to the staff of the protozoology section especially Kariuki Ndung'u, Mary Andole and Steven Okaye for doing what was beyond their duty to support this work. My thanks also go to Dr. Olaho-Mukani for scientific, moral as well spiritual support. I would also like to thank all the staff in room 103 as well as Bob Monroe at the CTVM for their assistance. For all the work in Chapter Eight which was done in the molecular biology laboratory at the Institute of Primate Research (IPR), Karen, I would like to thank the director for his kind permission as well as Dr. Rashid Aman who kindly availed his time and laboratory to me. I would also like to acknowledge all staff in the molecular biology laboratory at IPR.

In a special way, I would like to acknowledge Prof. John Marangu for developing in me an interest in scientific research especially in camels. I am indebted to Paul and the late Elizabeth Jennings, my "Scottish" parents, for providing a place where I was always welcome when I needed to momentarily forget my books. They provided a real home away from home. I am grateful to Pastor McFarlane who provided spiritual support during the writing-up.

Finally, my special thanks go to all my family who have supported me throughout. I would like to especially thank my dear parents Pastor and Mrs Wangai who taught me to trust in the Lord with all my heart, lean not upon my own understanding, in all ways acknowledge Him for He would direct my paths; my parents-in-law Rev. and Mrs Miano for their prayer and unwavering support; my sister Baxe for keeping me informed and going out of her way to "stand in" for me when I was not available, to my husband for constantly assuring me that I could do it and to our daughter for the innocence of a child.

DEDICATION

To Musi and Kai for sacrifice without measure.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

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Degree DOCTOR OF PHILOSOPHY (PhD) *Date* 1.6.93

Title of Thesis STUDIES ON SURAMIN RESISTANCE IN KENYAN STOCKS OF TRYPANOSOMA EVANSI

No. of words in the main text of Thesis 65,000

The work described in this thesis examined 44 stocks of *T. evansi*, 41 of which were isolated from camels in various areas of Kenya. These trypanosomes had a mean length of 25.5 $\pm$ 2.8 μm a mean prepatent period of 4.7 $\pm$ 3.4 days and fulfilled the classical criteria of this species in regard to these two parameters. Initially, the sensitivity of these trypanosome stocks was determined to four trypanocidal drugs active against *T. evansi*. The 44 stocks exhibited resistance to Berenil (57%), Samorin (7%), suramin (22%) and Trypacide (18%). It was noted that although Berenil is not recommended for use in camels due to its toxicity, more than half of the stocks were resistant to this drug. The malic enzyme patterns of these stocks were determined to investigate possible correlation with resistance to the trypanocides used. Most of the stocks (66%) possessed malic isoenzyme pattern II (66%), although patterns X (23%), IV (7%) and VII (5%) were also observed. Malic enzyme patterns IV and X had not been identified previously in this trypanosome species. No linkage was found between any of these patterns and resistance to the four drugs.

Further work concentrated on three trypanosome stocks which were highly resistant, of intermediate resistance or sensitive to the action of suramin. Investigations on the effect of trypanosome inoculum and timing of suramin administration in obtaining cures in *T. evansi* infected mice were carried out. The time of administration after infection was shown to be an important factor that influenced the outcome of suramin treatment. Mice which were treated immediately after infection had higher cure rates than those treated at the onset of parasitaemia. This was observed particularly in a trypanosome stock normally resistant to suramin at a dose rate of 160 mg/kg; if treatment was administered immediately after infection, most of the infected mice were cured. Except in the highly resistant stock, the role of trypanosome inoculum was not as important as timing of treatment in determining cures. The influence of both high trypanosome inocula and late administration of drug on failure of treatment was probably due to the opportunity provided for trypanosomes to invade the central nervous system. It is thus important to closely monitor animals that are exposed to trypanosomiasis in order to treat promptly those that are infected and thus increase the treatment success rate.

This study also investigated the interaction of suramin-sensitive and resistant trypanosomes in mixed infections. Drug-sensitive parasites were shown to interfere with the establishment of infection with the drug-resistant trypanosomes. Interference was not observed when both stocks were inoculated simultaneously. When a sensitive stock was inoculated first and allowed to establish infection in mice, subsequent inoculation of the resistant stock and treatment with suramin resulted in up to 60% cures suggesting that the resistant trypanosomes had not been able to establish infection in all instances. To a lesser extent, interference was observed in rats whereby treatment with suramin resulted in suppression of parasitaemia. If mice were inoculated with the resistant stock first, a temporary suppression of parasitaemia resulted. No interference was observed in the establishment of infection in rats.

Suramin sensitivities were determined in clones derived from four trypanosome stocks. All four stocks were shown to comprise clones with a wide spectrum of suramin sensitivities. Two of the cloned trypanosome stocks which were sensitive to 0.01 mg/kg of suramin were used in experiments to induce resistance by the administration of sub-curative doses in infected mice. The resistant clones were compared with their sensitive parents and shown to have slower growth rates in rats. This slower growth rate was especially marked in one stock of trypanosomes which had the higher level of resistance. Both the interference phenomenon and the slower growth rate of resistant trypanosomes may be important as a means by which resistant trypanosomes are selected out in mixed infections under field conditions, thereby limiting the potential spread of suramin resistance.

Suramin resistance at the molecular level was investigated by comparing the DNA of suramin-resistant stocks of *T. evansi* with that of the parent stocks from which they were derived. Endonuclease-digested trypanosome DNA was separated by electrophoresis and hybridised with a ribosomal probe and a p-glycoprotein probe. Hybridisation results showed that there were differences in DNA banding patterns which could be associated with suramin resistance. This preliminary study suggests that suramin resistance may be correlated to specific DNA banding patterns. These DNA differences may have a potential as markers for suramin resistance in *T. evansi*.

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CHAPTER ONE

INTRODUCTION AND OBJECTIVES

Although trypanosomes parasitise many different vertebrate hosts, their importance lies in their role in causing disease in man and his domesticated livestock in both the Old and New World. Trypanosomes of economic importance can be divided into two groups according to the mode of their transmission; cyclically transmitted trypanosomes have part of their developmental cycle in an insect vector; while for mechanically transmitted trypanosomes, the insect vector plays a purely physical role in transferring parasites from one host to another, without the parasite undergoing any developmental changes. In the Old World, *T. rhodesiense* and *T. gambiense* are two species of medical importance that cause East and West African forms of sleeping sickness respectively. These two trypanosome species are cyclically transmitted by tsetse flies of the genus *Glossina*. Amongst trypanosomes of veterinary importance *T. brucei, T. vivax* and *T. congolense* are cyclically transmitted and cause a disease in livestock known as Nagana. *T. vivax* is found also in South America where it is mechanically transmitted by biting flies. *T. evansi,* the cause of Surra, is transmitted by biting flies while, uniquely for trypanosomes, *T. equiperdum,* the cause of Dourine, is transmitted *in coitu*.

T. evansi (Steel, 1885; Balbiani, 1888) is the most widely distributed trypanosome species in the world, occurring in Central and Southern America, Africa, Arabia, Europe and Asia. The distribution of *T. evansi* in Africa is closely linked with that of camels, suggesting that throughout the world some 19.5 million camels (FAO, 1990) are at risk from infection with *T. evansi*. Other animals such as horses and other equids, buffaloes and dogs are also at risk. Along with camels, these animals develop infections which are fatal unless treated. Other domestic animals such as cattle, sheep, goats and pigs are susceptible to *T. evansi* infection but the disease they develop is usually less severe. This form of disease is however more important than is

usually recognised since an animal which is chronically infected can act as a carrier and thus be a potential reservoir from which other animals can be infected (Losos, 1980).

The importance of *T. evansi* in Kenya has been recognised by camel keepers for generations. Somali tribesmen, the original camel keepers in Kenya, have always kept camels as the livestock of choice in northern Kenya which forms a continuation of the camel belt from Somalia. After the great Sahelian drought of the mid 1980s, other Kenyan tribes such as the Gabbra, Rendille and the Turkana increased their camel herds although their most important livestock continued to be cattle, sheep or goats. This change in attitude by non-camel keepers was due to the observation that during the drought, while they lost most of their livestock, their camel-keeping neighbours were not affected since their animals not only survived the drought but kept on providing milk and meat. The government of Kenya has now recognised the important role of camel keeping in meeting the protein requirements of the nation.

The Republic of Kenya is situated in the eastern part of Africa and has a population estimated at 24 million people (FAO, 1990). By the turn of the century, this population is projected to number 35 million according to Sessional Paper No. 1 (1986). This government paper presented the economic situation of the country and set development strategies for the five-year period, 1989-1993. According to the projections, the meat consumption of the country was expected to triple by the year 2000. The total area of Kenya is 44.6 million hectares of which only 8.6 million can be classified to be of medium to high agricultural potential. In order to achieve the estimated meat production, emphasis needs to be placed on developing areas of low agricultural potential since the medium and high potential areas are already utilised almost to the maximum. To achieve this target, emphasis on increased production would need to be directed towards Arid and Semi-arid lands (ASAL) which make up 80% of the country, support 20% of the people and 50% of the livestock. The 1986 sessional paper further noted that ASAL were areas of fragile environment prone to degradation and thus any developmental programmes would need to address environmental conservation problems.

It is not coincidental that the camel is kept in these low potential and marginal areas since the low, unpredictable rainfall patterns and the harsh terrain are not conducive to other livestock animals or alternative agricultural activities. The camel is perfectly adapted to survive in these extreme conditions and of special importance is

its ability to go for long periods without drinking water. Although the camel is at times kept alongside cattle, sheep and goats in areas of medium potential, it does not compete with them for forage because it utilises a spectrum of vegetation which is either different, beyond reach or ignored. The camel is environmentally-friendly for it does not trample and harden the earth and its feeding is by browsing rather than stripping vegetation.

In view of these attributes, the camel is the livestock animal of choice not only in Kenya but also in other hot and cold deserts of the world. Seventy per cent of camels in the world are kept in the five countries of the "horn of Africa", Somalia, Ethiopia, Djibouti, Sudan and Kenya. The camel population of Kenya estimated to be 4% of the total world population (810,000) is distributed as shown on Figure 1.1 (KREMU, 1981). The exact camel population in Kenya is however not known due to the remoteness, inaccessibility and lack of security in the north-eastern part of the country. In order to meet the protein needs of Kenya at the turn of the century, a possible approach would be to maximise the potential of these lands by increased camel production. Although the camel as a source of milk and meat is a new concept to many Kenyans, it is possible that a decrease in the availability of protein products from other livestock would lead to the necessary adaptation.

The government of Kenya has realised the need to encourage camel-keeping by improving production in traditional camel-keeping areas and introducing camels to communities which do not normally keep them. These efforts are augmented by non-governmental organisations such as the Food and Agriculture Research Management (Farm) Africa. The most important constraint to this endeavour is trypanosomiasis (Olaho *et al* 1987) which is economically the most important camel disease (Richard, 1979). In order to increase camel-keeping and production in Kenya, cameline trypanosomiasis must be controlled or reduced to ensure that camel keeping is economically viable. In Kenya, camel trypanosomiasis is caused almost exclusively by *T. evansi* infection. Although tsetse-transmitted trypanosomes can infect the camel, camel owners recognise the importance of avoiding tsetse habitat in order to protect their animals from infection.

Despite the fact that *T. evansi* was the first trypanosome to be identified and is widely distributed in the world, relatively little research effort has been directed towards it. This apparent negligence has been due to several factors, the most important being a misconception that information gained from research on *T. brucei* could be applicable to *T. evansi.* This misconception has been based on the belief that

the two species are phylogenically very close to each other as suggested by the theory that *T. evansi* developed from *T. brucei.* The reasons for this theory were comprehensively discussed and summarised by Hoare (1972). The most important difference between the two species is in the area of transmission. The fact that *T. brucei* undergoes cyclical development in flies while *T. evansi* does not, indicates that *T. evansi* has lost specific characters that are important at the level of host-vector-parasite interaction. Studies on *T. brucei* can therefore not necessarily be extrapolated to *T. evansi* and only *T. evansi*-specific investigations can lead to an understanding of this species. Another reason for the negligence in *T. evansi* research is the relative lack of importance of the camel, the principle host of this parasite. The geographical distribution of camels is such that they are not found in parts of the world which are economically independent and this has led to a lack of camels being prioritised as far as resource allocation in research is concerned. It is evident that the overall economic importance of camels is not comparable to other livestock such as cattle, pigs, sheep and goats. This neglect of camels is not only in the area of trypanosomiasis but also in other areas such as breeding which could lead to increased camel production.

The importance of *T. evansi* has recently been recognised and more attention is now being placed on its research. An international working group on *T. evansi* formed in 1983 by the Office International des Epizooties (OIE) led to the first seminar on non-tsetse transmitted trypanosomiasis (NTTAT) in 1992 where *T. evansi* featured prominently. With this recent attention, increased *T. evansi* research will undoubtedly result to a better understanding of the parasite and hopefully lead to more efficient methods for its control.

The ideal method of control of trypanosomiasis would be by use of a vaccine which would confer protection to the hosts exposed to infection. To date, attempts to develop such a vaccine have been thwarted by the ability of the trypanosome to continually undergo the process of antigenic variation thus altering possible antigens which would serve as the vaccine's recognition site. The current control of diseases caused by trypanosomes, aims at disrupting the transmission cycle and can be divided into three broad areas; methods directed towards the parasite, methods directed towards the vector and methods involving the mammalian host. A number of trypanocidal drugs have been used either as prophylactic or therapeutic agents in the control of animal trypanosomiasis. Indeed, this has been the major method of control

for over 70 years. A major consideration in utilising this control approach is the need for qualified medical or veterinary supervision in order to ensure the proper use of drugs. Lack of proper supervision may lead to situations where drugs are misused and trypanosomes develop resistance to the drugs, thus rendering them ineffective. The development of drug resistance is especially important in situations where drugs are used repeatedly due to re-infections of animals which have been previously infected. The ideal approach to treatment would be where all animals in an area are treated thus destroying any possible source of re-infection. Although parasite control by the use of chemical compounds has been shown to be effective in the short term, this method may prove impractical in the long run due to the high expense needed to maintain it (Dwinger, 1985). Another difficulty encountered in this control approach is the presence of wild animal reservoirs which cannot be treated, and yet, due to their close proximity to treated livestock animals, act as a constant source of re-infection (Bruce, 1895; Hoare, 1972). While this is of special importance in relation to the tsetse-transmitted trypanosomes it is of less importance in *T. evansi*, except possibly in South America where capybaras are considered to act as reservoirs of this species. In Africa, other livestock animals have been suspected to play the role of *T. evansi* reservoirs from where treated camels can be re-infected. In instances where livestock populations are not isolated and are continuous into herds owned by different people or even move into different countries, a concerted effort in the synchronisation of mass treatments is necessary.

At the beginning of the study, suramin was the recommended drug for the control of cameline *T. evansi* infections in Kenya and other countries like Sudan, Somalia and Ethiopia. The production of this trypanocide has since stopped due to the high cost needed to update the production process to a level that would be environmentally acceptable. There are however large stocks of suramin still available which are estimated to be sufficient for five years. The fact that this trypanocidal drug is still preferred for the control of cameline *T. evansi* infections may lead to a re-evaluation of this situation. For instance, in 1974, the production of quinapyramine was stopped due to widespread occurrence of resistance. Ten years later, this drug was re-introduced due to a re-evaluation of the situation whereby it was decided that its production would be important in view of the small number of available trypanocides. There is thus hope that suramin may be re-introduced due to its high

demand not only as a trypanocide for the control of camel trypanosomiasis and early stages of human infections, but also as a drug against HIV infection and cancer.

Transmission of trypanosomes by tsetse flies is accomplished when a tsetse fly takes a blood meal from an infected vertebrate host, and ingested trypanosomes undergo development in the fly eventually maturing to an infective metacyclic stage. The parasites are then inoculated into a new host during a subsequent feed. A second approach to trypanosomiasis control directed at the vector has been used where the methods of control take advantage of the single vector involved in transmission. Studies into habits of tsetse flies have led to the development of both chemical and physical methods of control. Control measures that are vector-oriented aim at interrupting the host-fly-host interaction at various levels. The most indiscriminate method involves the destruction of the vertebrate host by the use of insecticides. This approach is not specific enough to destroy *Glossina* alone and is known to affect other non-target organisms. A more specific approach is based on the destruction of tsetse by the use of traps and targets. This method is more discriminative for it is based on the knowledge of visual and olfactory preferences of *Glossina.* Other methods of vector control are destruction of tsetse habitats by bush clearing to destroy areas where these flies live. A more sophisticated method involves the rearing, sterilisation and release of male tsetse flies. These sterile insect techniques (SIT) aim at providing competition between sterile males and the wild ones in order to reduce successful matings and thus decrease offspring. All these methods of tsetse control have met some success especially in areas where the flies are geographically isolated, and there is little likelihood of re-invasion from other areas. Some of these methods however are not acceptable now due to their adverse effect on the environment, causing flora and fauna degradation and chemical pollution. These methods are only suitable for the control of tsetse-transmitted trypanosomiasis and have little or no effect on mechanically transmitted infections like *T. evansi.* The mechanical transmission of trypanosomiasis can theoretically be achieved by any agent that can transfer the parasites from one animal to the other. Tabanids, *Stomoxys, Lyperosia,* ticks and vampire bats have all been implicated in the transmission of *T. evansi.* The control of these vectors is not as easy as that of *Glossina* due to variation in the habits of the different species involved.

Finally, trypanosomiasis control can be directed towards management of the vertebrate host. The traditional method used is the movement of animals to avoid areas that are inhabited by vectors of trypanosomiasis. This control method can only be used successfully in areas where animal movements are not restricted by individual land ownership systems or small farm sizes. The destruction of non-livestock vertebrate hosts which may act as reservoirs is another approach that has been used. It is however, not acceptable due to its role in the destruction of natural fauna. Another, possibly more effective, but essentially long term approach, is in the exploitation of known trypanotolerance of certain livestock breeds. It is known that some animals are able to control trypanosomiasis infection better than others. Such animals have been identified in West and East Africa (Stewart, 1951; Chandler, 1952; Griffin and Allonby, 1979; Murray *et al,* 1982; Njogu *et al,* 1985; Mwangi *et al,* 1991). Research is being carried out on trypanotolerance in order to understand its mechanisms and introduce animals with this trait in other trypanosomiasis-infested areas.

The major method used for the control of cameline trypanosomiasis in Kenya is by the use of trypanocides. At the start of this work, only two, suramin (Naganol[R], Bayer) and quinapyramine (Trypacide[R], Rhone Merieux) were available in the Kenyan market. Both of these drugs are active against *T. evansi* although their effectiveness has lately been reduced by the development of resistance to both drugs (Gitatha, 1980; Schillinger *et al,* 1985a). Although the extent of trypanocide resistance of *T. evansi* is not known, these isolated reports suggest that there may be a genuine problem. However, a new trypanocide, Cymelarsan, has been developed by Rhone Merieux for camel trypanosomiasis (Raynaud *et al,* 1989) and may soon be registered and available in Kenya. With the control of trypanosomiasis in Kenya, the areas which are currently under-utilised could be used for agricultural activities which would alleviate the problem of food shortages. Successful control of trypanosomiasis would also relieve the pressure on current trypanosomiasis-free areas leading to their better utilisation without the threat of overgrazing.

The number of trypanocides is limited and there is an urgent need to develop new ones. In view of the estimated £80 million and ten years needed to develop a new drug (Hyde, 1990), however, there is little possibility of developing new trypanocides. This is due to the comparatively small number of animals at risk, the relative

unimportance of the camel in world economy and the low purchasing power of camel-owners. It is therefore very important to exercise prudent use of available trypanocidal drugs in order to reduce development of resistance to them and ensure their continued usefulness.

The work described here was concerned with investigation of resistance of *T. evansi* in Kenya to trypanocidal drugs which are normally effective against this species. The trypanosomes used consisted of 41 stocks isolated from camels in different parts of Kenya, as well as two stocks from Sudan and one stock from Colombia, South America. The overall aim of these studies was to determine the extent of resistance shown by *T. evansi* stocks in Kenya and attempt to relate these findings with the biological characteristics of the different stocks. The specific areas of investigations were;

1. To determine the sensitivity of the 44 stocks to four trypanocidal drugs, Berenil, Samorin, suramin and Trypacide in relation to the geographic origin of the stocks; to determine the effect of the number of trypanosomes inoculated and the time suramin was administered on the outcome of chemotherapy.
2. To induce suramin resistance in some Kenyan *T. evansi* stocks using different methods and to determine the range of suramin sensitivities expressed by clones derived from parent trypanosome stocks.
4. To compare the replication rates of *T. evansi* parasites of different suramin sensitivities with a view to investigate any correlation between the two.
5. To investigate interaction of *T. evansi* parasites of different suramin sensitivities in mixed infections as compared to single infections.
6. To compare suramin-resistant trypanosomes and their sensitive parents at the molecular level and attempt to correlate any differences found with suramin resistance.

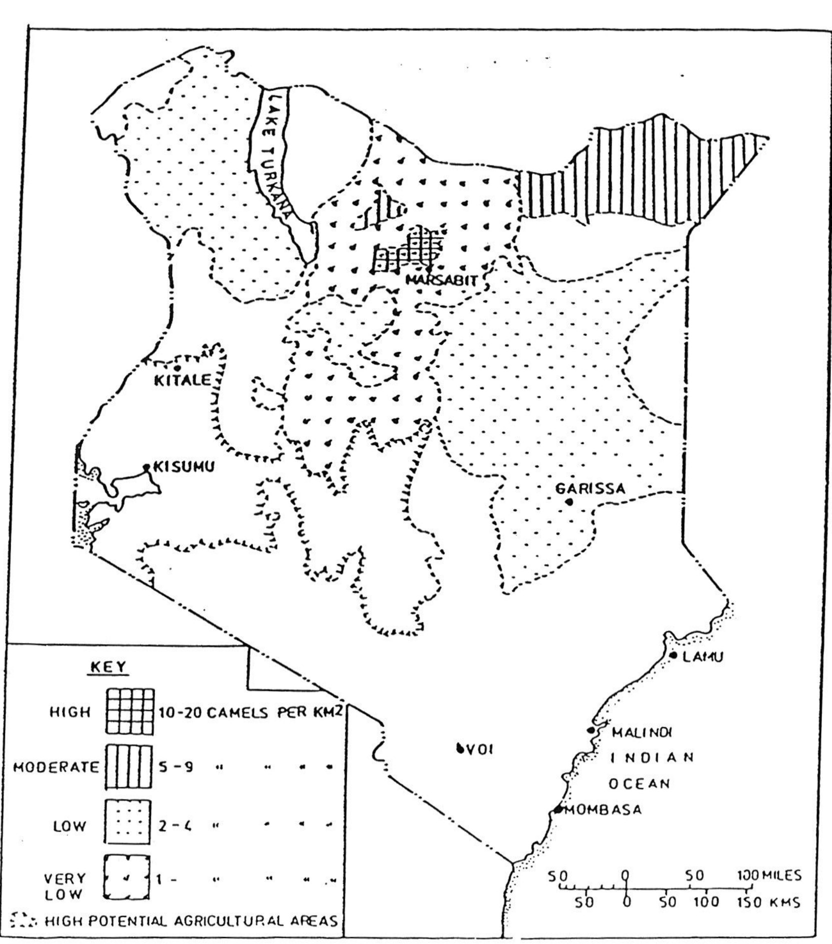


Figure 1.1 Regional differences in camel distribution density (KREMU, 1981).

CHAPTER TWO

LITERATURE REVIEW

2.1 BACKGROUND, CLASSIFICATION AND NOMENCLATURE OF TRYPANOSOMES

2.1.1 HISTORICAL BACKGROUND

Trypanosomes are haemoprotozoan parasites found in the blood and tissues of vertebrates and arthropods. It is believed that the first description of trypanosomatids was in 1680 when Antony Van Leewenhoek, the father of protozoology, saw organisms in the gut of horseflies. It was Valentin (1841), however, who observed and clearly described motile, elongated organisms in the blood of trout. Gruby (1843) further studied these organisms in detail and classified them as the genus *Trypanosoma.* For the next forty years, little attention was paid to these organisms until their parasitic nature was described in rats in India by Lewis (1878, 1879). The pathogenic nature of trypanosomes in animals was however recognised by Evans (1881) when he observed these organisms in the blood of horses and camels suffering from a disease commonly called Surra in India. In Africa, Bruce (1895) also recognised the pathogenic nature of trypanosomes and went a step further to observe that these parasites were transmitted between animals by tsetse flies. The role of insects in transmission of these parasites was confirmed by Rogers (1901) who, for the first time, was able to experimentally transmit *T. evansi* from dog to rabbit using tabanids. The discovery that trypanosomes were responsible for causing sleeping sickness in human beings drew further attention to them. Dutton (1902) observed trypanosomes, which he named *T. gambiense,* in humans suffering from sleeping sickness in West Africa. In East Africa, Castellani (1903) also observed similar parasites in human sleeping sickness patients. Although from then on workers continued to identify trypanosomes in various animal species, it is the importance of trypanosome species that are infective to man and his livestock that have received most attention and have been the subject of most of the work done to date.

2.1.2 TRYPANOSOME CLASSIFICATION AND SPECIATION

After Gruby (1843) created the genus *Trypanosoma*, various workers described trypanosomes in different animals and named them as they pleased. This led to confusion whereby the same trypanosome species was being reported independently by different people and assigned different names. A good example of such confusion is the trypanosome species causing disease in camels, horses and buffaloes which has borne different names in different parts of the world. It was reported as *T. equinum* (Voges, 1901), *T. venezuelense* (Mesnil, 1910) and *T. hippicum* (Darling, 1910) in Central and South America, *T. ninaekohlyakimovae* (Yakimoff, 1921) in Soviet Middle Asia; *T. cameli* (Pricolo and Ferraro, 1914) in Somalia; *T. soudanense* in Sudan; and *T. annamense* in Indo-China (Hoare, 1972). It is, however, now accepted that these names all refer to the same species, *T. evansi* (Hoare, 1972). There was thus a genuine need for consolidating names accorded to various trypanosome species. A major difficulty was encountered in determining speciation in trypanosomes in view of the classical concept of a species. This concept describes a species as a population of organisms which is isolated from all other populations by inability to breed with them and produce fertile offspring. This concept of a species does not accommodate organisms, like trypanosomes, which do not routinely reproduce sexually and for which a different criteria of defining a species is necessary. Hoare (1972) suggested that for trypanosomes, a species be recognised on the basis of organisms that share a common ancestor and are segregated from other organisms by their environment. According to this concept, the morphology, life cycle, host range, geographical distribution of the parasite and the disease it causes are important in assigning a trypanosome population to a particular species. The current classification of trypanosomes according to Hoare (1972) and Stephen (1986) is shown in Table 2.1. According to this classification, the method of trypanosome development in the arthropod host is used to divide the genus *Trypanosoma* into two sections. The section Stercoraria consists of trypanosomes whose whole development in the arthropod vector is exclusively in the gut culminating in transmission to the next host by faecal contamination. Some species in this section include *T. theileri*, *T. lewisi*, and *T. cruzi*. Except for *T. cruzi* which causes Chaga's disease in humans, all the other

species in this section are non-pathogenic. The second section, Salivaria, is descriptive of trypanosome species whose development is in the mouth-parts or in both the mouth-parts and gut of the insect vector and their method of transmission is by inoculation through deposition of saliva at feeding. This section also includes *T. evansi* and *T. equiperdum* which are mechanically-transmitted by salivary contamination without development of parasites in a vector. All trypanosome species important to man and animals causing African trypanosomiasis are found in this section.

2.1.3 NOMENCLATURE

The binomial system of nomenclature, where the genus and the species are quoted, is operational in all trypanosomes except those of the subgenus *Trypanozoon* where a trinomial system is sometimes used. The basis of this system is based on the concept that all species in this subgenus originated for *T. brucei*. According to this theory, *T. gambiense* and *T. rhodesiense* other than infecting animals, became specialised to infecting human hosts during the process of phylogenic divergence. *T. evansi* and *T. equiperdum* on the other hand specialised to being mechanically transmitted. The proposed phylogenic relationship (Hoare, 1972) where the genus, subgenus and species are used is shown in Figure 2.1. In some instances, a quadrinomial system is used for example *Trypanosoma (Trypanozoon) brucei evansi* (Zweygarth and Rottcher, 1986). This system of nomenclature is however not acceptable according to the International Code of Zoological Nomenclature (Uilenberg, 1987) since *T. brucei* was identified after *T. evansi*.

Table 2.1 Classification of *Trypanosoma* (Hoare, 1972; Stephen, 1986).

Kingdom:	Protista					
Subkingdom:	Protozoa					
Phylum:	Sarcomastigophora					
Subphylum:	Mastigophora					
Class:	Zoomastigophora					
Order:	Kinetoplastida					
Suborder:	Trypanosomatina					
Family:	Trypanosomatidae					
Genus:	Trypanosoma					
Section:	Stercoraria		Salivaria			
Subgenus						
Megatrypanum	Herpetosoma	Schizotrypanum	Duttonella	Nannomonas	Pycnomonas	Trypanozoon
e.g	e.g.	e.g.	e.g.	e.g.	e.g.	e.g.
T. theileri	*T. lewisi*	*T. cruzi*	*T. vivax*	*T. congolense*	*T. suis*	*T. rhodesiense*
			T. simiae			*T. gambiense*
						T. equiperdum
						T. brucei
						T. evansi

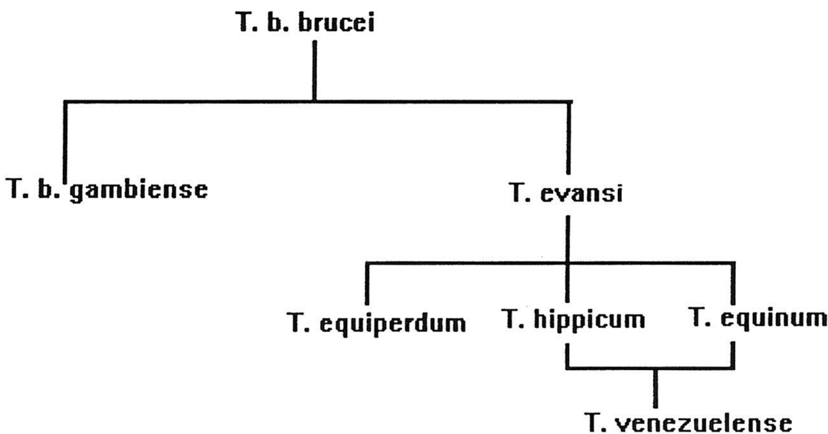


Figure 2.1 Phylogenic relations of trypanosome species of the *Trypanozoon* subgenus (Hoare, 1972).

2.2 CHARACTERISTICS, DIAGNOSIS AND CONTROL OF TRYPANOSOMES

2.2.1 TRYPANOSOME MORPHOLOGY

Trypanosomes are spindle-shaped organisms enclosed in a plasma membrane and have a rounded or pointed posterior end, a cytoplasm, a nucleus, a kinetoplast as well as a flagellum at the anterior end (Figures 2.2 and 2.4).

2.2.1.1 Trypanosome length

The length of the trypanosome is measured from the posterior end to the tip of the flagellum at the anterior end. This length varies depending on both the trypanosome species and the stage of development. Trypanosomes range in length from 8 μm in the subgenus *Nannomonas* to 130 μm in the subgenus *Megatrypanum* (Hoare, 1972). Within the same species, the mean length of individual trypanosomes at times differs resulting in distinct populations; a phenomenon first recognised in the subgenus *Trypanozoon*. These pleomorphic populations were first observed in *T. gambiense* and described as short stumpy and long slender forms (Dutton and Todd, 1903). A third form of intermediate length was later described by Minchin (1908). In tsetse-transmitted species, these differences in morphology have been shown to be related to the developmental stage of the trypanosomes. Trypanosome stages in the tsetse fly are predominantly of the short stumpy form while bloodstream forms in the vertebrate host are mostly long and slender. Long slender forms are related to a period when trypanosomes are rapidly dividing as in the rising parasitaemic phase in vertebrates. Whether these pleomorphic forms represent distinct populations or consist of a continuous range of trypanosome length can be determined by statistical analysis. If they are distinct populations, their distribution curve would have specific peaks correlating to the different populations whereas the distribution curve of a single population would have a single peak. *T. evansi* is similar in length to the other species in the subgenus *Trypanozoon* except for some differences which are related to its not being cyclically transmitted. In this species, there are no short stumpy forms and little evidence of pleomorphism is found. The predominant population of trypanosomes comprises long slender forms with a mean length of 24 μm and a range of 15 to 35 μm. Although this morphology of *T. evansi* is regarded as classical, variations have been observed. For example, a low frequency (0.03 and 0.4 %) of short stumpy forms were observed (Lavier, 1933; Hoare, 1956) but their rarity and proportions justifies

their being considered aberrant types and *T. evansi* is therefore monomorphic (Hoare, 1972).

The degree of parasitaemia and the antibody response of the host animal have also been shown to influence trypanosome length. Ashcroft (1957) observed that during the period of infection with *T. rhodesiense* preceding the production of antibodies by the host animal, long slender, dividing forms were predominant. Later, when the host mounted an antibody response, the predominant population consisted of short stumpy non-dividing forms. Another association that has been found is between the length of trypanosomes and their pathogenicity. In *T. congolense*, Chardome and Peel (1954) observed that the shortest trypanosomes stocks were infective only to mice, stocks of intermediate length were infective only to pigs while the longest stocks were infective to all laboratory animals susceptible to infection. Godfrey (1960) identified three distinct populations in *T. congolense* which he called congolense, intermediate and dimorphon according to their increasing length. Further work with cattle, sheep, dogs, rats and baboons revealed that stocks showing the congolense type, were characterised by infections of low parasitaemia and low pathogenicity while dimorphon types, caused infection of high pathogenicity and high parasitaemia (Godfrey, 1961). Although the intermediate trypanosomes produced infections characterised by high parasitaemia and low pathogenicity, their behaviour was not as well defined as the two other types. A similar relationship between trypanosome length and pathogenicity has been observed in *T. vivax*. In this species however, Fairbairn (1953) observed that the longer trypanosome stocks were less pathogenic than the shorter ones. The longer stocks were found mostly in East Africa while the shorter ones were from West Africa. Some *T. vivax* stocks from Kenya were, however, similar in length to the West African ones and were characterised by acute haemorrhagic infections.

2.2.1.2 The nucleus

With Giemsa's stain, this organ is seen as a densely-staining mass which is situated at different positions within the parasite. In bloodstream trypanosome forms, it is usually located at the centre or the anterior half of the body. The nucleus contains chromatin material which stores genetic information needed for all processes of the trypanosome.

2.2.1.3 The flagellum

This is the organ of locomotion found in all trypanosome species. It originates from the basal body situated near the kinetoplast in the posterior portion of the trypanosome. The flagellum follows the entire length of the trypanosome to the anterior end where it either terminates abruptly as in trypanosomes of the subgenus *Nannomonas* and some developmental stages of *Trypanozoon* or continues to form a free flagellum beyond the body of the trypanosome in other species (Table 2.2). The absence of a free flagellum can be used to identify trypanosomes belonging to the subgenus *Nannomonas*, or infective forms of the tsetse-transmitted members of the subgenus *Trypanozoon*.

2.2.1.4 The kinetoplast

This is another densely-staining organ in the trypanosome. Its size and location varies according to both the trypanosome species and the stage of development (Table 2.2). As mentioned earlier, the kinetoplast is closely associated with the basal body and its position also varies according to the stage of trypanosome development. The diameter of the kinetoplast ranges from 0.4 µm in the subgenus *Pycnomonas* to 1.4 µm in the subgenus *Duttonella* (Hoare, 1972). Due to its association with the basal body, the kinetoplast was thought to have a role related to that of the flagellum. The fact that this organ is densely-staining like the nucleus, however, led to indications that it contained nuclear-like material. It is now known that the kinetoplast is equivalent to the mitochondria of other cells and that it contains extra-nuclear chromatin material which is important in coding for enzymes used for energy production (Hoare, 1972). The complexity of this organ has been shown to be linked to its function and varies according to the developmental stage of the trypanosome (Vickerman, 1970). In cyclically-transmitted trypanosomes found in the bloodstream of animal hosts and also in *T. evansi*, this organ is small and simple while in trypanosomes found in the arthropod vector, it is large and complex. In cyclically-transmitted trypanosomes found in animal hosts and in mechanically transmitted species like *T. evansi*, trypanosomes only catabolise glucose, the major source of energy, partially to produce pyruvate and glycerol as the main end-products (Bowman and Flynn, 1976). This is because they have an abundant source of glucose from host blood. In the insect vector however, glucose is not as abundant and trypanosomes therefore need to produce energy more efficiently and further catabolise the pyruvate to carbon dioxide and more energy. The complex kinetoplast is thus important to the

trypanosome as an efficient energy producer in the insect vector while the simple one is adequate in the bloodstream of the animal host.

Some trypanosome stocks appear to lack a kinetoplast when seen under the light microscope. These dyskinetoplastic forms have been shown by electron microscopy studies to have the membranous envelop commonly found around this organ but to lack the central chromatin DNA material (Vickerman, 1963). Dyskinetoplasty has been shown to be an induced mutation which can be produced by maintaining field isolates in the laboratory by syringe passage or by treatment with compounds such as prothidium, Ethidium and Berenil (Ray and Malhotra, 1960; Killick-Kendrick, 1964; Hoare, 1972). Once the mutation arises, it is inherited as trypanosomes reproduce. This mutation has been observed in all trypanosome species with frequencies ranging from 0.001 to 10 per cent (Hoare, 1972). The highest proportion of the mutation is found in the subgenus *Trypanozoon,* especially in *T. evansi.* In this species, the occurrence of dyskinetoplastic forms is best exemplified by the two South American strains *T. venezuelense* and *T. equinum* which have either a high proportion of dyskinetoplastic forms or totally lack a visible kinetoplast. Since both these strains conform to the classical *T. evansi* identity in other respects, they are considered mutant aberrant forms and not as separate species (Hoare, 1972). These dyskinetoplastic strains are totally liberated from their life in the insect vector and, therefore, do not need a kinetoplast since, except for very short periods, they are found in the bloodstream of animals where there is an abundant supply of glucose.

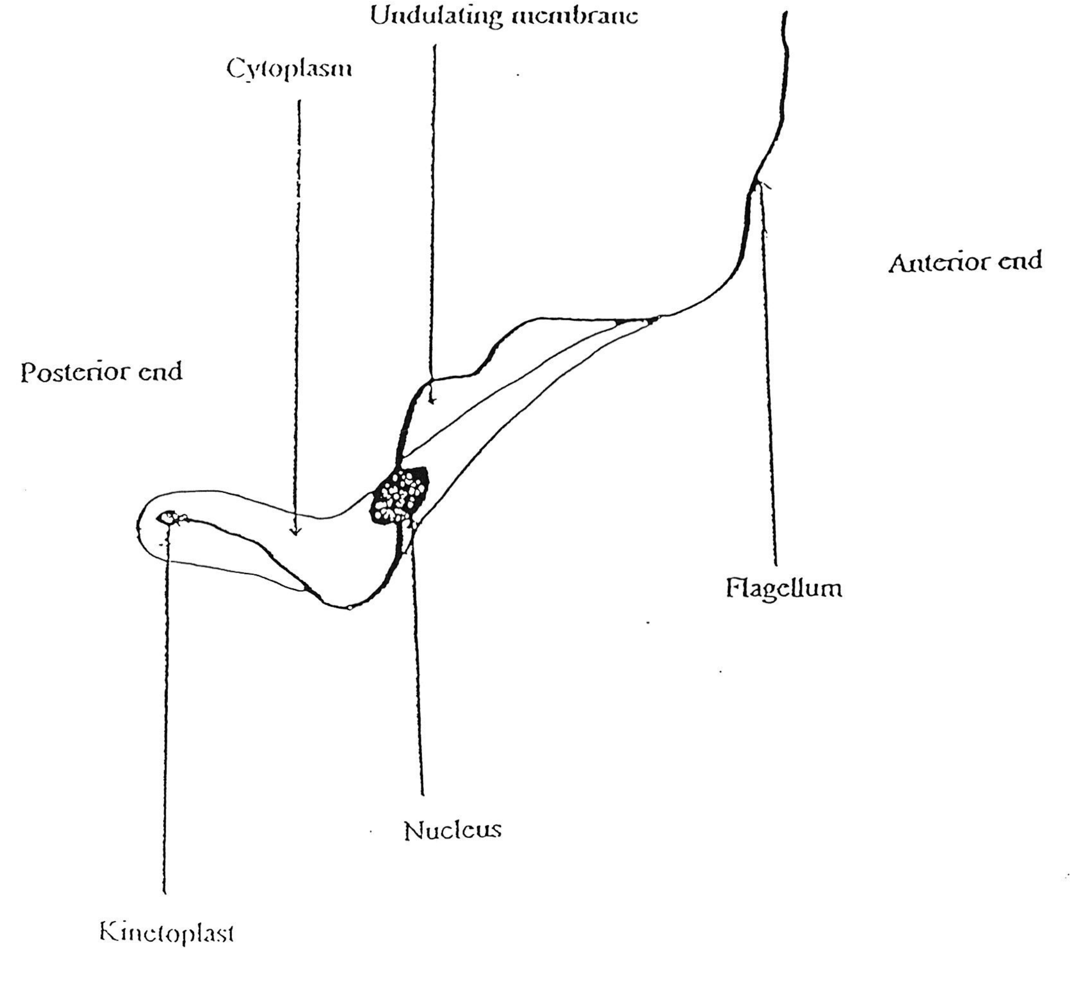


Figure 2.2 The morphological structure of a trypanosome.

Table 2.2 Morphological characteristics of African trypanosomes (Hoare, 1972).

Subgenus	Trypanosome mean length in μm	Kinetoplast position	Kinetoplast diameter in μm	Free flagellum	Undulating membrane
Duttonella	18-31	Terminal	Large (1.1)	present	inconspicuous
Nannomonas	8-24	Subterminal, marginal	Medium (0.7-0.8)	absent	inconspicuous
Trypanozoon	17-27	Subterminal	Medium (0.6-0.7)	present except in infective stages	conspicuous
Pycnomonas	9-19	Subterminal, marginal	Small (0.4)	present	inconspicuous

2.2.2 TRANSMISSION AND LIFE CYCLE OF TRYPANOSOMES

Trypanosomes are digenetic parasites whose life cycle involves two hosts; the definitive vertebrate host and the intermediate invertebrate host. These parasites spend most of their lives in the vertebrate host while the invertebrate host enables transfer of parasites from one animal to the other. Since Bruce (1895) established the tsetse fly's role in the transmission of trypanosomiasis, these haematophagous insects of the genus *Glossina* have been shown to be the most important intermediate hosts of African trypanosomes. Two types of transmission, cyclical and mechanical, are however now recognised. In cyclical transmission, trypanosomes undergo development in the insect vector before subsequent introduction into the vertebrate host. This type of transmission is most important in African trypanosomes of all subgenera. In cyclical-transmission, a fly ingests bloodstream trypanosomes when it takes a blood-meal. Once in the fly, trypanosomes undergo various stages of development, accompanied by changes in morphology, in the proboscis, salivary glands or gut of the fly. The site and duration of development in the fly depends on the species of the trypanosome (Table 2.3). The mature infective metacyclic trypanosomes find their way to the proboscis of the fly from where they can be inoculated into another host at the next feeding. The dependence of cyclically transmitted trypanosomes to the invertebrate intermediate hosts is indicated by the fact that the distribution of trypanosomiasis in Africa closely follows the distribution of tsetse (Figure 2.3).

The other type of transmission is mechanical whereby the insect vector merely transfers trypanosomes from one vertebrate host to the other. In this type of transmission first recognised by Rogers (1901), parasites do not undergo any development in the insect and remain infective in the mouth parts of the fly for only short periods of time before they are inoculated into a recipient host. For mechanical transmission to succeed, the fly involved must be interrupted in its feeding and then continue feeding on another host while the trypanosomes are still viable. This type of transmission has been recognised in *T. congolense* where blood-sucking insects like tabanids are involved (Barnett, 1947; Soltys, 1954). In *T. vivax*, mechanical transmission has also been observed and is considered important in this species for it is said to be responsible for the maintenance of this species in tsetse-free areas (Curasson, 1943; Hoare, 1947).

T. evansi is unique in being the only trypanosome species which is exclusively mechanically transmitted. Evans (1881) first suggested this kind of transmission by

dipteran flies when he noted that " the fly (tabanid) is able to go from one horse to another before the blood about its mouth is dry". The first scientific evidence of mechanical transmission was, however, provided by Rogers (1901). In his experiments, he interrupted flies feeding between infected dogs and healthy rabbits and dogs and observed that transmission was possible if the interval between the two feeds was less than one day. On dissecting the flies, no developmental stages were found, regardless of the interval after ingestion. It is now known that other that tabanids, *Stomoxys*, Haematobia, Chrysops, Lyperosia, mosquitoes, ticks and vampire bats can also transmit *T. evansi* (Cross, 1923; Barotte, 1925; Nielchultz, 1930; Curasson, 1943; Gatt-Rutter, 1967; Hoare, 1972). Other methods implicated in the transmission of this trypanosome species are carnivores eating infected meat (Galuzo and Novinskaja, 1960) and vertical transmission where the mother infects the offspring *in utero* (Sergent *et al,* 1919). In Kenya, vectors of *T. evansi* in field conditions have been identified as being *Hippobosca, Tabanus, Pangonia, Stomoxys* and *Brucomyia pentagera* (Ogonji-Oyieke, 1987, 1991).

The efficiency of mechanical transmission by tabanids has been shown to be dependent on several factors; the most important being the time lapse between feeding on the donor and recipient animals. This is due to the fact that in mechanical transmission, trypanosomes are not able to survive for long periods because they are digested in the gut of the vector (Hoare, 1940). In no case have live trypanosomes been found beyond a few days in a mechanically transmitting vector (Nieschultz, 1930; Hoare, 1940; 1972). Another factor which is important in mechanical transmission is parasitaemia; a high parasitaemia in the donor increases the chance of a vector ingesting trypanosomes and therefore increases the chance of transmission (Leese, 1912; Payne, 1989; Foil and Issel, 1991, 1992). Extensive investigations have determined that other factors important to increased transmission are the structure and size of the mouth parts of a particular vector which enable it to get a large blood meal; the attractiveness of the host; the proximity of the donor and recipient hosts and the degree of interruption at feeding (Payne, 1989; Foil and Issel, 1992). The number of trypanosomes inoculated in mechanical transmission of Surra is not known.

Table 2.3 The site of development of various trypanosome species in the tsetse fly.

Subgenus	Site of development in the fly		
	Proboscis	Salivary gland	Midgut
Duttonella	Trypanomastigotes, epimastigotes and metatrypanosomes		
Nannomonas	Trypanomastigotes, epimastigotes and metatrypanosomes		Trypanomastigotes
Trypanozoon (cyclically transmitted species)		Trypanomastigotes, epimastigotes and Metatrypanosomes	Trypanomastigotes
Pycnomonas		Trypanomastigotes, epimastigotes and metatrypanosomes	Trypanomastigotes

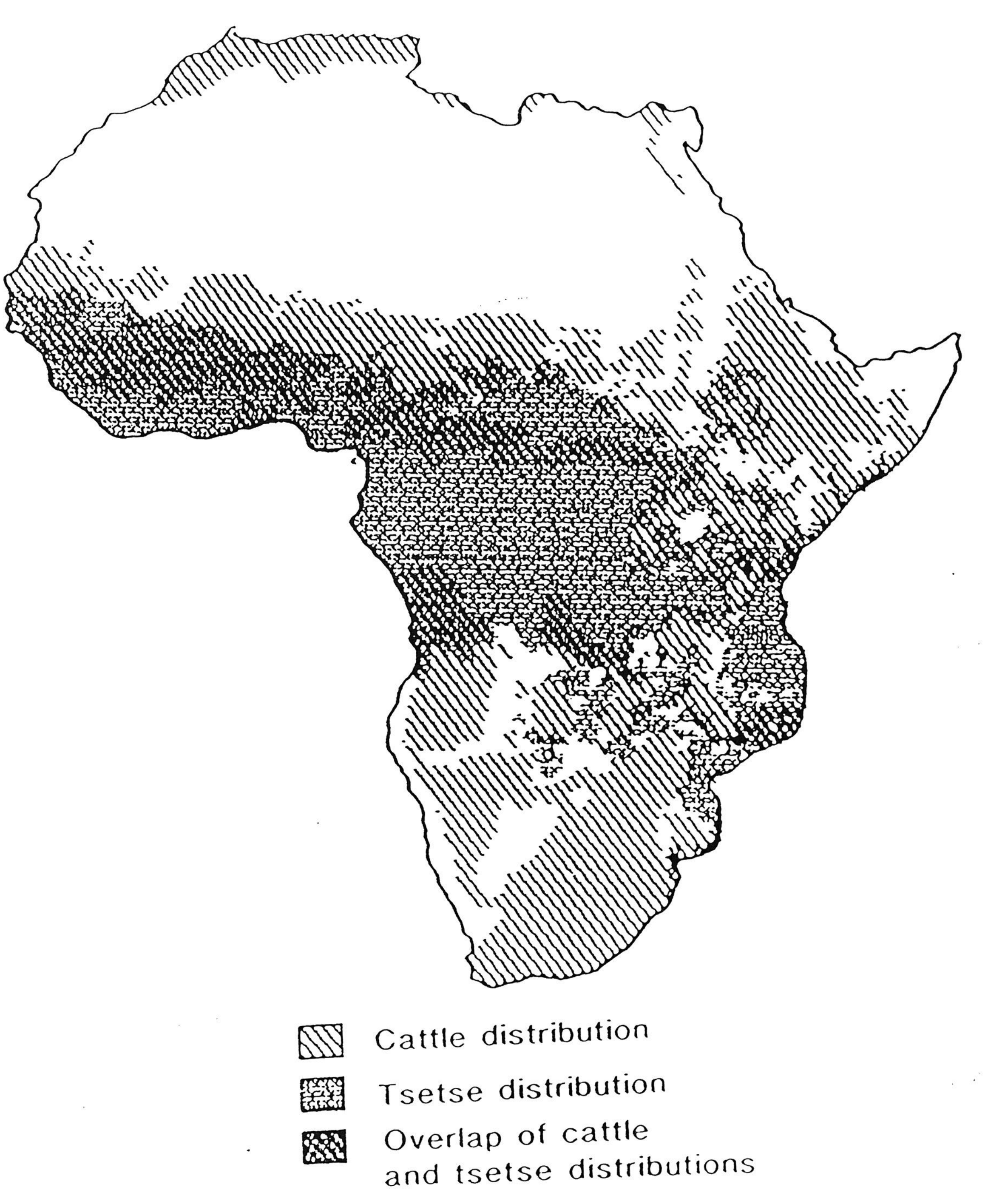


Figure 2.3 The distribution of tsetse flies and cattle in Africa (Peregrine, 1989).

2.2.3 TRYPANOSOME REPRODUCTION

Reproduction in *Trypanosoma* is by binary fission. The first indication of division is the partition of the kinetoplast followed by emergence of a daughter basal body and flagellum near the parent ones. The new flagellum grows in length as the nucleus divides and the kinetoplasts separate. The final stages of division involve the separation of a fully formed daughter trypanosome from the parent with each one having fully formed organelles. These steps of binary division are illustrated in Figure 2.4 (k-r).

It is generally believed that the long slender trypanosome forms are the ones involved in reproduction at a rate which varies according to the different trypanosome species. In Stercorarian trypanosomes, the period of reproduction is limited to the stage when parasites are in tissues while in salivarian trypanosomes, reproduction is continuous (Hoare, 1972). The rate at which trypanosomes divide has been shown to differ between the different species as well as between different morphological forms. Another factor that has been shown to be related to growth rate of trypanosomes is the variable surface antigen type (VAT) presented by the trypanosome at a given time (Van Miervenne *et al,* 1975; Clayton, 1978). Since binary fission occurs mainly among the long slender morphological forms which predominate the early stages of infection (Ashcroft, 1957) and antigenic variation is mainly in these long forms (Gray and Luckins, 1976), the VATs expressed early in infection can thus be related to periods of high reproduction rates. It also follows that when the host mounts an immune response, reproduction may be inhibited as shown by the predominance of the short, stumpy non-dividing forms. Several workers have suggested that there is a relationship between the growth rate of trypanosomes and their trypanocide sensitivities and this will be discussed later

There have been suggestions of sexual reproduction in trypanosomes. Plimmer and Bradford (1899) thought that conjugation was taking place when they observed two trypanosomes closely associated with their cytoplasm apparently fused but with separate kinetoplasts, nuclei and flagella. Minchin *et al,* (1906) were of the opinion that the slender trypanosome forms were male while the shorter ones were female. Culwick *et al,* (1951) observed that mixing two trypanosome strains of different lengths, *in vivo* or *in vitro,* resulted in parasites whose length was unlike that of any of the parents. This apparent evidence of sexuality in trypanosomes was dismissed by other workers who showed that the "conjugated" trypanosomes were actually trypanosomes in the final stages of binary division and the morphological differences were due to developmental stages. The possibility of sexual reproduction was further

disproved by Amrein (1965) who mixed drug-sensitive and resistant strains as well as kinetoplastic and dyskinetoplastic ones. In his experiments, no evidence of recombination suggestive of sexual reproduction was obtained.

More recent observations, however, indicate that there may be a stage of sexual reproduction in trypanosomes. Nyindo *et al,* (1981) observed that there were large and small parasites forming pairs in the midgut and salivary glands of tsetse flies and believed that this indicated the existence of sexual forms. Tait (1980) analysed several isoenzyme electrophoretic patterns of *T. brucei* stocks and showed that the observed frequencies of the main and hybrid enzyme patterns corresponded to the expected frequencies as determined by the Hardy-Weinberg equilibrium. Jenni *et al,* (1986) also used isoenzyme activity to detect hybrids that implied sexual reproduction. In their study, these workers infected flies by feeding them on blood which contained trypanosomes having different isoenzyme patterns. Trypanosomes recovered from mature infections in the flies were then cloned and analysed for isoenzyme activity. The results suggested that there was genetic exchange between trypanosomes when they were in tsetse flies. Further evidence for sexual reproduction in *T. brucei* was suggested by measurement of DNA content of trypanosomes in the tsetse fly and those in the bloodstream of mammals (Zampetti-Bosseler *et al,* 1986). Results suggested that trypanosomes in the fly were haploid while those in mammals were diploid. Tait *et al* (1989) using isoenzyme and restriction fragment length polymorphisms, however, found evidence suggesting that metacyclic forms of *T. brucei* were diploid. In reviewing the status of sexual reproduction in kinetoplastids, Tait (1983) concluded that such a system would be important in providing an opportunity for mutations to spread as well as providing variation and diversity in the genetic pool of trypanosome strains. This would avoid the harmful effects of the continuous cloning provided by asexual reproduction. On the other hand, sexual reproduction would also provide an opportunity for the spread of mutations like drug resistance. So far, no work has suggested that similar genetic exchange suggestive of sexual reproduction exists in mechanically transmitted *T. evansi.* This may be due to the absence of an insect developmental stage in this species; a stage which would provide opportunity for genetic exchange as has been observed in cyclically transmitted trypanosomes.

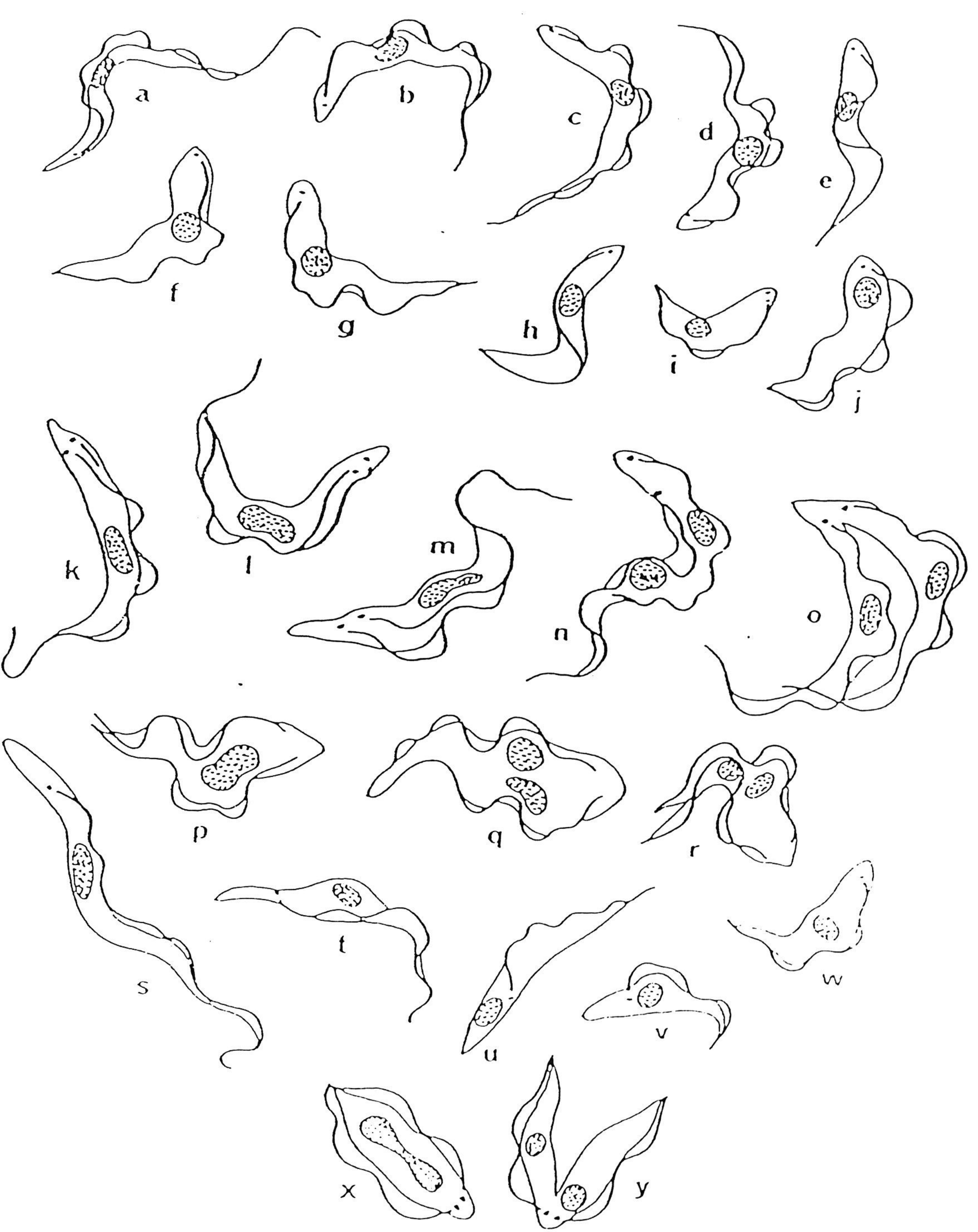


Figure 2.4 Reproduction in *T. brucei* showing binary fission (Hoare, 1972).

2.2.4 HOST RANGE OF TRYPANOSOMES

In the subgenus *Trypanozoon, T. gambiense* and *T. rhodesiense* are animal infective species whose host range includes humans compared with the host ranges of *T. brucei* and *T. evansi* which are restricted to animals and *T. equiperdum* restricted only to equidae. *T. vivax* infects a range of ungulates including cattle, sheep , goats, horses and camels. The host range of *T. congolense* is even wider than that of *T. vivax* and includes every species of livestock that is susceptible to trypanosome infection (Hoare, 1972). *T. simiae* and *T. suis* infect pigs although there have been recorded *T. simiae* infections of camels, cattle and horses (Culwick and Fairbairn, 1947; Pellegrini, 1948; Killick-Kendrick and Godfrey, 1963). Wild suids such as warthogs, bush pigs and forest pigs are also infected by *T. simiae* and can act as carriers from which transmission to domestic pigs is achieved. A whole range of wild animals have been shown to be infected with all the tsetse-transmitted trypanosome species. The severity of disease in wild animals varies although their role as symptomless carriers which act as reservoirs of infection to livestock is well recognised.

The definitive host of *T. evansi* is the camel although infections have been observed in other domestic animals such as horses, donkeys, mules, dogs, cats, sheep , goats, buffaloes, cattle, deer and the Indian Elephant (Hoare, 1972; Stephen, 1986; Luckins, 1988; Lun *et al*, 1993). The importance of the camel as the main host of *T. evansi* is emphasised by the fact that the world geographic distribution of this parasite overlaps the distribution of camels (Figure 2.5). Other hosts of this trypanosome species are horses in Latin America and buffaloes in South East Asia. In addition, a whole range of wild ruminants, canines and felines are infected by this trypanosome species although their role as reservoirs is considered minimal (Hoare, 1972). An exception to this is in Central and South America where capybaras act as reservoirs (Morales *et al*, 1976; Reveron *et al*, 1992). In Africa, the reservoir role of other domestic animals in maintaining infection from where camels are infected is suspected but not properly documented.

2.2.5 DISEASES CAUSED BY TRYPANOSOMES

The diseases caused by trypanosomes were recognised in different parts of the world long before the discovery of trypanosomes. These diseases were classified according to symptoms shown by infected animals and given local names. The pathogenicity of these diseases vary considerably and is determined by host-parasite relationships. It is believed that in hosts that have had a long association with a particular trypanosome species, an equilibrium is reached whereby the two co-exist in harmony. The high pathogenicity of trypanosomes in domestic animals thus reflects an imperfect host-parasite relationship of recent origin (Hoare, 1972). The severity of disease in different animal species, shown in Table 2.4, is determined by the infecting trypanosome strain and the susceptibility of a particular animal. The susceptibility of the host is influenced by factors such as its immunological status, its nutritional status as well as environmental conditions. In animals with a compromised immune system, the disease is more virulent leading to fast deterioration and quick death.

Nagana is the term given to diseases caused by tsetse-transmitted *T. brucei, T. congolense* and *T. vivax* in a whole range domestic animals. These diseases can be acute or chronic depending on the animal species infected (Table 2.4). Nagana is characterised by intermittent fever, anaemia, oedema, emaciation and sometimes mucosal haemorrhages as well as abortions in pregnant females. In trypanosomes of the subgenus *Trypanozoon*, the parasites can invade the central nervous system (CNS) as was first observed by Castellani (1903) in sleeping sickness patients in Uganda. This CNS involvement results in detectable pathological changes. In most cases, Nagana is fatal unless treated.

The disease caused by *T. simiae* in pigs is severe and causes death within a short period after the parasites are detected in peripheral blood. The virulence of this species in other domestic animals such as camels varies from being hyper acute to chronic (Pellegrini, 1948). For *T. suis*, the disease in pigs is more chronic than that caused by *T. simiae* and takes longer to kill the host (Stephen, 1966).

Dourine is the disease caused by *T. equiperdum* in equidae. This disease is chronic with symptoms appearing one week to 4 months after sexual infection. Death may occur as long as 4 years after infection.

The two human infective trypanosome species cause sleeping sickness. This disease is so named because it is characterised by periods of drowsiness during the late stages of infection when trypanosomes invade the CNS. The two species can be differentiated by the severity of disease they cause (Table 2.4). In *T. gambiense* infections, parasites are patent about 10 days after infection followed by chronic disease characterised by intermittent parasitaemia in the peripheral blood and bouts of fever. The parasites invade the CNS sometime during this chronic stage and are believed to be a source of re-invasion of the peripheral blood (Apted, 1980). If untreated, Gambian sleeping sickness leads to death several years after its onset. The disease caused by *T. rhodesiense* is similar to that caused by *T. gambiense* except that it is acute, with death occurring within a year of infection.

The disease caused by *T. evansi,* Surra, was the first trypanosomiasis to be recorded. This disease is severe in camels, horses, elephants and dogs but mild and often symptomless in bovines, goats and pigs (Table 2.4). Like other *Trypanozoon* species, *T. evansi* invades the CNS from where parasites can be a source of relapsing infection (Innes and Saunders, 1962; Seiler *et al,* 1981). In camels, Surra usually takes a chronic form characterised by intermittent fever, anaemia, emaciation, abortions in pregnant females and finally death. Trypanosomes appear in the blood about a week after infection at which stage there are no signs of clinical disease. About a month later, progressive weakness and loss of condition sets in. The camels' coat becomes rough and staring as appetite declines and anaemia sets in. Pregnant females may abort or give birth to weak calves and the milk yield of lactating camels is reduced. Recurrent bouts of fever are observed and other clinical signs including subcutaneous oedema, haemorrhages of the mucosa and discharge from the eyes may be observed. The camel dies months or even years after the initial infection (Leese, 1927; Boid *et al,* 1985). Fazil (1977) thus described Surra in the camel as a slow wasting disease which may take up to four years before the animal dies. Some cases of symptomless and cryptic *T. evansi* infections (Sergent *et al,* 1918; Killick-Kendrick, 1968) as well as spontaneous recovery in well managed camels have been observed (Mukasa-Mugerwa, 1981).

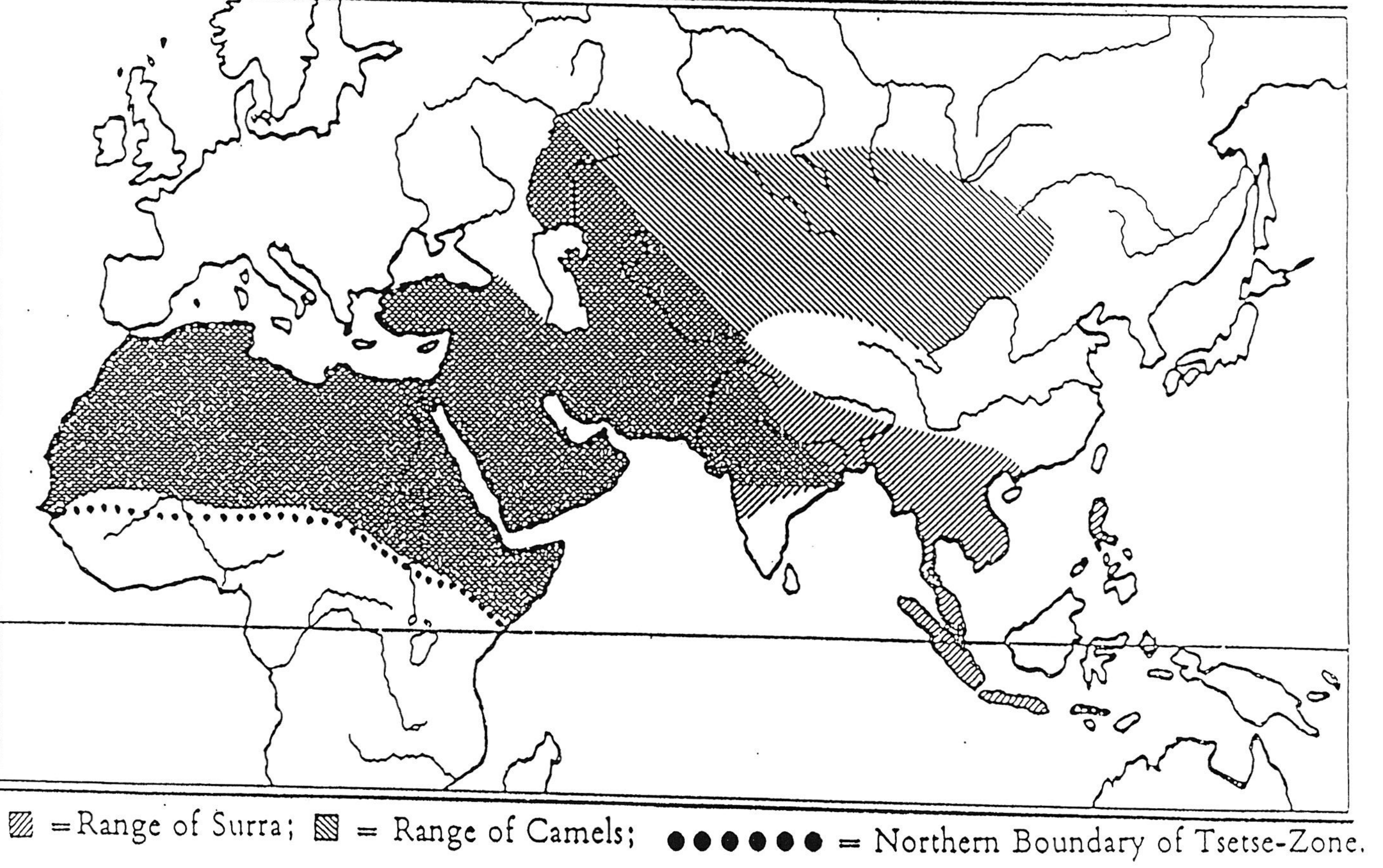


Figure 2.5 The geographical distribution of Surra and camels (Hoare, 1972).

Table 2.4 Some characteristics of diseases caused by African trypanosomes (Hoare, 1972).

Species	Disease	Animal hosts	Average pre-patency	Severity
T. brucei	Nagana	equidae	6-10 days	*
		dogs	10 days	*
T. gambiense	Sleeping sickness	humans	10 days	Chronic
T. rhodesiense	Sleeping sickness	humans	10 days	*
T. congolense	Nagana	cattle	2-3 weeks	*
		pigs	6-15 days	*
		horses	17-19 days	*
T. vivax	Nagana	sheep and goats	2-3 weeks	*
		horses	3 weeks	*
		cattle	9-59 days	*
		camels	4-8 days	Benign
T. simiae	-	pigs	very variable	Acute
		camels	6-17 days	*
T. suis		pigs	about 1 week	Chronic
T. evansi	Surra	camels	4-9 days	Chronic*
T. equiperdum	Dourine	equidae	1 week to 3-4 months	Chronic

* - Varies according to infecting trypanosome strain.

- No general name recognised.

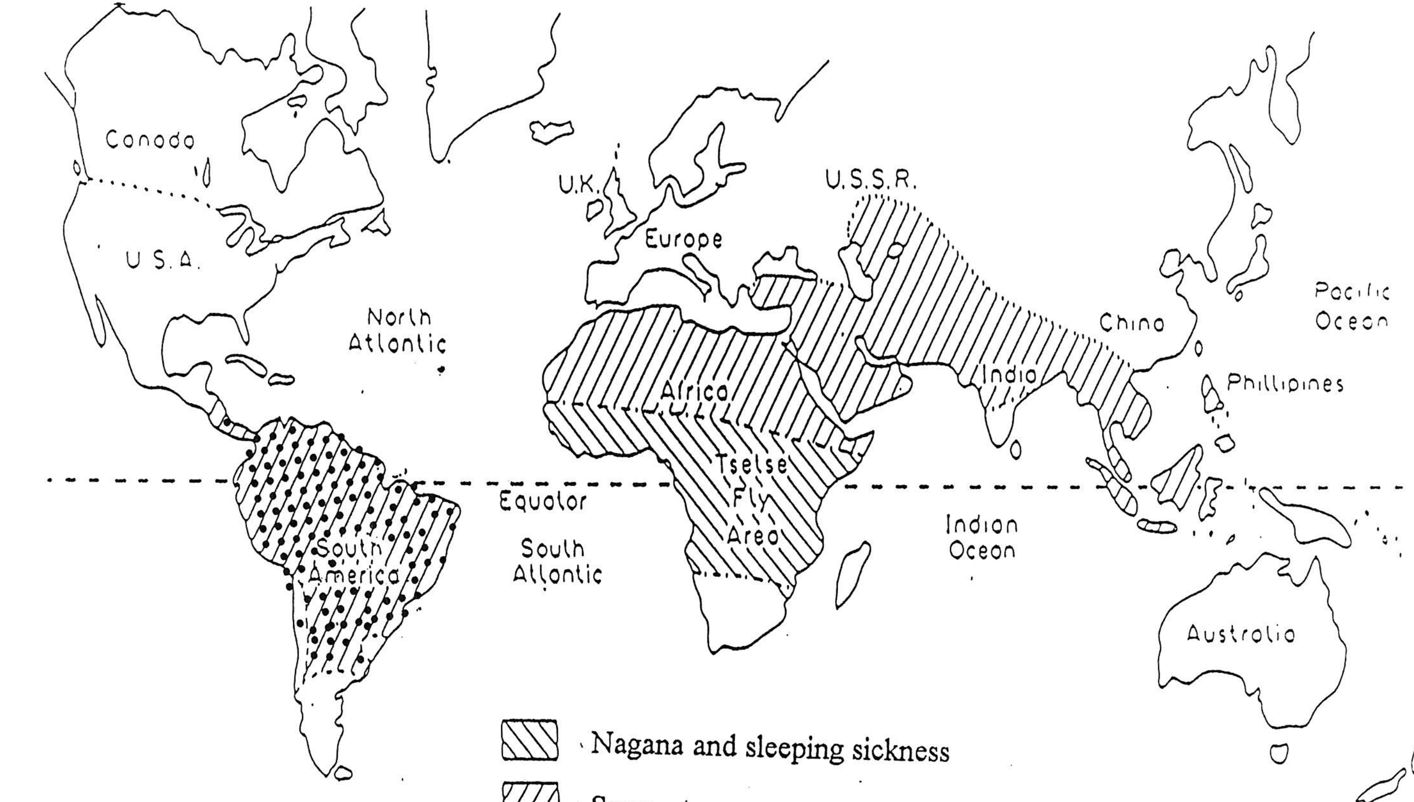


Figure 2.6 The distribution of Surra and Camels in the world (Hoare, 1972).

2.2.6 GEOGRAPHIC DISTRIBUTION OF AFRICAN TRYPANOSOMOSES

African trypanosomes are distributed throughout the continent, their main limitation being the availability of suitable host species and appropriate vectors. Due to the wide host range of *T. congolense, T. brucei* and *T. vivax*, the distribution of Nagana is limited by the availability of suitable habitats for the *Glossina* species which transmit these trypanosomes. Nagana is thus found throughout Africa wherever there is a suitable tsetse habitat. The extent of the problem of tsetse transmitted trypanosomiasis in Africa is not known, but the fact that 10 million square kilometres are tsetse inhabited (Jordan, 1986) gives a broad indication. The distribution of diseases caused by *T. simiae* is as wide as that of Nagana although there is a limitation on the availability of both domestic and wild suids. Indications suggest that the disease caused by *T. suis* is found mainly in East and Central Africa although the exact distribution is not well known (Hoare, 1972). The distribution of Dourine is not known although it is believed to be cosmopolitan with infections caused by *T. equiperdum* found all over Africa wherever equidae are found. The distribution of sleeping sickness is generally limited to scattered foci within suitable tsetse habitats; the Gambian form of the disease generally being found in West and Central Africa while the Rhodesian form is found in East Africa. The two disease zones overlap in a small area of Central Africa where both Gambian and Rhodesian sleeping sickness have been reported.

T. evansi has the widest distribution of trypanosome species in the world and is a cause of Surra in both the Old and New Worlds. The distribution of Surra ranges from Central and Southern America, the Middle East, Eastern Europe, Central Asia as well as the Far East (Figure 2.6). Since *T. evansi* is mechanically transmitted by a wide range of vectors, the distribution of Surra is not as limited by the vector as are diseases caused by tsetse-transmitted trypanosomes. As earlier indicated, the distribution of *T. evansi*, and therefore Surra, closely follows that of its principle host, the camel (Figure 2.5).

It is believed that *T. evansi* originated in tropical Africa from where it spread to the extent found today. According to the "*T. brucei* origin of *T. evansi*" theory (Leese, 1927; Godfrey and Killick-Kendrick, 1962), camels were infected with *T. brucei* when they were temporarily brought into the tsetse belt. When these camels left the tsetse belt, the trypanosomes became adapted to a mechanical means of transmission in the absence of tsetse flies. Camels having acquired chronic infection, spread *T. evansi* as they travelled in caravans of ancient Egyptians, across sub-Saharan Africa, the Arabian peninsula, Asia minor and India (Currason, 1943). The spread of

Surra can thus be attributed to the camel being used as a beast of burden for transport in trading and military missions in Africa and the Middle East. Other livestock such as horses, buffaloes, and cattle were subsequently infected when they came into contact with infected camels. Further spread of Surra into areas such as the American continent as well as islands like Mauritius, the Philippines and Indonesia can be traced to the importation of infected livestock from endemic areas. The spread of Surra provides a good example of how human beings in their movements can play a role of introducing and distributing disease.

2.2.7 TRYPANOSOME DIAGNOSIS AND CHARACTERISATION

It is very important to diagnose trypanosome infections and characterise the species involved. The need to accurately identify parasites is not just for the purpose of assigning them to their correct taxonomic groups, but for reasons of helping the medical doctor or veterinarian to improve treatment in the quest for controlling infections (Godfrey, 1978). All factors that have been previously reviewed; morphology, life cycle, host range, disease and geographic distribution, can be used in isolation but mostly in combination as characterisation tools. In order to get more accurate identity of infecting trypanosome species, however, more refined techniques have to be used. The ideal diagnostic and characterisation technique would be a simple specific and sensitive pen-side test which can be performed quickly and cheaply without the need of sophisticated equipment. Of all the available techniques used for characterising trypanosomes, none has met all of these specifications because with increased specificity and sensitivity, the simplicity and speed of performing the test are decreased and the cost increased.

2.2.7.1 Parasite detection

Disease is often diagnosed by observation of symptoms which are readily recognised by herdsmen in pastoral systems. In addition to the presenting symptoms, the absolute evidence of infection to a clinician is the demonstration of parasites in blood or other body fluids. The most direct method of trypanosome demonstration is microscopic examination of peripheral wet blood films (Baker, 1970). The paucity and fluctuating nature of parasitaemia in chronic trypanosomiasis may, however, hamper the demonstration of parasites in the circulation by this method even though there may be many parasites in other tissues (Losos and Ikede, 1972). By concentrating blood using microhaematocrit centrifugation (Woo, 1970; Murray *et al,* 1977), the probability of detecting the parasites is improved. In this method, trypanosomes are concentrated just above the "buffy coat" which consists of leucocytes. The parasites

are then looked for by either inspecting the buffy coat and its adjoining areas using a phase-contrast microscope or by breaking the tube at the buffy coat, extruding the contents of the adjacent areas followed by microscopic inspection of the extruded matter. The efficiency of this concentration method in detecting *T. brucei, T. evansi* and *T. rhodesiense* has been estimated at 85% (Woo and Rogers, 1974). After demonstration of parasites, characterisation of the particular trypanosome species is on the basis of morphology as shown in Table 2.2.

At times, these direct methods of detecting parasites do not work especially when the parasitaemia is low or the infection cryptic. The sub-inoculation of blood into rodents has been shown to be effective in detecting infections otherwise missed by direct microscopic examinations. Rodent blood is then regularly examined microscopically for the presence of parasites. This method provides opportunity to expand a trypanosome population to a magnitude that is detectable by microscopy. Paris *et al,* (1982) evaluated the various parasitological tests available and found that for *T. brucei,* microscopic examination of wet blood film could detect 8.3×10^3 while mouse sub-inoculation could detect as few as 1.25×10^2 parasites. For *T. evansi,* the mouse sub-inoculation test has been determined to be the best direct diagnostic method (Godfrey and Killick-Kendrick, 1962; Pegram and Scott, 1976; Payne *et al,* 1990). In *brucei* group infections involving the CNS, examination of cerebral-spinal fluid either microscopically or by rodent sub-inoculation may increase the chances of parasite detection. This is, however, mainly done in human trypanosomiasis for the acquisition of this fluid is laborious, difficult and may even be dangerous. Even with these modifications of the basic microscopic methods, parasitological methods are sometimes inadequate and fail to detect non-patent infections (Killick-Kendrick, 1968) and thus more sophisticated indirect tests must be resorted to. The most important tests currently used for diagnosis and characterisation will now be discussed.

2.2.7.2 Serological tests

Franke (1905) was the first person to observe and document the agglutination of trypanosomes in a serological reaction. Since then, there have been many *in vitro* tests that use basic antigen-antibody reactions in the detection of trypanosome infection. Most of these tests are based on the fact that trypanosomes release antigens when they die and disintegrate. The range of serological tests for diagnosis of various trypanosomes reviewed comprehensively by a number of authors (Killick-Kendrick, 1968; Molyneux, 1975; WHO, 1976; Nantulya, 1990; Luckins, 1992) will be discussed according to the serological reaction involved. Although in some instances tests

appear to detect interspecific differences, the value of many as diagnostic tests is considered poor (Lumsden *et al,* 1973; Gray and Luckins, 1976).

Agglutination was the first serological reaction to be described for trypanosomes (Franke, 1905) and various agglutination tests were thereafter developed (e.g Soltys, 1957a; Cunningham and Vickerman, 1962; Ross, 1971). Modifications of the basic agglutination tests are the indirect haemagglutination test (Gill, 1964), the card agglutination test for trypanosomes, CATT (Croft, 1985) and the procyclic agglutination trypanosomiasis test, PATT (Pearson *et al,* 1986). Lysis tests involve reactions between antigens on the parasite and antibodies produced in animals with long standing infections. Trypanolysis tests usually depend on the presence of complement for the reaction to proceed leading to the destruction of parasites. The original test described by Schilling (1904) has undergone modification to produce the current micro-technique which is done using micro-titer plates (Lumsden *et al,* 1973). This test is complement dependent and was initially developed for detecting Dourine in horses (Watson, 1925). Mayer (1905) first observed that antigen-antibody reactions could lead to the precipitation of trypanosome material. A recent test based on precipitation is double diffusion in agar gel whereby the antigen and antibody in agar diffuse to form detectable areas of interaction (Gray, 1961). Neutralisation tests are based on the ability of serum from an infected animals to neutralise trypanosomes leading to a reduction or even loss in their ability to infect. The first neutralisation test was developed by Soltys (1957b) and is used to date with modifications (Lumsden *et al,* 1963). Although these serological tests have been used to detect infections caused by various trypanosome species they are used mainly for detecting antigenic differences of trypanosome strains in the laboratory (Molyneux, 1975; Gray and Luckins, 1976).

The most significant recent improvement in available serological diagnostic assays is in the application of tests which directly measure the interaction between antigen and antibody without relying on a secondary reaction consequent to binding. One of the first primary binding assays for the detection of trypanosomal antibodies was the indirect immunofluorescent antibody test (IFAT). Antigens are prepared from blood smears fixed in formaldehyde or acetone and stained with a flourescent conjugate. Blood smears are then examined by the use of a fluorescent microscope and results read and graded according to the intensity of fluorescence. Such assays have been used to detect trypanosomal antibodies in cattle and camels although they may not discriminate between different trypanosome species (Luckins and Mehlitz, 1978; Luckins *et al,* 1979; Luckins, 1992). The IFAT test requires expensive and sophisticated equipment, relies on subjective comparison of results and only indicates

that an animal has been infected with a trypanosome without enabling determination of its current status.

The enzyme linked immunosorbent assay (ELISA) was first developed as an indirect assay to detect trypanosome-specific antibodies in the blood of animals. In this assay, test sera is reacted with a trypanosomal antigen which is adsorbed onto polystyrene plates. This is followed by incubation of the resulting antigen-antibody complex with enzyme-conjugated antiglobulin to the IgG fraction of the particular animal host species. The test is visualised by the addition of enzyme substrate and chromogen, with the resulting colour change allowing photometric interpretation. The original tests were not species-specific since they did not distinguish between *T. congolense, T. brucei* and *T. vivax* although they did not cross react with *T. theileri* or with *Theileria, Babesia* and *Anaplasma* (Luckins, 1977). Although these tests are superior to the IFAT in that they can be read objectively, like the IFAT, their major disadvantage is that they do not differentiate between current and past infection. Positive ELISA results due to persistent antibodies may thus be detected months after an animal has been successfully treated (Nantulya, 1990). An alternative approach to ELISA detection tests involves the use of assays which detect trypanosomal antigen in the blood of infected animals. In antigen ELISA tests, a trypanosome-specific antibody is used to coat a polystyrene plate. Test serum is then added onto the plates and the antigen in serum captured by the coating antibody. Since the trapped antigen has several combining sites, a second antibody which is enzyme-labelled and introduced into the plate will bind to free sites on the captured antigen. Like the antibody ELISA test, the reaction is revealed by addition of substrate and chromogen (Figure 2.7). The tests developed initially used a polyclonal antibody to coat the plate (Rae and Luckins, 1984) although improved systems developed later used monoclonal antibodies. Nantulya *et al,* (1987) and Nantulya and Lindqvist (1989) developed antigen ELISA systems which could distinguish the various trypanosome subgenera. A further development of similar tests enabled the distinction of the various species in *Trypanozoon.* Such tests which have been developed aim at identifying *T. evansi* (Nantulya *et al,* 1989a; 1989b; Olaho-Mukani, 1989; Luckins, 1991). Although there is a period early in the infection when antigen ELISA may not detect antigens in infected animals, the antigen ELISA test correlates well with other tests like microscopy and mouse sub-inoculation (Nantulya *et al,* 1989a). These apparent false negatives observed early in the infection are attributed to the fact that antigens are produced only after the first parasitaemic wave when trypanosomes are destroyed and antigens released. The antigen ELISA test appears to be the best available serological test for the detection of current infection.

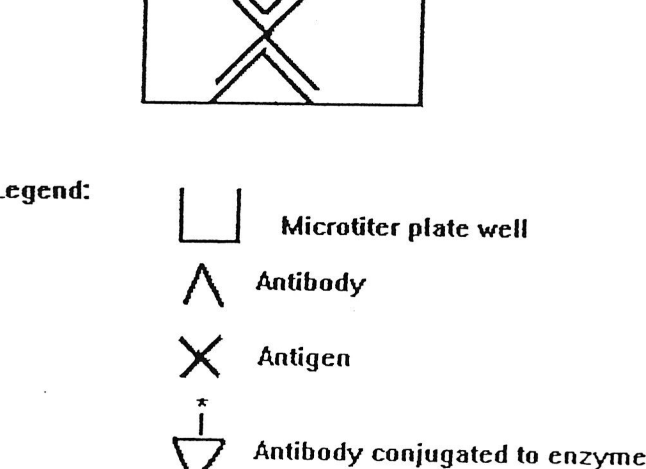


Figure 2.7 A diagrammatic illustration of antigen ELISA.

2.2.7.3 Human infectivity and blood incubation infectivity test (BIIT)

Two methods are used to differentiate *T. rhodesiense* and *T. gambiense* from all other species by their ability to infect humans. Several attempts were made to directly use the human-infectivity criteria by inoculating human volunteers with suspected human-infective parasites (Heisch *et al*, 1958; Onyango *et al*, 1966). Although the ability to infect volunteers is a positive means of identifying human-infective trypanosomes, the reverse is not the case since the inability to infect humans does not necessarily indicate that the stock is *T. brucei*. This is because there are other factors such as the pathogenicity of the infecting trypanosome stock and the susceptibility of an individual which may prevent the establishment of infection. Such experiments are no longer acceptable due to ethical considerations.

Another characterisation system used to identify human-infective parasites is the blood incubation infectivity test (BIIT). This test designed by Rickman and Robson (1970) is based on the observation that *T. rhodesiense* is resistant to human serum whereas human serum is trypanocidal to stocks of *Trypanozoon* which are not infective to humans (Laveran, 1902). As a characterisation tool, this test broadly separates the two human-infective trypanosome species from the non-infective ones. This test however has its shortcomings for though a positive BIIT status of a stock positively indicates that it is human-infective, the reverse is not necessarily the case (a negative BIIT does not positively identify a stock as being non human-infective). This is due to the fact that well known human-infective stocks were shown to be BIIT negative while other stocks were observed to vary in their BIIT status with passage (Hawking, 1973).

2.2.7.4 *In vitro* cultivation of trypanosomes

The cultivation of trypanosomes in culture medium has been done since the beginning of this century (Novy and MacNeal, 1903). The media and temperatures used in *in vitro* cultivation of trypanosomes are chosen to mimic growth requirements of these parasites in the insect vector. It was initially believed that *in vitro* cultivation of mechanically transmitted *T. evansi* and *T. equiperdum* was not possible and could thus serve as a tool for characterisation (Hoare, 1972). This inability to grow in culture was attributed to a loss of the ability of these species to develop in the invertebrate host and therefore the subsequent loss of the ability of cultivation in culture at below-body temperatures (27°C). Systems to culture *T. evansi in vitro* have, however, now been developed whereby parasites are cultivated at 37°C, a

temperature similar to that which metacyclic and bloodstream forms are normally exposed (Baltz *et al*, 1985; Zweygarth and Rottcher, 1986; Zweygarth *et al*, 1989).

Lately, trypanosome cultivation in specific culture medium has been shown to distinguish *T. evansi* from *T. brucei*. In this test, bloodstream forms of *T. brucei* differentiate into procyclics in culture while *T. evansi* die within 7 days (Zweygarth and Kaminsky, 1989). As a characterisation assay, this test may have a potential although it is still being developed and tested.

2.2.7.5 Isoenzyme analysis

Isoenzymes are multiple molecular forms of given enzymes. These proteins have similar properties but differ in their electrophoretic mobilities due to differences in charge caused by variations in their primary, secondary or tertiary molecular structures. Harris (1969) characterised the primary variations as structural and the secondary and tertiary variations as post-translational. Structural changes occur at the level of transcription when the genetic codes of various polypeptides that make up an enzyme, are determined. The occurrence of multiple gene loci coding for the polypeptide chains that make up an enzyme has been considered as one source of structural differences. Mutations in the various loci accumulated in the course of evolution would lead to changes in the enzymes concerned. Such structural changes lead to interspecific enzyme differences. Further studies into the various loci have led to the assignment of enzymes as either cytoplasmic or mitochondrial according to cellular region of their synthesis or action (Harris and Hopkinson, 1976). Another primary structural source of isoenzymes is the occurrence of multiple alleles at a single gene locus which leads to either homozygous or heterozygous phenotypes. Various combinations of the alleles are thus possible; the complexity of which depends on the number of alleles at a particular locus. These structural changes result in intraspecific differences.

Post-translational changes are due to modifications which occur after the polypeptide chain has been synthesised and lead to the final form of the enzymes. Such changes could be secondary in the form of deaminations, acetylations, oxidations or other modifications of the primary chemical structure. Secondary structural changes, such as cleavage of the polypeptide chain at certain locations due to formation of hydrogen bonds, can also be conformational. Aggregations and polymerisations of the initial polypeptide chains are tertiary changes which are classified as post-translational changes as well. Structural and post-translational variations may not change the function of the resulting enzymes although they may result in a change in the overall

net charge leading to differences in electrophoretic mobilities (Harris and Hopkinson, 1976).

Knowledge concerning enzyme electrophoretic mobilities together with the high degree of enzyme substrate specificities has led to the development of characterising systems. In these systems, crude tissue preparations are used to detect the presence of different isoenzymes. In order to detect enzymatic activity, staining methods are used which indicate the products of enzymatic reaction by a detectable colour, fluorescent or radioactive change. The use of staining systems which result in a colour change are preferred as they are comparatively cheaper and less hazardous (Harris and Hopkinson, 1976). Isoenzyme systems and their electrophoretic mobility differences in starch gels have been used to characterise different species in *Entamoeba* (Reeves and Biscoff, 1968), *Plasmodium* (Carter, 1973), *Theleria* (Musisi, 1978; Melrose, 1983) as well as *Leishmania* (Chance *et al*, 1978) and *Trypanosoma* (Kilgour and Godfrey, 1973; Murray, 1982; Godfrey *et al*, 1987).

In the subgenus *Trypanozoon*, isoenzyme analysis has been particularly important in differentiating parasite species which are morphologically indistinguishable (Gibson *et al*, 1980; Gibson and Wellde, 1985; Godfrey *et al*, 1990). These studies have defined eleven enzymes in this subgenus which are useful in distinguishing the various *Trypanozoon* species. These enzymes are; alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), isocitrate dehydrogenase (ICD), malate dehydrogenase (MDH), malic enzyme (ME), threonine dehydrogenase (TDH), nucleoside hydrolase (NH) and two peptidases 1 and 2 (PEP 1 and PEP 2). For cameline *T. evansi,* five enzymes; ASAT, PGM, ME, PEP1 and PEP2; vary among the different isolates studied and have been used to differentiate this species from others in the *Trypanozoon* subgenus (Gibson *et al*, 1980). The patterns of the malic enzyme so far found in *T. evansi* stocks are patterns 2, 4, 7, 10 and 24 (Gibson, 1983; Boid, 1988) as illustrated in Figure 2.8.

Other than characterising the various trypanosome species, attempts have also been made to use isoenzymes as markers for drug resistance. Dukes (1984), working with *T. gambiense* sought, without success, to find a correlation between patterns of ALAT, ASAT, ICD, PEP 1, PEP 2, PGM and ME and resistance to Trypamidium, melarsoprol, pentamidine and Berenil. Boid *et al*, (1989) working with *T. evansi,* however, suggested that a particular ME pattern (7) could be used as a marker for suramin resistance. The latter workers based their suggestion on the observation that there was a correlation between this ME pattern and suramin resistance in *T. evansi*

stocks from Sudan and Egypt. This link between suramin resistance and ME pattern 7 has not been observed in stocks from any other parts of the world and its value as a marker is therefore uncertain.

According to migration on a starch electrophoretic gel, ME of *Trypanozoon* parasites has been shown to have 28 variant patterns found in 12 locations (Table 2.8, Godfrey *et al*, 1990). In man, this enzyme is coded for at two autosomal loci to produce the soluble and mitochondrial forms. Both of these forms migrate anodally with the latter remaining close to the origin (Cohen and Omenn, 1972; Povey *et al*, 1975). The ME catalyses a reaction associated with glycolysis whereby L-malate and oxidised nicotinamide adeninedinucleotide phosphate (NADP) are converted to pyruvate, carbon dioxide and reduced NADP (NADPH). This reaction is important to trypanosomes because they derive their energy from the breakdown of glucose found in their hosts.

In order to detect the activity of the malic enzyme, an electron transfer staining system, using either methyl thiazolyl tetrazolium (MTT) or 3-amino ethyl carbazole dyes, is used. The MTT staining method is preferred because it is more sensitive (Harris and Hopkinson, 1976). In this reaction illustrated in Figure 2.9, phenazine methosulphate (PMS) acts as an intermediate catalyst where MTT, an acceptor for dehydrogenase reactions, is reduced to dark blue insoluble formazan by electron donors produced as NADP is reduced to NADPH.

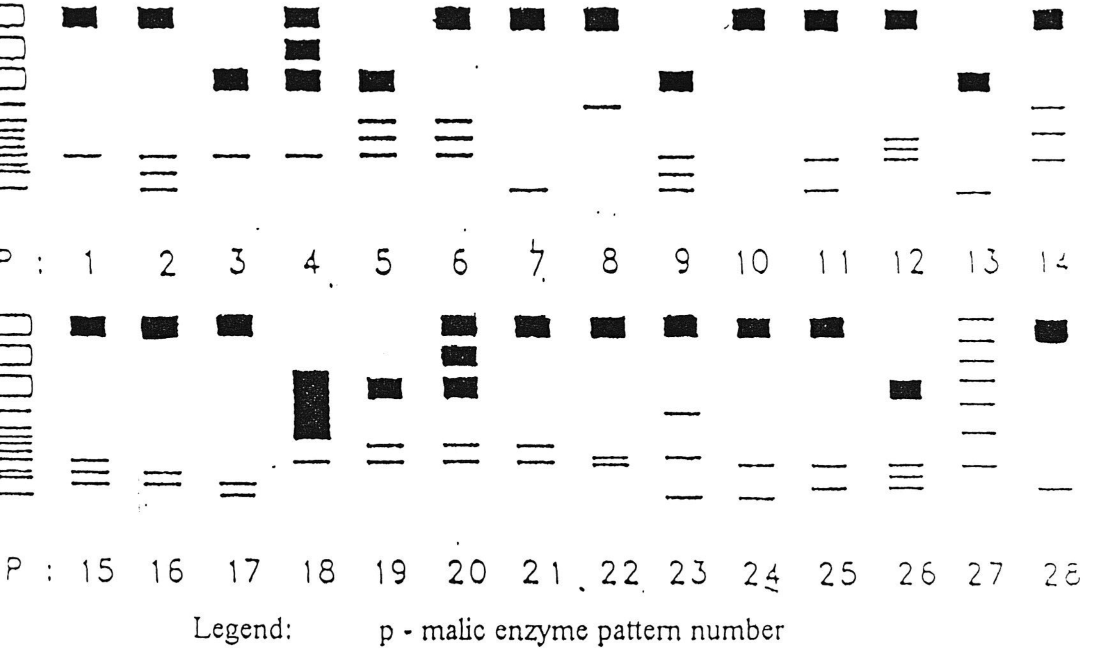
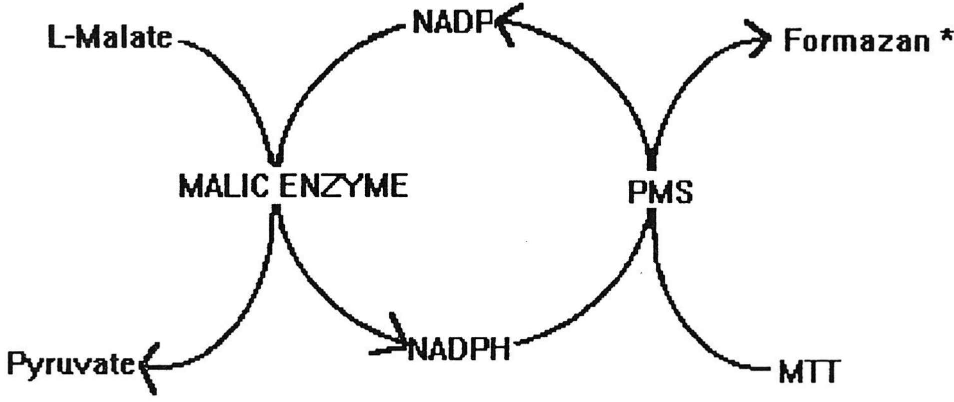


Figure 2.8 Enzyme patterns found in *Trypanozoon* (Godfrey *et al*, 1990)



Legend: NADP - Nicotinamide adeninedinucleotide phosphate
NADPH - Reduced nicotinamide adeninedinucleotide phosphate
PMS - Phenazine methosulphate
MTT - Methyl thiazolyl tetrazolium

Figure 2.9 The malic enzyme reaction leading to formation of a blue insoluble formazan dye.

2.2.7.6 Molecular Methods of Characterisation

Of all the methods currently available, the most specific and efficient method of characterising trypanosomes is at the level of the trypanosome genetic material. The available methods are based on the fact that each trypanosome species must have differences in its genetic material, deoxyribonucleic acid (DNA), which distinguishes it from all other species. These variations at the DNA level might be reflected in phenotypic characteristics resulting in variations in infectivity to vertebrate hosts, morphology and life cycles. The identification of such differences at the molecular level would have the advantage of directly analysing trypanosome DNA as opposed to indirect methods that detect phenotypic characteristics resulting from DNA changes.

Specific DNA fragments (probes), have been shown to be important in characterising parasites. These fragments are labelled by radioactive material or enzymes in order to determine the degree of similarity in test DNA by hybridisation reactions . So far, species-specific probes are available which detect the three subgenera of African trypanosomes (Massamba and Williams, 1984; Kukla *et al,* 1987; Gibson *et al,* 1988). *Duttonella* and *Trypanozoon* require only one probe each for identification but three probes are required to detect *Nannomonas* parasites. These different probes have been developed to detect savannah, forest and Kenya coast *T. congolense* types.

Molecular methods have also been developed which have been used to distinguish *T. evansi* from other members of the *Trypanozoon* subgenus. Borst and Hoeijmakers (1979) and Borst *et al,* (1987) observed that there were differences in the kinetoplast DNA of this *T. evansi* which distinguish it from other species in the subgenus. The kinetoplast of cyclically transmitted *Trypanozoon* parasites contains two DNA components consisting of 50-100 homogeneous DNA maxi circles and 5000-100,000 heterogeneous mini circles. Mechanically transmitted *T. evansi* and *T. equiperdum* differ from the other species in the subgenus in that although they have the same number of minicircles, they are homogeneous. Another difference is that *T. evansi* completely lacks maxi circles while *T. equiperdum* has maxi circles which are not functional (Borst and Hoeijmakers, 1979). The absence of maxi circles and the homogeneity of mini circles in *T. evansi* can thus be used to distinguish this species from other morphologically identical trypanosomes.

More recent molecular methods of characterising *T. evansi* involve the use of species-specific DNA fragments. Masiga and Gibson (1990) identified two kinetoplast DNA (kDNA) sequences which were present in all *T. evansi* stocks tested

but were absent from all other trypanosome species. In this test, a trypanosome sample on a blot is hybridised with a probe to determine whether the trypanosome species involved in the infection is *T. evansi*. Another DNA-based characterisation method is based on the polymerase chain reaction (PCR); an *in vitro* technique used to amplify a segment of DNA that lies between two regions of known sequences (Mullis and Faloona, 1987; Saiki *et al,* 1988). These known sequences (primers) are found on the DNA template and are recognised by polymerase enzymes as regions from where replication is initiated. Species-specific primers have been identified in *T. congolense* and *T. brucei* (Moser *et al,* 1989) as well as in *T. evansi* (Masiga *et al,* 1992; Wuyts *et al,* 1992). In the PCR, a DNA test sample is denatured (the two strands separated) by heating and incubated with a species-specific primer in the presence of DNA polymerase. If the sample is from trypanosomes of the appropriate species, the polymerase enzyme recognises the primer, replication is initiated and a new strand of DNA is synthesised. By raising the temperature to over 90^{0}C, the DNA template is denatured again as well as the newly synthesised DNA. When the temperature is lowered to a level that allows replication, more DNA is synthesised using both the original and the newly synthesised DNA fragments as templates. This cycle is repeated and thus DNA amplified to an extent that depends on the number of times the temperature is varied as well as the availability of both the primer and the polymerase. The reaction mixture is then run on an electrophoresis agarose gel where the intensity of the DNA band seen compared to the original test sample determines whether DNA has been amplified.

DNA-based methods have great potential as characterisation tools which directly analyse specific trypanosome differences between various trypanosome species. These tests have also shown their potential in classification and speciation of trypanosome species and suggest that the present system may need revising for it classifies trypanosomes, like *T. congolense*, in groups which are more heterogeneous than was initially believed.

2.2.8 ANTIGENIC VARIATION IN TRYPANOSOMES

The first reference to antigenic variation was by Franke who observed *T. equinum* parasites in a monkey were not affected by antibodies from the same animal (Gray and Luckins, 1976). This was later identified as being due to antigenic variation a phenomenon now documented in trypanosomes of all species infective to man and animals. Antigenic variation is caused by a change in the glycoprotein surface coat of trypanosomes leading to the presentation of varying antigen types to the host (Soltys,

1963; Vickerman, 1968). At infection, trypanosomes present a particular antigen to which the host produces antibodies. This should lead to the destruction of all parasites. By the time these antibodies are produced by the host, however, a small number of parasites have changed their surface coat and are thus not destroyed by the antibodies. The destruction of the majority of parasites leads to a detectable drop in parasitaemia. The parasites that remain proliferate, leading to an increase in parasitaemia and the presentation of a different antigenic type to the host. The host produces more antibodies which are specific to this new antigen and the sequence of events is repeated. This presentation of antigens by the parasite and the accompanying antibody production, characterised by a rise and fall in parasitaemia, leads to a situation where the trypanosome is always a step ahead of the host in its attempt to eradicate the parasites. This is repeated over and over again until the parasite finally overwhelms the host and kills it.

The variable antigen types (VATs) produced by a particular trypanosome stock have been shown to be a characteristic of the stocks. Although each trypanosome population is capable of producing a large number of different VATs, the types and order of these variants, the VAT repertoire, stabilise irrespective of the species of the host animal (Gray and Luckins, 1976; Cross, 1975, 1978). This leads to a tendency of a particular basic VAT being produced first, at infection, followed by a sequence of other types. At any given time during an infection, there is one predominant antigen type although there are other VATs which are simultaneously presented by the trypanosomes. The number of VATs that trypanosomes can produce would appear to be inexhaustible as estimated by the identification of over 1000 genes for different VSGs (Cross, 1990; Barry and Turner, 1991). The maximum number documented, however, is about 100 in *T. equiperdum* (Capbern *et al*, 1977). It has been observed that there is a difference in antigenic variation between trypanosomes in the bloodstream, tissue and fly with tissue VATs differing from bloodstream ones (Seed, 1964; Le Ray, 1969; Seed and Effron, 1973). When the trypanosomes are ingested by a fly, they loose their surface coat only to regain it in the final stages of development in preparation for infecting a host (Vickerman, 1968).

As with other trypanosomes, *T. evansi* exhibits antigenic variation. Although this species does not have a basic antigen type due to the absence of an insect developmental stage, the development of its VAT repertoire is similar to that of *T. brucei* (Jones and McKinnell, 1984). Studies involving the VAT repertoire of this species, identified fewer VATs than in other species with two predominant VATs present in all 15 stocks isolated from the Sudan which were examined (Jones and

McKinnell, 1985a, b). The limited number of VATs in this species is probably due to a lack of exchange of genetic exchange during cyclical transmission (Tait, 1980). Other than the use of evading the immune mechanisms of the host, antigenic variation is the major barrier to the development of a vaccine for the control of trypanosomiasis. The fact that *T. evansi* has fewer VATs suggests that the development of a vaccine to prevent infections by this species is more likely than with other trypanosome species.

Although the VAT repertoire can be used to characterise specific trypanosome stocks, its use as a method of species characterisation has not been developed.

2.2.9 TRYPANOSOMIASIS CONTROL

Methods used in the control of trypanosomiasis can be divided into three according to their target; methods involving the host as well as methods directed against the vector and the parasite. Control of trypanosomiasis has been a concern since this disease was first recognised at the beginning of this century. Control methods of transmissible diseases are aimed at breaking transmission cycles in the belief that this will result in the restriction of parasite movements between hosts.

2.2.9.1 Control methods directed against the vector

The earliest control method for trypanosomiasis was aimed at both the vector and the host. This was the avoidance, by animal movements, of areas known to be of high trypanosomiasis risk; a method still practised by pastoralists. It is based on a knowledge of vector habitats as well as the seasonal variations in vector prevalence. In Kenya, pastoralists move their animals to lowland grassland areas during wet seasons in order to avoid tsetse habitats in the high ground which have dense vegetation. During dry seasons, the animals are moved to high ground areas which are then devoid of undergrowth and thus have few tsetse flies. For this method to be effective, there must be enough grazing land available with different ecological zones, to allow the livestock owner such movements. Communally owned land in pastoral communities or large ranches are especially suited to this kind of control.

Another method of vector control is the destruction of tsetse habitat. The most drastic form of this control method involves total clearance of vegetation in areas tsetse flies are known to inhabit. This kind of total bush clearing aims at replacing all trees and shrubs with grassland, resulting in the disappearance of tsetse. A less radical type of bush clearing involves the destruction of only certain components of woody vegetation known to harbour tsetse flies. In this method, only the understorey of the forest is removed, leaving tall trees untouched. This method of selective bush clearing

has at times been unsuccessful for tsetse flies have been known to adapt to habitats other than their traditional ones. An example of this is the adaptation of *Glossina fuscipes* to peri-domestic bushes of *Lantana camera* in Kenya as their traditional habitats were destroyed (Jordan, 1986). These methods are presently not acceptable because they degrade the environment and interfere with ecological balances necessary for the maintenance of fauna and flora. Although these methods of tsetse control were practiced in campaigns in many parts of Africa, the establishment of human settlements in virgin territories accomplishes the same results. For the control of *T. evansi,* these methods are not appropriate for invertebrate vectors of this species have varying habits and habitats.

Another method of tsetse control is by the use of mechanical devices which act as baits. The main device that has been used for tsetse control is the trap. Although there are many traps of varying shapes and sizes which have been developed, the basic trap design involves the construction of a device which flies enter easily but cannot leave. Traps are made of a metal or wooden frame with a cloth whose colour combination are white, blue or black; colours which have been shown to attract tsetse. Traps are used as monitoring devices to determine the presence or density of flies in an area. Another mechanical device for tsetse control is the target which consists of a piece of black cloth, flanked by netting which is mounted on a metal frame (Vale *et al,* 1988). The role of traps and targets in the control of tsetse is however minimal unless used in conjuction with insecticide as will be described later.

The use of traps and targets for Surra control have been more difficult to apply than in the tsetse-transmitted trypanosomiasis. This is because the vector involved in other trypanosome species is of the same genus *Glossina* as opposed to different genera and even families in the case of *T. evansi*. Although tabanids and other flies which may transmit *T. evansi* may enter traps, the use of these devices as a control measure for Surra have not been evaluated. The use of traps and targets in the control of vectors of *T. evansi* is thus not as well developed as in the control of tsetse as the visual and olfactory preferences of the different vectors have not been investigated and determined.

Of all methods of vector control, chemical control has been most widely used employing insecticides which are usually pyrethroids or organochlorines. The first insecticide used for the control of tsetse-flies was dichlorodiphenyltrichloroethane (DDT). Other organochlorines that have been used are dieldrin and endosulfan. Although natural pyrethroids have been shown to have activity against tsetse, it is the

synthetic pyrethroids, deltamethrin, cypermethrin and permethrin, which have been widely used for tsetse control (Jordan, 1986).

The methods of insecticide application vary. Initially, ground spraying of vegetation either indiscriminately or in suspected tsetse habitats was routinely used. Spraying was done using knapsack, motorised or aerial sprayers. Logistic problems associated with ground spraying mean that this method is labour-intensive and also involves the destruction of vegetation to provide access to the sprayers. Aerial spraying from aircrafts results in indiscriminate application of insecticide in an area. Other problems associated with aerial spraying are the high costs of ground operations of the aircraft as well as the tendency of some insecticides to remain on the forest canopy without penetrating it to reach underlaying tsetse habitats. Both ground and aerial spraying of insecticides have the undesirable effect of killing not just tsetse, but also non-target insects, mammals, birds, fish and reptiles. Some of the insecticides used have been shown to leave residues in the tissues of birds and fish as long as one year after spraying (Jordan, 1986); a side-effect which is not acceptable due to the involvement of these animals in the food chain.

Different chemical methods have been used to control some insects which act as vectors of *T. evansi* (Soulsby, 1968). The removal of moist bedding material in animal houses and clearing the yard around it of food wastes, weeds, grass and vegetable refuse has been used to control *Stomoxys*. The application of kerosene over water in breeding areas as well as regular insecticide spraying in animal houses has been used to control *Tabanus, Stomoxys, Lyperosia* and *Haematobia*. Regular insecticide spraying however, poses a danger by risking the development of resistance to the insecticide applied as has been reported in *Lyperosia* (Soulsby, 1968). These methods are applicable where animals are housed but are not useful in the control of cameline *T. evansi* in pastoral systems.

Recent methods of chemical control involve the baiting of tsetse flies by the use of odours that are known to be attractive. Known attractants include octanols, phenols, acetone and carbon dioxide, which were identified as a result of observations that ox breath and cow urine are attractive to tsetse. The use of such attractants near traps and targets have been shown to increase the number of tsetse entering them (Laveissiere *et al*, 1989; Vale *et al*, 1988). For increasing the killing efficiency of traps and targets, insecticides are usually impregnated onto the cloth so that tsetse landing on the cloth receive a lethal dose of insecticide. This method results in an increase of the efficiency of traps as control devices (Vale and Hargrove, 1979). Insecticide impregnation on targets together with the use of attractants has been used to control or

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eradicate tsetse populations in Zimbabwe (Vale *et al,* 1986) and Kenya (Opiyo *et al,* 1987).

The application of insecticides on animals as a means of vector control in trypanosomiasis has received attention recently. A photostable pyrethroid is applied by dipping the animals or as a pour-on applied on the dorsal midline of the animal. The use of this method not only as a control for tsetse and other flies like tabanids which may act as vectors of trypanosomiasis, but also for ticks, has met reasonable success although it is still being evaluated (Munga, 1992; personal observation).

Biological methods of trypanosomiasis control have been used to reduce tsetse fly populations. These methods take advantage of the fact that tsetse flies have a low reproduction rate and females mate only once in their life times. The sterile insect technique (SIT) is such a method which has been successfully used in controlling tsetse flies in isolated foci (Kipling, 1982). In this technique, laboratory bred male flies are sterilised before their release. These sterile males compete with normal wild males for mating with wild females. Mating with sterile males results in a lack of offspring and thus a loss of one generation. The success of this method depends on the use of another method of control which first reduces the tsetse density to such a low level that the sterile males out-number wild ones. Problems associated with this technique are mainly associated with laboratory production of tsetse *en masse.* This method has been used to control tsetse flies in Burkina Faso, Tanzania and Zimbabwe (Jordan, 1986). So far, a similar method of biological control has not been developed for different vectors of *T. evansi.*

2.2.9.2 Control methods involving the host

As earlier indicated (2.2.7.1) the earliest method of trypanosomiasis control involved the movement of animals to avoid areas known to harbour flies. This method is still used by camels owners who avoid areas of thick vegetation which may harbour tsetse flies. As a method of avoiding vectors of *T. evansi*, however, animal movements are not effective because of the many different habitats of these vectors. The destruction of animals known to act as reservoirs of trypanosomiasis is another method that has been used for vector control. This involves the killing of wild animals inhabiting trypanosomiasis foci closely associated with human settlements. The destruction of these animals is meant to disrupt the transmission cycle by destroying sources of infection which act as reservoirs of domestic animal and human infections. This method may not be important for the control of *T. evansi* since transmission is believed to be from one domestic animal to the other with a limited role of wild

animals as reservoirs. It may however be applicable in South America where capybaras have been implicated as reservoirs of *T. evansi* by being found to be naturally infected without showing any clinical signs (Morales *et al*, 1976; Reveron *et al*, 1992). However, as with bush clearing, this method of vector control is no longer acceptable due to disruption of ecological balances.

Another control method that involves the host is trypanotolerance. This method is based on the observation that some animals are able to control trypanosomiasis infection better than others. Such animals have been identified as the N'Dama, Muturu and other shorthorn cattle in West Africa (Stewart, 1951; Chandler, 1952; Murray *et al,* 1982), the Orma Boran and Maasai Zebu in East Africa (Njogu *et al,* 1985; Mwangi *et al,* 1991) as well as some indigenous sheep and goats in both East and West Africa (Griffin and Allonby, 1979). Although there are indications that some camels are not as susceptible to trypanosome infections as others (personal observations), trypanotolerance in camels has not yet been properly defined. Trypanosomiasis control methods in the area of trypanotolerance are aimed at increasing the production of these animals with a view of introducing them into other trypanosomiasis infested areas as well as research into the mechanism underlying it. Of the three approaches of trypanosomiasis control, this is most hopeful since it involves fewer activities which are environmentally unacceptable.

2.3 CHEMOTHERAPY

Chemotherapy is a term that covers the use of chemical substances to treat disease as well as the ability of these compounds to destroy infective organisms without harming the animal host (Hawking, 1963a). The potential for using chemical compounds to control protozoan infections was realised first by Ehrlich and his group, who when studying the staining properties of dyes noticed that one of them, methylene blue, was active against human malaria (Ehrlich, 1907). Further work showed that another dye, trypan red, could cure infections with *T. equinum* in mice. This led to work on arsenious oxide and various other arsenic compounds which were found to destroy trypanosomes but were unfortunately, also very toxic to the animal hosts (Thomas, 1905). Another arsenic preparation, atoxyl, proved to be less toxic. Atoxyl consists of a benzene ring with arsenic as well as other groups attached to it (Figure 2.10). The addition of different side chains, including amino or hydroxyl groups, to this benzene ring-arsenic structure led to the development of compounds which had varying levels of trypanocidal activity and toxicity to the host. A need to balance

curative activity and toxicity of these compounds, was of utmost concern to these workers and led to the concept of high chemotherapeutic index: a high ratio of curative activity to toxicity (Hawking, 1963a). In an attempt to explain toxicity, Ehrlich suggested that the curative effect and toxicity of a chemotherapeutic agent depended on its distribution in the body. He referred to toxic drugs as being organotropic if they bound to the organs of the host. Parasitotropic drugs, however, bound to the parasite resulting in their curative action. For a drug to be effective, Ehrlich and other workers thus looked for compounds which would selectively destroy pathogens without harming host mammalian cells.

An ideal drug is one that is able to inhibit growth of a pathogen without having any harmful effect on the host. This selective toxicity can be achieved by several methods. A drug that is able to permeate the pathogen in concentrations that would affect it and yet not permeate host cells to similar levels would have selective toxicity. Such selective permeability could be accounted for by differences in the cell membranes of the pathogen and the host. If, on the other hand, the drug is able to permeate both the pathogen and the host cells but its target is unique to the pathogen, then this would achieve the desired discrimination. For example, a pathogen may have a specific enzyme that is not found in the host cells. It is also possible to have a drug that is in a non-toxic form when administered and needs to be activated for it to be useful. If this activation can occur only in the pathogen and not in the host cell, then although the drug may permeate both, its action would only be in the pathogen. This selective toxicity can be achieved by using a drug that needs to be metabolised to an active form using a pathway unique to the pathogen. Another possibility for achieving selective toxicity is in instances where the target of the drug, though found in both pathogen and host, is of greater importance to the pathogen than to the host. This is possible where the drug targets a pathway which is necessary for the survival of the pathogen but not the host. Alternatively, the host, unlike the pathogen, might have more than one pathway and thus be able to use an alternative one when the other is targeted by the drug. A drug may achieve discriminative toxicity by several of these methods at the same time.

Other qualifications of an ideal drug are related to the method by which it is administered. In developing a drug, early consideration must be given to the host animal species in which the drug will be used particularly in relation to the presentation as well as the route and ease of drug administration. Thus, where the host is a stubborn camel owned by a nomad in the remote arid north of Kenya, a drug presented in a form requiring no refrigeration, given as a single parenteral dose would be

preferred because repeated administration of the drug is difficult or impossible. Another consideration in applying chemotherapy is the use of the animal. Animals used for milk or meat production would require a drug that does not persist in the tissues or leave residues that would be undesirable for human consumption. The price of a drug must be within the purchasing power of the animal owners for it to be of any use to his animals. Similarly, the price of the drug must be in a range where the owners value their animals enough to justify the spending. In this respect, the cultural value of camels to the traditional camel keepers in Kenya would justify the purchase of any drug but, ultimately, purchasing power would determine whether or not it was used. Although these considerations are important, the chemical composition of the drug, its target and development dictate many of these conditions.

There are several stages in the development of a chemotherapeutic compound. A biological study of the organism concerned is important in order to understand its life cycle, its metabolism and its interactions with the host. Where the organism is not infective to a host that is suitable for experimental purposes, a related organism might then be used in anticipation that the results obtained could be applicable to the pathogenic organism. The final phase of drug development involves making and testing a therapeutic compound believed to destroy the organism. This includes the screening of existing compounds for their action and on finding one, its large scale production to enable the carrying out of clinical trials. At first, clinical trials are carried out in laboratory animals which are used as models for the disease in the host animal. Further trials then involve the host animal. It is only when these stages of development have been completed that the mode of action of the drug is investigated. In some of the earliest cases, the anti-microbial activity of a compound was discovered by chance. Later, related compounds were tested for action against specific parasites. This was the situation in Ehrlich's laboratory where dyes, which were already available, were tested for anti-microbial action. The trypanocides in use today were developed using this traditional method and thus their modes of action are only now being investigated and are only partially understood. Another method of drug development is the testing of drugs which have already been targeted for a particular organism or condition, to a range of other organisms. This was the case of difluoromethylornithine (DFMO) which was developed for cancer therapy and has lately been tested for treatment of human trypanosomiasis (Bacchi, 1993).

A better method of drug development is by a rational approach which involves studying the parasite and the host for differences and then developing compounds that would selectively target the parasite. This method of drug development is both

expensive and time consuming and it has been estimated to take ten years and £80 million to develop a drug in such a manner (Hyde, 1990). To develop such a drug, the manufacturers must be convinced that its market would justify production in a manner that developmental costs can be recovered and profits made.

2.3.1 MECHANISMS OF DRUG ACTION

It is important to ensure that drug action lies in areas that are necessary for the survival of pathogens. These vital areas are mostly energy metabolism and are a key area targeted by many trypanocides as well as other anti-microbial drugs. This is due to the absolute necessity for all organisms to produce energy in the form of adenosine triphosphate (ATP) which is needed for maintaining their integrity as well as for locomotion, growth and reproduction. In the mammalian host, this form of usable energy is produced by hydrolysis of macromolecules which have been ingested as food. This hydrolysis is either extracellular in the stomach and intestines or intracellular in the lysosomes and produces glucose as the end product. By either aerobic or anaerobic means, glucose is then catabolised in the cytoplasm and the mitochondria of cells to produce ATP. Different enzymes are needed within various pathways to catalyse reactions involved in producing this final product. This process of catabolising glucose to energy in mammals is illustrated in Figure 2.11. Nicotinamide adenine dinucleotide (NAD) is reduced to NADH both in the glycolytic and pyruvate oxidation pathways. This reduced form, NADH, is later reoxidised to NAD and recycled to further participate in catabolism. In trypanosomes, ATP is produced in a similar manner except for differences in the enzymes involved. A major difference is in the reoxidation of the NADH to its original form, NAD, which was first described by Grant and Sargent (1960). In trypanosomes, this is catalysed by L-α-glycerophosphate dehydrogenase in the cytoplasm and L-α-glycerophosphate oxidase in the mitochondria. The first enzyme is also found in mammalian cells but the latter is not found in mammals and is unique to salivarian trypanosomes. Suramin inhibits L-α-glycerophosphate oxidase and thus its activity selectively discriminates against pathogenic trypanosomes (Fairlamb and Bowman, 1977, 1980).

In some enzyme reactions, oxygen is reduced to various reactive species such as the superoxide anion, singlet oxygen, hydrogen peroxide and hydroxyl radicals which can cause oxidative stress and result in irreversible damage to cells (Docampo and Moreno, 1984). In mammals, these oxidants are changed to non-toxic substances by a tripeptide glutathione γ-glutamyl-cysteinylglycine (GSH) and its ancillary enzymes glutathione reductase, glutathione peroxidase and glutathione-S-transferases. These

enzymes are also involved in the pathway for the synthesis of polyamines, which are small molecules important for differentiation and division of cells as well as maintenance of their function. Other functions of polyamines are conformational regulation of DNA, initiation and elongation of protein synthesis, translational fidelity, as well as structure and function of ribosomes and tRNA (Pegg, 1986; Marton and Morris, 1987). The most important polyamines in mammalian cells are putrescine, spermidine and spermine, which are synthesised from ornithine by ornithine decarboxylase and S-adenosyl-methionine decarboxylase (SAM DC) and several other enzymes found in the polyamine pathway. In trypanosomes, the polyamine pathway is similar to those of mammalian cells but there are important differences. Unlike mammalian cells, trypanosomes do not synthesis spermine nor do they transport polyamines between cells (Gutteridge and Coombs 1977; Bacchi *et al,* 1983). In polyamine metabolism, glutathione reductase is absent and instead trypanosomes have trypanothione spermidine (SPD) which maintains the reduction-oxidation balance (Fairlamb and Cerami, 1985). The role of glutathione reductase and peroxidase in removing hydrogen peroxide and other alkylperoxidases is taken over by trypanothione spermidine and trypanothione peroxidase (Penketh and Klein, 1986; Henderson *et al,* 1987). Another difference is in the synthesis of trypanothione from amino acids ornithine, methionine, glutamate, cysteine and glycine. The initial stages of trypanothione synthesis, from ornithine to GSH and spermidine, is similar to mammalian cells (Figure 2.11). Beyond this, the formation of trypanothione via glutathionylspermidine is unique to trypanosomatids (Fairlamb *et al,* 1986). Two classes of drugs, the fluorinated analogues of ornithine, and diamidines, target this pathway of trypanothione synthesis by inhibiting two rate-limiting enzymes. DL-α-difluoromethylornithine (DFMO) inhibits ornithine decarboxylase, the first enzyme in the biosynthesis of spermidine from ornithine while Berenil and pentamidine inhibit S-adenosyl-methionine decarboxylase (Bacchi and McCann, 1987; Fairlamb, 1989) as illustrated in Figure 2.11. Another group of drugs, the arsenicals, such as melarsoprol and cymelarsan, have trypanothione as their target and interact with this polyamine to form a stable inactive complex leading to cell lysis (Fairlamb, *et al,* 1989). These drugs are therefore selective because they have targets unique to the trypanosomes.

Another target for drugs is nucleic acid synthesis. Nucleic acids are polymers of nucleotides linked to a nitrogen base and a phosphate group. There are purine nucleotides; adenosine and guanosine, and pyrimidine nucleotides; cytidine, thymidine and uridine. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are the two nucleic acid classes which differ from each other by the consistuent pyrimidines

(thymidine in DNA and uracil in RNA) and the sugar (ribose in RNA and deoxyribose in DNA). The synthesis of nucleic acids is at two levels; first the acquisition of purine and pyrimidine bases by either *de novo* synthesis or a salvage mechanism, second, polymerisation of the nucleotides to nucleic acids. In both mammals and salivarian trypanosomes *de novo* synthesis of pyrimidines is from glutamine, bicarbonate and aspartate to make uridine monophosphate (UMP) which can then be converted to other pyrimidines. Another source of pyrimidines is from a salvage mechanism whereby bases and nucleosides (nucleotides lacking the phosphate group) are obtained from broken down pyrimidines found in blood. The *de novo* synthesis of purines from glycine, formate, CO_2, glutamine and aspartate found in mammalian cells is however absent in all African trypanosomes making them dependent on their hosts for their purine needs. Polymerisation of nucleic acids, which involves a nucleotide primer where polymerisation starts, is catalysed by DNA and RNA polymerases.

Potential drug targets of nucleic acid synthesis can be at three levels; at the purine/pyrimidine synthesis, at the nucleotide salvage mechanism or at the polymerisation stage. Polymerisation is targeted by phenanthridines such as ethidium bromide which intercalate with DNA inhibiting the function of DNA and RNA polymerases. Aromatic diamidines like Berenil and pentamidine also bind to DNA and interfere with nucleic acid synthesis (Gutteridge and Coombs, 1977). The action of these trypanocides is directed to DNA irrespective of its type (whether nuclear or kinetoplast) or origin (trypanosomal or mammalian host). Their trypanocidal action is due to selective membrane permeability whereby drugs penetrate trypanosomes more readily than host cells.

Another class of trypanocidal drugs targets protein synthesis. Protein synthesis starts with DNA in the nucleus which serves as a template. Messenger RNA (mRNA) is transcribed from nuclear DNA and passes to ribosomes on the endoplasmic reticulum found in the cytoplasm. Here, the DNA code consisting of three nucleotides for each amino acid is recognised and the appropriate amino acids, transported by transfer RNA (tRNA), aligned in the proper order. Amino acids are then polymerised by formation of peptide bonds by the process of translation. Quinapyramine targets this process by displacing magnesium ions and polyamines, which are among the many necessary requirements of this process, from ribosomes. This results in aggregation of ribosomes leading to their inactivation and subsequent blocking of protein synthesis (Gutteridge and Coombs, 1977). Selective toxicity of this trypanocide is by selective permeability.

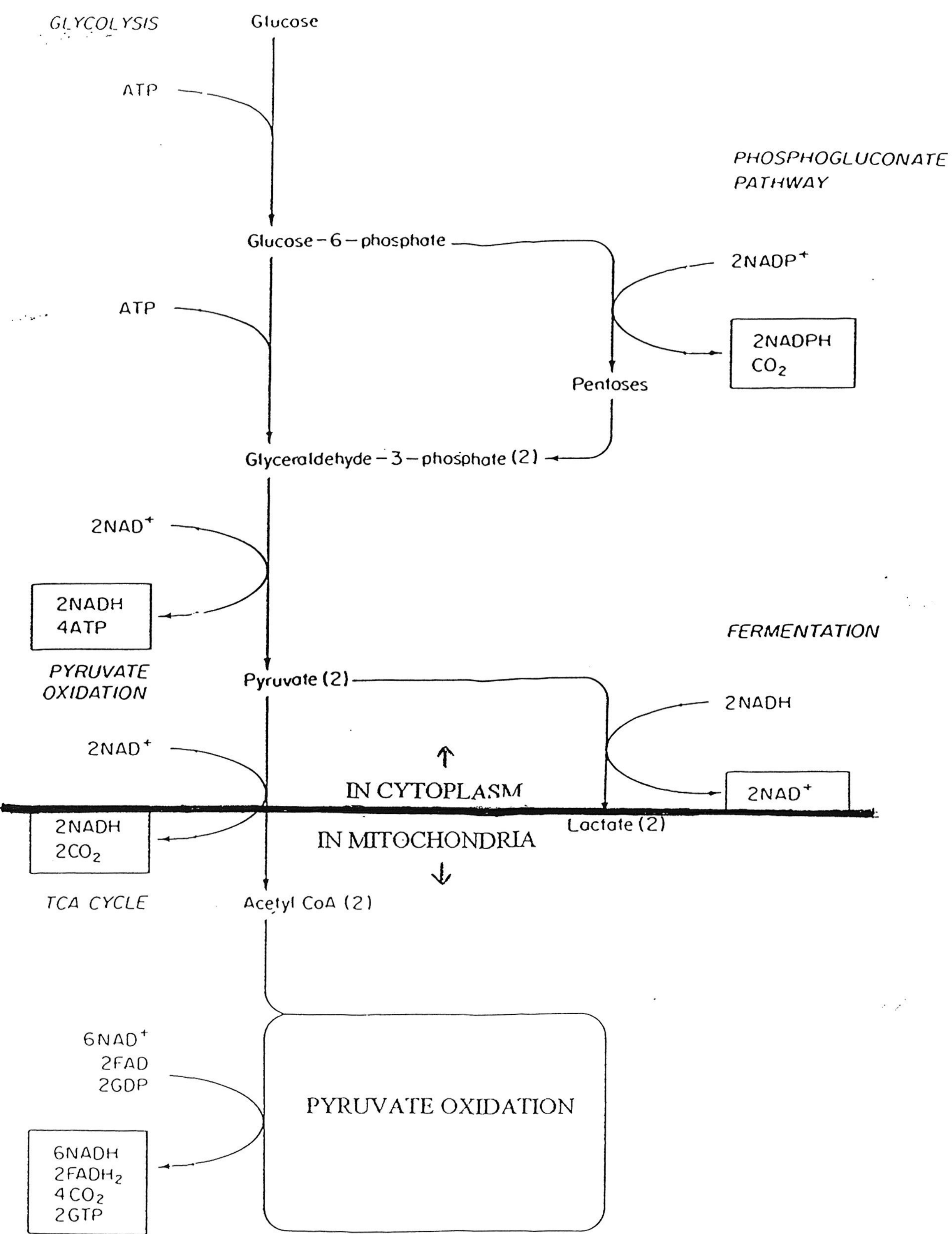
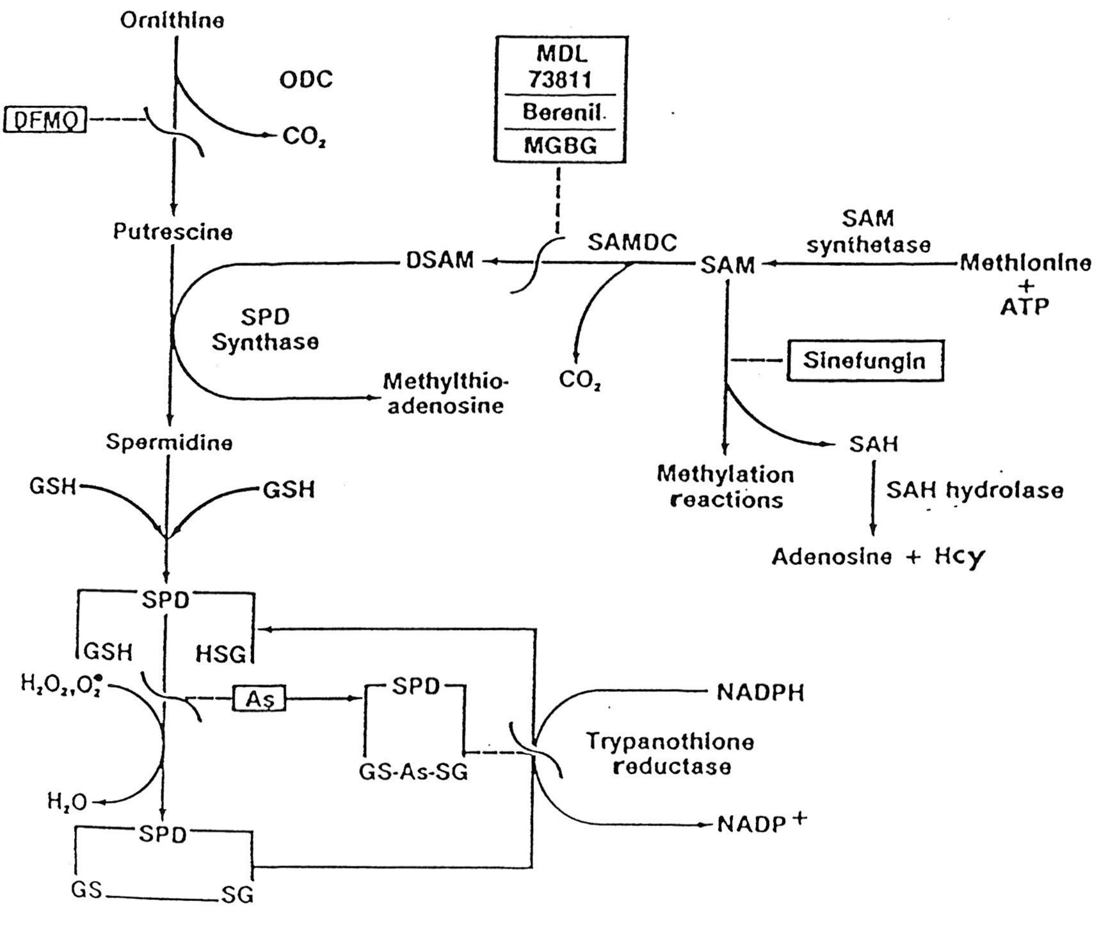


Figure 2.11 Glucose catabolism in mammalian cells (Gutteridge and Coombs, 1977)



Legend: DFMO- α-difluoromethylornithine
SAM - S-adenosylmethionine
SAM DC - SAM decarboxylase
SPD - Spermidine
GSH - Reduced glutathione
As - Arsenical drugs
MGBG - Methylglyoxal *bis*
ODC - Ornithithine decarboxylase
GSH-SPD-GSH - Trypanothione
----- Inhibition by a chemotherapeutic agent

Figure 2.12 Polyamine biosynthesis and related reaction in African trypanosomes (Bacchi, 1993).

2.3.2 TRYPANOCIDAL DRUGS

Since Thomas (1905) first demonstrated that atoxyl could cure rats infected with trypanosomes, the search for drugs that would be less toxic but still maintain chemotherapeutic activity has continued. Compounds that are now used for treatment of trypanosome infection vary in their chemical composition, their activity against different trypanosome species and their effect on different animal hosts.

2.3.2.1 Drugs interfering with energy metabolism

I. Arsenicals

The mode of action of this group of drugs reviewed by Gutteridge and Coombs, (1977) is believed to be due to their association with proteins containing sulphydryl groups. Many enzymes fall into this group of proteins and are targets for these compounds which can produce a marked inhibitory effect on their activity. Likely target enzymes for these compounds include glycolytic kinases, especially pyruvate kinase. As trypanocidal drugs, arsenical compounds are especially important due to the fact that bloodstream forms depend exclusively on glycolysis as a source of energy (section 2.2.1). Recent work has indicated that trypanothione is a prime target for arsenicals and interact with it leading to trypanosome lysis (Fairlamb, *et al,* 1989). After intravenous administration of arsenicals, drug concentration in blood reaches a maximum within 24 hours and then decreases due to excretion so that no drug is detectable after 4 days (Hawking, 1963a). These drugs also cross the blood-brain barrier which makes them important in the treatment of infections caused by *Trypanozoon* parasites such as *T. evansi* which invade the CNS.

The selective toxicity of this group of compounds is due to their greater permeability in trypanosomes, the greater dependence of these parasites on glycolysis as a means of energy production, the greater sensitivity of trypanosomal pyruvate kinase to inhibition as well as their having a unique target, trypanothione spermidine, which is found in trypanosomes but not in the host cells (Gutteridge and Coombs, 1977; Fairlamb, *et al,* 1986).

A. Tryparsamide

After atoxyl, tryparsamide was the next arsenical developed for the treatment of trypanosomiasis. This compound, Sodium N-phenylglycinamide-*p*-Arsonate, first described by Jacobs and Heidelberger (1919), was shown to be useful for the cure of *T. gambiense* infections in human beings and has been used for its control (Hawking,

1963a). Tryparsamide, whose chemical structure is shown in Figure 2.13, is a water-soluble colourless salt given to sleeping sickness patients as an intravenous solution in doses of 2-3 g per week for 10 to 12 doses. Tryparsamide is inactive *in vitro* for it needs to be reduced to a trivalent form by the host before its trypanocidal action is apparent. This need for activation explains why there is a delay of up to six hours between the administration of the drug and the disappearance of trypanosomes from blood (Hawking, 1963a). The disadvantages of this drug are the possibility of causing blindness and the long duration of the treatment course. For these reasons, this drug is not regularly used any more.

B. Melarsen

The melarsen group of compounds were developed by Friedheim starting from 1941. They consist of the basic benzene ring-arsenic structure, an additional 2,4-diamino group and other side chains. The basic compound produced by Friedheim (1941) was melarsen oxide whose structure is shown in Figure 2.14. This compound proved to be toxic and so a related compound, melarsoprol, 2-*p*-(4,6-diamino-1,3,5-triazin-2-ylamino) phenyl-4-hydroxymethyl-1,3,2-dithia-arsoline (Figure 2.15), was developed. Melarsoprol, marketed as Mel B or Arsobal, is not soluble in water but is soluble in propylene glycol. Other than their action on trypanothione, the action of melarsen compounds is also believed to be partially due to antagonism by a competition of their diamino structure to the amino hydroxyl group of folinic acid (Hitchings, 1952).

Melasoprol is active both *in vivo* and *in vitro* against *T. gambiense* and *T. rhodesiense.* In human sleeping sickness patients, melarsoprol is administered intravenously at a dose rate of 3.6 mg/kg body weight for three days and repeated once after some interval. This drug is the only one that can cure cases of *T. rhodesiense* which involve the CNS and is still the drug of choice in the treatment of late stage sleeping sickness. The disadvantage of this drug is its toxicity resulting in encephalopathy in about 1% of the patients (Hawking, 1963a).

Cymelarsan (Mel Cy) is a water-soluble melarsen compound recently developed by Rhone Merieux. This compound, melarsenoxyde cysteamine, which is the first melaminyl-substituted phenylarsonate designed for animal use, has a chemical structure of as shown in Figure 2.16. This arsenical is used against trypanosomes of the *brucei* group with special emphasis on *T. evansi* infections (Raynaud, *et al,* 1989). Studies in Ethiopia (Zelleke, *et al,* 1989), Mali (Tager-Kagan, *et al,* 1989) and Kenya (Otsyula, *et al,* 1992) indicate that Mel Cy is active against cameline *T. evansi*

infection at dose rates of 0.3 to 1.25 mg/kg body weight administered subcutaneously. Although the recommended dose for this drug is 0.25 mg/kg, both Zweygarth *et al,* (1992) and Lun *et al,* (1991) found that this dose was not effective in curing infections involving the CNS in goats and buffaloes.

II. Suramin

Suramin is a water-soluble trisodium sulphonated naphthylamine whose chemical structure is 8,8-[3",3"-ureylene bis (3""-benzamido-4""-methyl-benzamido)]--bis-1,3,5-naphthalenetrisulfonic acid (Figure 2.17). Development of this compound started from trypan red, trypan blue and Afridol violet. It took eight years for Bayer workers in Germany to develop and produce this compound in 1916 before it became available in 1920 for the treatment of human and cameline trypanosomiasis (Knowles, 1925; Williamson, 1978; Apted, 1980; Leach and Roberts, 1981). Suramin (Naganol^R, Bayer) has also been available under several trade names such as Germanin^R, Bayer 205^R, Antrypol^R, Moranyl^R, Fourneau 309^R, Belganyl^R, Napharide^R and Naganin^R.

Strongly anionic suramin was observed to persist in the bloodstream after administration due to its ability to bind with plasma proteins, especially serum albumin, with its naphthylamine-trisulfonic groups (Dangerfield *et al,* 1938; Spinks, 1948; Muller and Wollert, 1976). The ability of this drug to bind with plasma proteins may explain the inactivity observed in early *in vitro* systems, which did not include proteins in the medium, although it was active *in vivo* against *Trypanozoon* (Hawking, 1963a). After administration into an animal, excretion is very slow since the bound suramin persists in blood resulting in a prophylactic activity for a period of up to 3 months (Findlay, 1930).

The mode of action of suramin is still not fully understood after more than 70 years of production. It has been shown to inhibit the activity of a number of enzymes including hyaluronidase, fumarase, urease, hexokinase, thymidine kinase, DNA and RNA polymerases (Hawking, 1963a; Chello and Jaffe, 1972; Ono *et al,* 1988). The major activity of suramin is, however, believed to be related to glycolysis where it targets the pathway involving reoxidation of NADH to NAD. In trypanosomes, this reaction is catalysed in the mitochondria by L-α-glycerophosphate oxidase. Suramin inhibits this enzyme leading to a depletion of the cofactor NAD and thus a suspension of glucose catabolism. The selective toxicity of suramin is believed to be due to its action on unique targets such as, L-α-glycerophosphate oxidase, which are found in the trypanosome but not in the mammalian host.

There is a difference in the action of suramin *in vivo* and *in vitro*. Trypanosomes exposed to suramin *in vitro* show a reduced ability to infect animals inspite of the fact that they may not killed (Hawking, 1963a). *In vivo* however, trypanosomes are destroyed by suramin, although trypanocidal action is observed after a delay of about 24 hours. Roehl (1926) and Leach and Roberts (1981) suggested that the apparent inactivity of suramin *in vitro* is a need for *in vivo* activation due to the dependence of this compound to the defence mechanisms of the host for it to be fully effective. This effect can, however, now be explained from the fact that suramin needs to bind to proteins before it can be active. This activity has been demonstrated by more recent *in vitro* assays (Zinsstag *et al*, 1991; Zhang *et al*, 1992; Sutherland *et al*, 1993). The delayed action of suramin can be explained by its enzyme inhibitory activity. Thus, after its action on the target enzyme, the effect can only be detected when all NAD produced before suramin administration is depleted due to a lack of NADH reoxidation.

Although suramin is active against infections caused by *Trypanozoon* parasites, it is inactive against *T. vivax, T. simiae* and *T. congolense*. For human-infective trypanosomes, this drug is recommended for intravenous use in early stage sleeping sickness, before the CNS is involved. It is especially effective in the treatment of infections caused by *T. rhodesiense* (Hawking, 1963a). For animal trypanosomiasis, suramin is recommended for curative use at 10 to 12 mg/kg body weight and for prophylactic use at 5 mg/kg body weight administered intravenously as a 10% solution in camels and equids infected with *T. evansi, T. brucei* or *T. equiperdum*. It is believed that highly polar suramin does not cross the blood-brain barrier (Apted, 1980) although there was one report which indicated that it could enter the CNS when the barrier was damaged by infection (Raseroka and Ormerod, 1985, 1986). Attempts to reproduce this phenomenon of suramin crossing the blood-brain barrier were, however, unsuccessful (P. D. Sayer, personal communication). Suramin is relatively toxic with this effect being seen especially in the kidneys (Hawking, 1963a). Due to the slow excretion of the drug, toxicity is cumulative in respect to time.

2.3.2.2 Drugs interfering with nucleic acid synthesis

Drugs in this group exert their action either by interacting with DNA primers, thereby blocking DNA or RNA synthesis or by interfering with synthesis of the polymerase enzymes. When DNA or RNA is synthesised, a primer is recognised by the polymerase enzyme in order for polymerisation of the nucleotides to start. If this primer is blocked, polymerisation cannot be initiated since the polymerase does not have a binding site and, therefore, no DNA or RNA can be synthesised. If, on the other hand, the production of the polymerase enzyme is inhibited, again,

polymerisation is not initiated and ribonucleotide synthesis is not possible. Two groups of trypanocidal drugs interfere with nucleic synthesis.

I. Phenanthridines

This group of compounds was first discovered by Browning *et al*, (1938). Although the original compounds, phenidium and dimidium were shown to be active against trypanosomes, they had severe delayed toxicity in cattle. Further work on this group of compounds involved changes of the original compounds without interfering with the general structure (Figure 2.18) leading to the development of homidium and prothidium. These drugs have both *in vivo* and *in vitro* activity against *T. congolense* and *T. vivax*. Phenanthridines are active against *brucei* type parasites only during the early stages of infections, for once the CNS is involved, they are not able to cross the blood-brain barrier (Jennings *et al*, 1977). Phenanthridines are, however, not active against *T. evansi* infection (Gitatha, 1979; Rottcher and Heising, 1980). These drugs first cause loss of infectivity followed by trypanosome death after a delayed period. Their mode of action is by intercalation with the DNA helix thereby interfering with its function as a primer for nucleic acid synthesis.

A. Homidium

Homidium (REthidium, Novidium), was developed by the substitution of the methyl group with an ethyl one in the general phenanthridine structure which reduced its toxicity (Watkins and Woolfe, 1952). Homidium, 2:7 Diamino-9-phenyl-10-ethyl phenanthridinium bromide, has the structure shown in Figure 2.19. The use of this drug is recommended at a dose rate of 1 mg/kg body weight intramuscularly for the cure of infections caused by *T. congolense* and *T. vivax*. Homidium also has some activity against *T. gambiense* although its effect was not shown to be sufficient to warrant its use in sleeping sickness (Hawking, 1963a). Once administered, this drug is rapidly excreted from the body. Some toxic effects in cattle are characterised by local irritation and tissue necrosis.

B. Prothidium

Prothidium was developed from the basic phenanthridine by the addition of a pyrimidine moiety (Watkins and Woolfe, 1956). The chemical name of this drug is 2-amino-7-(2-amino-6-methyl-pyrimidin-4-ylamino)-9-*p*-amino-phenyl phenanthridinium 10,1-dimethobromide with a chemical structure as shown in Figure 2.20. Unlike homidium, this compound is not quickly eliminated from the body for it binds strongly

to proteins, thus resulting in a prophylactic activity (Taylor, 1960). It is recommended for use as a single intramuscular or subcutaneous dose of 2-4 mg/kg body weight which provides prophylactic activity for 6-8 months (Hawking, 1963a). Like homidium, local irritation at the site of injection have been observed as well as delayed reactions resulting in deaths several months after treatment.

II. Diamidines

Since trypanosomes require blood sugar for their metabolism, it was believed that compounds which reduced blood sugar would have some trypanocidal effect. This theory led to the testing of synthalin, a compound that lowers blood sugar. Work on synthalin and allied compounds resulted in the identification of compounds observed to have *in vivo* trypanocidal activity (Von Jancso and Von Jancso, 1935a). Further work led to the production, by the May and Baker group, of many diamidines some of which have been shown to be useful in the treatment of human and animal trypanosomiasis. All diamidine compounds used in trypanosomiasis consist of a basic aromatic structure (Figure 2.21) and are water-soluble. The trypanocidal activity of these compounds is limited to infections that do not involve the CNS for they do not cross the blood-brain barrier. Their mode of action relates to their ability to bind to DNA. Since they bind to DNA irrespective of species, their selective toxicity against trypanosomes is believed to be as a result of selective permeability (Gutteridge and Coombs, 1977).

A. Pentamidine

This drug has a chemical structure as shown in Figure 2.22 and its chemical name is 4,4-Diamidino-1,5-diphenoxypentane. It is used for the control of early *T. gambiense* infections in man and is given as an intramuscular dose of 3-5 mg/kg body weight. Pentamidine is excreted slowly, resulting in a prophylactic activity of up to 6 months when administered as an intramuscular injection. The action of this drug is by interference with nucleic acid synthesis as well as inhibition of S-adenosyl-methionine decarboxylase during polyamine synthesis (Gutteridge and Coombs, 1977; Fairlamb, 1989).

B. Diminazene aceturate

The chemical name of this compound is $4,4^{1}$-Diamidino-diazo amino benzol diaceturate with a structure as shown in Figure 2.23. The drug, marketed as Berenil

(R Hoechst), was shown to have activity against *T. congolense T. evansi, T. vivax* and to a lesser extent *T. brucei* (Hawking, 1963a; Williamson, 1970). It is recommended for use at a dose rate of 3.5 to 7 mg/kg body weight administered through the intramuscular route. Berenil has little prophylactic activity for it is either excreted quickly or metabolised within 24 hours of administration (Hawking, 1963a). It is commonly used to control trypanosomiasis in cattle, sheep and goats. Its use however is contraindicated in the dromedary camel because it is highly toxic and believed to be ineffective (Leach, 1961). Its mode of action is similar to that of pentamidine.

C. Isometamidium chloride

Isometamidium chloride (RSamorin) is a drug that was synthesised by combining the aminobenzamidine moiety of diminazene with homidium in the presence of sodium acetate to produce metamidium (Wragg *et al,* 1958). The resulting compound consisted of two isomers; red and purple. Although both isomers showed some trypanocidal activity, it was the more active red one developed by Berg (1960) which is currently available as Samorin. The chemical name of this red isomer is 7-(m-amidinophenyldiamino)-2-amino-10-ethyl-9-phenylphenanthridinium and it has the structure shown in Figure 2.24. Isometamidium is indicated for *T. congolense* and *T. vivax* infections at a dose rate of 1-2 mg/kg body weight administered intramuscularly and is prophylactic for a period of 2 to 3 months. Although this drug has been shown to have activity against *T. evansi* at 1.0 mg/kg body weight administered intramuscularly (Petrovskii, 1974), its use in camels has been reported to result to toxic reactions (Schillinger and Rottcher 1984; Schillinger *et al,* 1985b; Ali and Hassan, 1986). When administered subcutaneously or intramuscularly, pain and lameness are observed while intravenous administration results in pronounced shock or other fatal systemic symptoms. As with the two groups of compounds from which it was developed, Samorin does not cross the blood-brain barrier. It is effective against trypanosomes of the *brucei* group only during the early stages of infection, for once the CNS is involved, trypanosomes may only be removed from blood for a short period before relapses occur (Jennings *et al,* 1977; Ali and Hassan, 1986). Its mode of action is through inhibition of nucleic acid synthesis in a similar manner to that of phenanthridines and diamidines.

2.3.2.3 Drugs interfering with protein synthesis

These drugs belong to the quinaldine and quinoline group of compounds and work by interfering with the translational process thus preventing the synthesis of proteins. Only one trypanocide, quinapyramine, has this mode of action.

A. Quinapyramine

This compound was synthesised by Barrett *et al*, (1953) from the original quinoline, surfen C, which had been shown to have trypanocidal activity. Quinapyramine was then developed by Imperial Chemical Industries (I.C.I.) and introduced for cattle trypanosomiasis in the early 1950s (Curd and Davey, 1949). The chemical structure of quinapyramine, 4-amino-6-(2-amino-6-methylpyrimidyl-4-amino) -quinaldine-1,1-dimetho (methyl sulphate or chloride) is shown in Figure 2.25. It is available as a sulphate used for therapy or as a sulphate-chloride combination used for prophlylaxis. Quinapyramine is active against *T. congolense, T. simiae, T. vivax* as well as trypanosomes of the *brucei* group (Williamson, 1970). Against this latter group of trypanosomes, quinapyramine is only effective during the early stages of infection because it does not cross the blood-brain barrier. When first produced by I.C.I., quinapyramine sulphate (^{R}Antrycide) was recommended for use at a dose rate of 4.4 mg/kg body weight subcutaneously. The initial prophylactic compound consisting of a 3:4 sulphate-chloride ratio was recommended for use at a dose rate of 11 mg/kg body weight subcutaneously. A revised formula was developed which consisted of only half the original chloride and was recommended for use at 7.4 mg/kg body weight. The sulphate is soluble in water up to 33% while the chloride is only slightly soluble. I.C.I. withdrew quinapyramine from the market in 1974 due to widespread resistance developed by trypanosomes. In 1984, quinapyramine was however re-introduced by May and Baker (now Rhone Poulenc) as Trypacide^{R}. The prophylactic compound, Trypacide Prosalt, has a 3:2 sulphate-chloride ratio. Both the sulphate and the Prosalt are recommended for subcutaneous administration at 5 mg/kg. Since 1984, Rhone Poulenc has placed special emphasis on the use of quinapyramine in the control of *T. evansi* infections of camels. After subcutaneous administration, quinapyramine is quickly removed from the bloodstream and may be localised in the liver and kidneys (Spinks, 1958). The prophylactic action of the Prosalt is due to the low solubility of the chloride leading to a depot from where the compound is slowly released. Toxic systemic reactions are sometimes observed after administration of quinapyramine in cattle and to a larger degree in camels and equidae. These are

characterised by excessive salivation, sweating, tremors and even collapse and are attributed to the sulphate (Hawking, 1963a). Quinapyramine displaces magnesium ions and polyamines, which are among the many necessary requirements of translation, resulting in the aggregation and inactivation of ribosomes and subsequent blocking of protein synthesis (Gutteridge and Coombs, 1977).

2.3.2.4 Dl-α-difluoromethylornithine (DFMO)

Although DFMO is not presently used for chemotherapy, it will be included in this review because of its potential use as a drug whose mode of action is different from current trypanocidal drugs. Since its chemical structure (Figure 2.26) is unlike that of any of the other trypanocidal drugs, it may have an important role in the management of infections which are resistant to other drugs. The drug inhibits the activity of ornithine decarboxylase, the first enzyme in the biosynthesis of spermidine from ornithine as shown in Figure 2.12 (Bacchi and McCann, 1987). This is a unique target mechanism since the enzyme it inhibits is not found in mammals.

DFMO was first developed as an anti-tumour drug by Merrell Dow Research Institute in the 1970s. This drug has now been shown to be effective in treating late-stage infections of *T. gambiense* in human beings although it is not as effective against *T. rhodesiense* (Bacchi, 1993). The recommended administration of this drug is by the intravenous route at a dose rate of 400 mg/kg/day for two weeks followed by oral administration of a further 300 mg/kg/day for four weeks (Bacchi *et al*, 1989). This regimen is both stringent and cumbersome and is the major disadvantage of using this drug in treating sleeping sickness patients.

A Figure 2.10 The chemical structure of atoxyl.

B Figure 2.13 The chemical structure of tryparsamide.

C Figure 2.14 The chemical structure of melarsen oxide.

D Figure 2.15 The chemical structure of melasoprol.

E Figure 2.16 The chemical structure of cymelarsan.

H_2N — (benzene ring) — As(OH)(=O)(ONa)

A

NH_2COCH_2NH — (benzene ring) — As(OH)(=O)(ONa)

B

H_2N, H_2N — (triazine ring: C, N, C, N, C, N) — NH — (benzene ring) — AsO

C

H_2N, H_2N — (triazine ring: C, N, C, N, C, N) — NH — (benzene ring) — As(S—CH_2)(S—CH—CH_2OH)

D

NH_2, NH_2 — (triazine ring: N, N, N) — NH — (benzene ring) — As(S-CH_2-CH_2-NH_2)(S-CH_2-CH_2-NH_2) x 2 HC

E

Figure 2.17 The chemical structure of suramin.

A Figure 2.18 The general chemical structure of a phenanthridine compound.

B Figure 2.19 The chemical structure of homidium.

C Figure 2.20 The chemical structure of prothidium.

R, R′, R″, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, C=N+, Y, Halogen

A

H_2N, NH_2, N^+, C_2H_5

B

R = —HN— (pyrimidinium: CH_3, ^+N–CH_3, N, NH_2, Br)

R′, R″ = NH_2
Y = Me

C

A Figure 2.21 The general chemical structure of a diamidine compound.

B Figure 2.22 The chemical structure of pentamidine.

C Figure 2.23 The chemical structure of diminazene aceturate, Berenil.

Am = —C₆H₄—C(=NH)NH₂

NH_2

C

NH

A

NH_2 C NH —N=N—NH— NH C NH_2

B

NH_2 C NH —O$(CH_2)_5$O— NH C NH_2

C

Figure 2.24 The chemical stucture of isometamidium chloride, Samorin.

Figure 2.25 The chemical structure of quinapyramine.

$$\begin{array}{c} \qquad\qquad\qquad\qquad\qquad\qquad\qquad CHF_2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad | \\ H_2N-CH_2-CH_2-CH_2-C-COOH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad NH_2 \end{array}$$

Figure 2.26 The chemical structure of dl-α-difluoromethylornithine, DFMO.

2.4 DRUG RESISTANCE

Although chemotherapeutic compounds have been developed which are proven to be active against specific organisms, there are instances where some parasite populations do not respond, as expected, to these compounds. These populations, which are unaffected by the action of the compounds are considered to be "fast" or resistant to the particular drug. This phenomenon of drug resistance which was first systematically studied in trypanosomes (Ehrlich, 1907) has been observed in protozoa, bacteria, fungi, viruses and neoplastic cells. Drug resistance is a transitory or permanent loss of the initial sensitivity of micro-organisms or mammalian cells to the effect of substances which interfere with vital functions of these cells (Schnitzer, 1963). According to this definition, resistance manifests itself after exposure to the chemotherapeutic agent *in vivo* or *in vitro*. A simplified definition of resistance was given by Bryson and Szybalski (1955) as the temporary or permanent capacity of a cell or its progeny to remain viable and multiply under environmental conditions that would destroy or inhibit other cells. This definition recognises the heritable nature of drug resistance and does not limit the phenomenon solely to its appearance after exposure to a drug.

2.4.1 MECHANISMS OF DRUG RESISTANCE

Most of the information available today on the subject of drug resistance and mechanisms that lead to its development has been obtained from extensive work carried out with disease conditions which are important to human beings. As drug resistance is a heritable character (Bryson and Szybalski 1955), its development involves changes in the DNA (mutations), which can be passed on from one generation to the other. When cells which exhibit this mutation are exposed to the drug against which resistance is developed, they are at a selective advantage as compared to cells without the mutation. This leads to selective pressure whereby the non-mutant cells are destroyed by the drug while the mutants survive and continue to thrive and reproduce. Evidence for the mutational theory of drug resistance is found in *Plasmodium* where resistance to pyrimethamine is shown to arise from a single-step mutation (Diggens *et al*, 1970) while resistance to chloroquine arises from a point mutation (Foote *et al*, 1990). Neoplastic cells have also been shown to develop drug resistance through a single genetic event (Ling, 1982).

A major controversy has been whether the mutants arise because of the presence of the drug or whether the drug simply selects pre-existing mutants. So far,

evidence suggests that drug resistance can arise by both of these mechanisms. A situation in which trypanosomes tolerate drugs to which they have had no previous exposure to, is recognised and called drug tolerance or natural resistance (Leach and Roberts, 1981). Evidence for this kind of resistance is derived from observations that trypanocidal effectiveness differs among various subgenera, species or even subspecies of trypanosomes (Lumsden and Ketteridge, 1979). Further evidence is the observation that different trypanosome strains derived from a single clone have been found to have varying trypanocide sensitivities without themselves ever having been exposed to any drug (Peregrine, *et al,* 1980). The fact that these strains are derived from a clone means that any variations in the resulting strains must arise from mutations. Certain events are associated with the development of drug resistance which may give indications concerning its development. The first indication of resistance in an organism is the reduction of sensitivity to a particular drug. Other events associated with this reduced sensitivity will now be discussed.

2.4.1.1 Decreased drug penetration into cells

Schnitzer and Grunberg (1957) suggested that development of resistance in protozoa and spirochetes is partly due to a change in the biological character of cells which leads to a decrease in drug accumulation in the cells. This results into a situation where the drug is prevented from interacting with its target in the cell. Such a decrease in drug accumulation can either be due to a decreased influx of the drug or its increased efflux. An increased drug efflux would involve a kind of over-efficient pumping-out mechanism while decreased influx would involve a deficient system for the entry of drugs into cells. Most studies on drug resistance indicate that there is a decreased drug accumulation in resistant cells although a decrease in drug influx or an increase in efflux have only been identified in some cases. In *Entamoeba histolytica,* resistance to emetine was determined to be due to an increased drug efflux (Samuelson *et al,* 1990). In resistant cancer cells, Fojo *et al,* (1985) observed that there was a reduced drug accumulation while Kartner and Ling (1989) determined that this was due to an increased drug pumping-out mechanism. *Plasmodium* parasites resistant to chloroquine have been shown to have a decreased accumulation of the drug (Fitch *et al,* 1975) due to increased efflux (Krogstad *et al,* 1987). In the kinetoplastida, a similar reduction of drug concentration in resistant organisms has been observed. In *Leishmania,* resistance to methotrexate has been shown to be due to an increased drug efflux (Ouellette and Papadopoulou, 1993). Experiments on *brucei* type trypanosomes have suggested that there was a decreased uptake of pentamidine in resistant *T. brucei*

resistance to Samorin was related to reduced accumulation as shown by uptake of the radio-labelled drug (Sutherland *et al*, 1991, 1992a). A mathematical model, however, did not demonstrate that reduced accumulation of the drug was due to its reduced uptake (Sutherland *et al*, 1992b).

2.4.1.2 P-glycoproteins and the multidrug resistant (mdr) phenotype

A possible reason for the increased drug efflux in resistant cells could be due to the presence of an energy-dependent extrusion pump, which removes a drug from cells so efficiently that the drug does not have an opportunity to interact with its cellular target. This pump is related to the presence of P-glycoproteins which span the lipid bi-layer of cell plasma membranes (Bradley *et al*, 1988). P-glycoprotein molecules have been observed to function as extrusion pumps by forming channels across the plasma membrane which are used to actively transport drugs using energy derived from ATP hydrolysis. A fact that led to the association of these molecules with drug resistance is that although P-glycoproteins are found in cells that are drug sensitive, they are overproduced in many drug resistant cells (Riordan and Ling, 1979).

The multidrug resistant (mdr) phenotype led to the identification of P-glycoproteins and their role in drug resistance (Ueda *et al*, 1986). This phenotype was recognised from observations that resistance developed by one drug at times leads to resistance against other structurally and functionally unrelated drugs (Shen *et al*, 1986). The cellular targets as well as the mode of action of these drugs are different and this is, therefore, not simply cross-resistance. In all instances of the mdr phenotype, there was over-expression of the genes that code for P-glycoproteins leading to an overproduction of these proteins and subsequent increase in the extrusion of a variety of drug molecules (Bradley *et al*, 1988). The mdr phenotype has been shown to arise from a single genetic mutational event (Ling, 1982) lending further support to the mutational origin of drug resistance.

The P-glycoprotein gene has been sequenced and shown to be highly conserved in such phylogenically dissimilar species as bacteria, yeast, *Drosophila, Plasmodium,* hamster, mouse and human (Gros *et al*, 1986a, b, c; Bradley *et al*, 1988; Ouellette *et al*, 1990). There is thus a large degree of homology in the DNA sequence of this gene across a diverse range of species. The over-expression of this gene, resulting in over-production of P-glycoproteins, has been shown to be the most consistent biochemical alteration of multidrug resistant cells. The mdr gene, however, is also expressed and produces low levels of P-glycoproteins in many normal mammalian cells in tissues such as liver, pancreas, kidney and colon. The presence of this protein in tissues which are

specialised for secretory processes provide further evidence of the excretory role of these proteins in drug resistant cells.

2.4.1.3 Gene Amplification

Another factor that has been correlated to the development of drug resistant is chromosomal aberrations like deletions, inversions, rearrangements and translocations (Stark *et al,* 1989). Changes in the DNA of methotrexate resistant cells were shown by Schimke *et al,* (1978) to be due to gene amplifications. These amplifications caused by unscheduled DNA synthesis or excessive DNA duplications were located at the P-glycoprotein gene locus although other regions adjacent to this gene may also be involved (Stark *et al,* 1989). Such amplification results in high copy numbers of the P-glycoprotein gene, an over-expression of this gene and an over-production of P-glycoproteins which pump-out drugs from cells. Gene amplifications related to the development of drug resistance have been observed in *Plasmodium* (Peters, 1987b; Foote *et al,* 1989), cancer cells (Martin *et al,* 1987) and in *Leishmania* (Ouellette and Papadopoulou, 1993). Work done on *Leishmania,* however, shows that amplification of the P-glycoprotein gene does not confer resistance to unrelated drugs as observed in the mdr phenotype (Ouellette *et al,* 1990). This suggests that the mechanism of drug resistance in this species is different from that observed in the mdr phenotype; a suggestion also supported by the fact that its P-glycoprotein gene is divergent when compared to those of mammalian cells, yeast, *Plasmodium,* and *Drosophila* (Ouellette *et al,* 1990).

2.4.1.4 Transfer of Resistance Between Cells

In addition to the transfer of resistance from parent to offspring, it is possible to transfer resistance from one organism to another in a manner that does not involve reproduction. This has been observed especially in antibiotic resistance of bacteria. Mutations responsible for resistance are at times in extrachromosomal DNA portions called plasmids. It has been determined that drug resistance genes can be transferred from one bacterium to another using mobile DNA portions called transposons (Gentry, 1991; Bennet and Hawkley, 1991). These transfers resulting in conferring antibiotic resistance from a donor bacterium to a recipient one can be between bacteria of different species and thus causing a dispersal of this trait.

2.4.2 DRUG RESISTANCE IN TRYPANOSOMES

Trypanocide resistance is evidenced by a failure of treatment in which an animal with demonstrable parasites is not cured by drug administration at a normally curative dose level. Failure of treatment can be observed as an extreme condition where parasites do not disappear after drug administration or a less extreme situation where they only temporarily disappear. Davey (1950) working with Antrycide suggested that under field conditions, it is wise to assume that all relapse strains are potentially resistant. It is, however, difficult to distinguish relapses from re-infections under field conditions. Under such conditions, a relapse is assumed to have occurred when there is a recrudescence of parasitaemia after treatment within a period considered insufficient to allow a new infection to develop.

Although failure of treatment may indicate resistance, there are other factors which could account for a reduction in the efficacy of chemotherapy. For instance, drug tolerance may occur when an inappropriate drug is used i.e. a particular drug which is not active against the infecting trypanosome species. An example of this is the use of phenanthridines in the treatment of *T. evansi* which has been shown to be insensitive to these compounds (Gitatha, 1979; Rottcher and Heising, 1980). Another reason for apparent treatment failure is the use of drugs which do not gain access to trypanosomes in body compartments into which these parasites have invaded. Treatment failure of this nature can be observed in the use of trypanocides that do not cross the blood-brain barrier for treatment of late stage infections caused by trypanosomes of the *Trypanozoon* subgenus. Trypanocide resistance can therefore only be demonstrated unequivocally where trypanosomes are exposed to a drug known to be active against that particular species and which has access to the parasites in whatever body compartments they may invade.

In the field, there are examples of trypanosome resistance to all trypanocidal drugs currently in use. Resistance to Samorin has been observed in *T. vivax* and *T. congolense* stocks isolated from East and West Africa (Kupper and Wolters, 1983; Pinder and Authie, 1984; Rottcher and Schillinger, 1985; Schonefeld *et al,* 1987; Nyeko *et al,* 1989). There have been cases of Berenil resistance in *T. brucei, T. congolense, T. vivax* (Gray and Roberts, 1968; Mbwambo *et al,* 1988; Rottcher and Schillinger, 1985;) as well as *T. evansi* (Gill, 1971a). Resistance to homidium has been observed in stocks of *T. vivax, T. congolense* and *Trypanozoon* (Gray and Roberts, 1968; Scott and Pegram, 1974; Mwambu and Mayende, 1975). The case of Antrycide provides an example of how widespread trypanocide resistance can occur for this drug

was withdrawn in 1974 due to unacceptable levels of resistance in trypanosome stocks infecting cattle (Schillinger and Rottcher, 1986).

Resistance to the two trypanocidal drugs used to control *T. evansi* has been reported from a number of countries. Resistance to quinapyramine was observed by Auguadra (1963). Suramin resistance has been more widespread and was observed as early as 1927, two years after the drug's introduction (Knowles, 1927). Resistance to suramin was observed in buffaloes infected with Surra in Indonesia (Kraneveld and Mansjoer, 1941), infected ponies in India (Gill, 1971a) as well as infected camels in Sudan (Evans, 1946; Leach, 1961; Luckins *et al*, 1979; Boid *et al*, 1985, 1989), the former USSR (Petrovskii and Khamiev, 1974) and in Kenya (Gitatha, 1980; Schillinger *et al*, 1985a). There have been instances where stocks of *T. evansi* have been shown to be resistant to both quinapyramine and suramin (Mahmoud and Osman, 1979; Schillinger *et al*, 1984). The extent of *T. evansi* resistance to trypanocidal drugs recommended for its control is not known.

2.4.2.1 Detection of resistance

When trypanocide resistance was first recognised, the role of the trypanosome host in the development of resistance was investigated. Mesnil and Brimont (1908) observed that resistance to atoxyl varied according to the infected host animal. In their experiments, they noticed that a trypanosome stock which was resistant in mice, was sensitive when passaged into rats but re-acquired resistance when it was passaged once more in mice. Dale (1923) observed a similar situation where a strain was resistant in a rat, sensitive in a mouse and resistant in a dog. These observations led to the belief that the trypanosome host played an important role in drug resistance (Findlay, 1930; Browning, 1931). Yorke and Hawking (1932), however, disagreed with this view and considered that trypanosomes retained their drug resistance in the different hosts and it was the minimum effective dose which was different for each species. Ehrlich (1907) too considered that although drug resistance was inherent in a trypanosome, it was also a relative character. This relative nature of trypanocide resistance is evident even today for a stock can only be described as resistant when compared to another. A need therefore exists for a reference point, considered as a standard curative dose. Drug resistance can then be described in relation to deviation from the standard dose . This brings the concept of a minimum curative dose (MCD), defined as the minimum amount of drug which will cure 80% of infected animals (Walker, 1964; Gill, 1971b). The MCD is different from the minimum effective dose (MED) which is a dose that removes parasites from circulation, though temporarily, in 80% of infected animals.

Resistance, being relative, requires quantification in order to compare the degree of resistance between different trypanosome populations. The ratio of drug dose required to cure a resistant test strain over the dose required to cure a normal strain; resistance factor (Gill, 1971b) is often used as an indicator of the degree of resistance. When this ratio is greater than 2, the test strain is considered to be drug-resistant. Again, this concept of resistance is relative for it can only be standardised by using a reference trypanosome strain. Another system that has been used to quantify the degree of resistance is the one recommended by the World Health Organization (WHO, 1973) for malaria. In this system an increased severity of resistance is classified from R1 to R3 whereby: R1, is a situation where after treatment, parasitaemia is cleared but returns in less than 21 days; R2, a situation where after treatment parasitaemia drops to a lower level as compared to the period prior to treatment but does not clear; and R3 a situation where there is no drop in parasitaemia after treatment. Although this classification refers to malaria, it can also give a broad indication of resistance and its severity even in other diseases.

Both concepts of an MCD and a resistance factor require comparison with a reference trypanocide dose which is considered normal in a particular animal species. There is therefore a need to convert this recommended drug dose for use in another animal species or even *in vitro*. Manufactures usually recommend drug doses according to the weight of a particular animal. Thus, drug dosage is quoted as milligrams or grams per kilogram body weight (mg or g/kg). Due to differences in animal species, the amount of drug needed per animal weight is not acceptable when determining the drug dosage needed in a species other than the one referred to by manufactures. Moore (1909), working on drugs in the treatment of trypanosomiasis in mice and monkeys recommended that a better conversion ratio would involve the use of surface area instead of weight. He suggested that the surface area of animals was directly proportional to their respective weight raised to the power of two-thirds (Figure 2.27). Trypanocidal drugs could then be administered according to proportions of the surface areas of the host animals irrespective of species. Bushby (1963) further developed this concept and suggested a formula for the conversion of drug dosage from one animal to another. He suggested that the relationship between drug doses of two animals with different weights (W) a and b is $W_a^{0.7} : W_b^{0.7}$. The difference in using surface area instead of weight for conversion of drug dosage in animals was shown by Bushby to get larger as the weight differences of two animal increased (Figure 2.27). So far, this suggested conversion has not been adopted for trypanosomiasis..

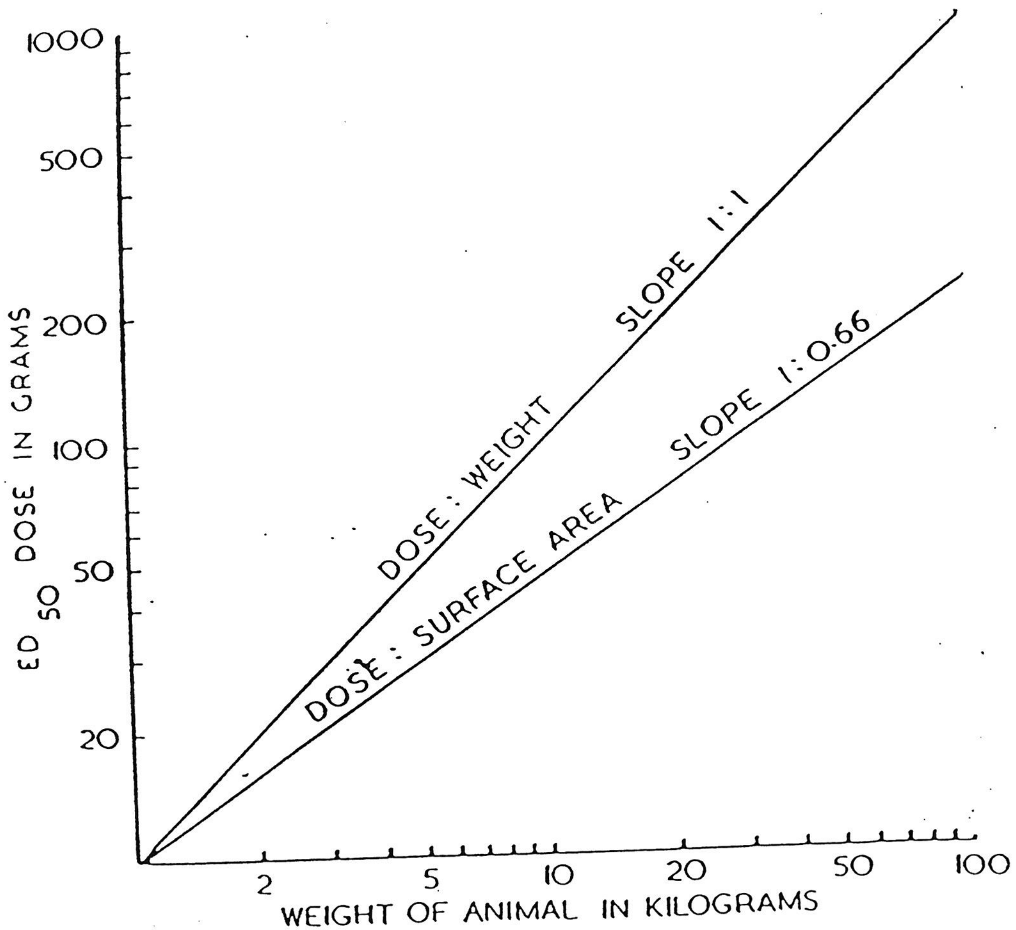


Figure 2.27 The difference between the use surface area and weight in the conversion of drug dosage in animals of different weights (Bushby, 1963).

2.4.2.1.1 *In vivo* methods

Due to the cost and technical difficulties of maintaining large animals in laboratory conditions, the traditional method of testing for drug resistance has been by the use of rodents (Godfrey and Killick-Kendrick, 1962). This is, however, limited to trypanosomes of the *Trypanozoon* and *Nannomonas* subgenera, which are rodent-infective. Parasites are inoculated into the rodents mainly by the intraperitoneal route at an inoculum dose ranging from $1x10^5$ to $1x10^6$ trypanosomes (Abebe *et al*, 1983; Schillinger *et al*, 1985a; Peregrine *et al*, 1990; Zhang *et al*, 1992). The duration between infection and treatment varies from immediately after infection, up to 48 hours post-infection (Jones and Folkers, 1966; Abebe *et al*, 1983; Schillinger *et al*, 1985a; Peregrine *et al*, 1990; Zhang *et al*, 1992). The rodents are then bled regularly and the blood examined microscopically for parasitaemia using microscopic examination of wet blood smears or the haematocrit centrifugation technique (Woo, 1970). Persistence of parasitaemia or relapse after treatment is taken to be an indication of resistance to the trypanocide used.

In using this system for testing resistance, there are variations in the methods used whose effect on the success of treatment have not been properly investigated. Variables such as the parasite inoculum dose and the timing of treatment may be important, especially in *T. brucei* family parasites which invade the central nervous system and the drugs used do not cross the blood-brain barrier.

2.4.2.1.3 *In vitro* methods

Trypanocide sensitivities can also be determined *in vitro*. The tests basically involve exposure of parasites to a drug *in vitro* and assessment of their viability. In one method, trypanosomes are maintained in a drug-containing medium for 3-4 hours after which their motility is observed as compared to the period before trypanocide exposure. A drug incubation infectivity test (DIIT) is then performed where rodents are inoculated with the drug-treated trypanosomes and regularly checked for parasitaemia; an indication that parasites have retained their infectivity (Williamson and Scott-Finnigan, 1978). A modification of this method described by Borowy *et al*, (1985) involves the establishment of a culture system where trypanosomes are grown on feeder-layer cells. Once trypanosomes are established and multiplying in culture, a trypanocide is then introduced and parasite multiplication assessed.

A different method used in the determination of drug resistance *in vitro* is the determination of trypanosome viability by the assessment of an appropriate physiological process. The original culture systems depended on the growth of

trypanosomes in medium containing feeder layer cells. However, recent improvements in the *in vitro* culture of trypanosomes in the absence of feeder layers has enabled a number of assay systems which have been used in the determination of drug resistance. In one type of test where the incorporation of a radiolabelled nucleic acid precursors is assayed, the viability of trypanosomes pre-incubated with a test drug is assessed by their ability to synthesise nucleic acids from hypoxanthine (Desjardins *et al,* 1980). The radiolabel of this nucleic acid precursor is used to detect its incorporation by the parasites. This method has been successfully used in trypanosomes of the *brucei* group as well as *T. congolense* (Brun and Kunz, 1989; Ross and Taylor, 1990; Kaminsky *et al,* 1993). A similar test described by Zinsstag *et al,* (1991) determines pyruvate production of trypanosomes as an assay of trypanosome growth. This test is based on the fact that trypanosomes produce pyruvate in glycolysis and their viability can be determined by its production. Pyruvate production as a method of determining resistance has been used for *T. brucei* and *T. evansi* (Zinsstag *et al,* 1991; Sutherland *et al,* in press). On analysing *in vitro* drug tests, Kaminsky (1990) concluded that the drug sensitivities of trypanosomes in culture correlated well with those observed in rodents and cattle. Advantages of using *in vitro* tests to determine drug sensitivities are that they are cheaper and more rapid than *in vivo* tests, require only small amounts of drugs and the effects of the drugs can be visualised easily. However, a major limitation of these *in vitro* tests is that they depend on the adaptation and maintenance of trypanosome stocks in culture, procedures whose effect on drug sensitivity may change the biological properties of trypanosomes and influence the outcome of drug sensitivity results (Zweygarth *et al,* 1991). These tests also determine the drug sensitivity of trypanosomes outside the host and therefore eliminate host-related factors such as immune response, which might influence drug sensitivity.

2.4.2.2 Induction of resistance

Information pertaining to how drug resistance develops can be derived from experimental induction of resistance. Franke and Roehl (cited by Ehrlich, 1907) observed that in *T. brucei*-infected mice given a trypanocidal dye parafuschin, trypanosomes disappeared only temporarily. Repeated administration of the dye resulted in the dye having no effect on the trypanosomes.

2.4.2.2.1 Experimental induction of resistance

Resistance can be experimentally induced in trypanosomes *in vivo* or *in vitro* by administration of trypanocidal drugs in subcurative dose levels. There are two basic

methods of inducing trypanocide resistance *in vivo* (Schnitzer and Grunberg, 1957). The method commonly used to induce drug resistance in large animals is by repeated exposure of trypanosomes to subcurative doses of a trypanocidal drug in a single animal. In this method, once an animal is infected, the trypanocidal drug is repeatedly administered in increasing doses whenever parasites reappear in peripheral blood. In small laboratory animals, however, the trypanocidal drug is usually administered once in an animal either after a relapse of parasitaemia, after a passage, or immediately after trypanosome infection without reappearance of parasites. The induction of trypanocide resistance can be hastened by preventing the host animal from mounting an immune response by splenectomy, irradiation or administration of immunosuppressive chemical compounds like cortisone and cyclophosphamide (Von Jancso and Von Jancso, 1934, 1935b; Schnitzer *et al,* 1946; Osman *et al,* 1992).

Experimental induction of resistance has been accomplished with various trypanocidal drugs. Early work on inducing trypanocide resistance involved atoxyl and tryparsamide (Ehrlich, 1907; Yorke and Hawking, 1932). Other studies performed later on *T. rhodesiense* involved inducing resistance to various acridines, antimonials and suramin (Yorke *et al*, 1932). A lot of work was done by Gill (1971b) on the development of resistance to quinapyramine, stilbamidine, tryparsamide and suramin in *T. evansi.* This work determined the ease with which resistance was induced to the various drugs by observing the time and passages it took to reach maximum resistance, a drug dose level above which no further resistance could be induced. For suramin Gill (1971b) observed that it took 80 passages in 85 weeks to induce maximum resistance in *T. evansi* using mice. Von Jancso and Von Jancso (1935b) had earlier observed that in suramin, the maximum dose reached after 12 passages was not necessarily maximum resistance but was the maximum tolerated dose (MTD) of mice to suramin. The MTD, a drug dose level which is toxic to 80% of a particular animal species (Walker, 1964; Gill, 1971b), has been determined for suramin in mice to be 150 mg/kg (Abebe *et al,* 1983).

It is also possible to induce trypanocide resistance *in vitro* by culturing trypanosomes in gradually increasing concentrations of the drug. Using this method, *T. evansi* populations with a 1000 fold level of resistance to suramin higher than parent trypanosomes have been induced (Sutherland, D. V., personal communication).

2.4.2.2.2 Natural development of resistance

Drug resistance under natural conditions develops when trypanosomes are exposed to subcurative doses of a trypanocide. When an animal is treated with a subcurative dose of a trypanocide, only a proportion of trypanosomes sensitive to that dose are destroyed. This leaves a proportion of trypanosomes as survivors which thrive and multiply to maintain the infection. Continued exposure to higher subcurative doses results in the selection of parasites with higher resistance which leads to a situation where the surviving trypanosomes tolerate higher levels of trypanocidal drugs than the original population. This can occur when drugs are used for prophylactic purposes where, over a time period, there is a decrease of active substance, until the drug level falls below the level that destroys all parasites. If by irregular application, the drug level falls to subcurative levels in an animal which is still exposed to infection, trypanosomes are then exposed to subcurative doses leading to the development of resistance.

Other ways in which trypanosomes could be exposed to subcurative doses results from underdosage. This can arise from underestimation of animal weight, a situation which could easily occur under field conditions. During mass treatment of animals, an average weight is used to determine the amount of drug each animal should get without weighing each individual. Another situation which may result in drug underdosage is the misuse of drugs. This especially happens when drug treatments are administered by unqualified people who may not understand the importance of using correct drug dosages and in their attempt to save money, over-dilute a drug so that it can treat more animals.

2.4.2.2.3 Selection processes

After development of resistance either by natural or experimental means, resistance is maintained in the presence of the drug by selective processes. Any mutations resulting in the loss of this resistance are quickly selected against by trypanocide destruction of trypanosomes displaying the mutation. In the absence of drug pressure, however, a situation might arise whereby the acquired resistance wanes. Resistance is described as stable when it is maintained *in vivo* or *in vitro* irrespective of drug presence and is unstable when it wanes. Studies on the stability of trypanocide resistance *in vivo* have produced contradictory results; stable resistance has been observed in trypanosomes resistant to the action of homidium, diminazene aceturate, isometamidium chloride, and cymelarsan (Osman *et al,* 1992). In this study, resistance to these drugs was experimentally induced in *T. evansi* and tested for

stability in immunocompetent mice. Unstable resistance has been observed in quinapyramine (Hawking, 1963b) and suramin (Morgenroth and Freud, 1924; Hawking, 1963a). Amrein and Fulton (1959) observed stable suramin resistance in which resistance in *T. rhodesiense* was still observed after 28 years of passage in mice. DFMO resistance was observed to be stable by Phillips and Wang (1987). In this study, *T. brucei* mutants induced *in vitro* were passaged 10 times and observed to have the same DFMO sensitivity as those before the passages. The *in vitro* stability of resistance to Berenil, Samorin, quinapyramine and melasoprol in *T. brucei* was investigated by Kaminsky and Zweygarth (1989). In this study, resistant parasites in mice transferred and maintained in culture for up to 275 days and transferred back to mice showed no change in their drug-resistance. Zweygarth *et al,* (1991) however observed that Berenil resistance decreased with maintenance of *T. evansi* in culture for 14 months although resistance to Samorin, quinapyramine and suramin remained the same. These contradictory reports on the stability of trypanocide resistance suggest that resistance may be of different kinds i.e. a trypanosome might develop resistance to a particular drug by different mechanisms resulting in either stable or unstable resistance. A similar a situation has been observed in malaria whereby chloroquine resistance may develop by different mechanisms; some related to the mdr phenotype while others are not (Peters, 1987a; Foote *et al*, 1989, 1990; Wellems *et al*, 1990).

Hawking (1963b) suggested a means of predicting the stability of an acquired resistance on the basis of trypanosome growth rates. In his prediction model, if the resistant mutants multiplied faster or at the same rate as the non-mutants, then the resistance exhibited will be stable. Mutants that multiply more slowly than non-mutants, however, develop unstable resistance.

2.4.2.3 Cross resistance

Trypanosomes which acquire resistance to one trypanocide can also develop resistance to another trypanocide. Unlike the mdr phenotype, the drugs involved are usually chemically related or target the same pathway. This type of resistance is usually, but not exclusively, reciprocal, i.e. resistance to one compound results in resistance to other members of similar chemical group and vice versa.

No cross-resistance has been observed between suramin, quinapyramine and diminazene aceturate (Gill, 1971a; Finelle, 1973) since these trypanocides are chemically different and have different modes of action. Cross resistance has however been observed between the diamidines and arsenicals as well as between phenanthridines and isometamidium (Hawking 1963a). Although arsenicals and

diamidines are chemically unrelated, this action could be due to both drugs having polyamine synthesis as a target. It is fortunate that the two drugs used in the control of camel trypanosomiasis caused by *T. evansi* are chemically unrelated and their modes of action are different and thus do not cross-react. There, however, are observations of cross-resistance between Berenil and Mel Cy, (Zweygarth and Kaminsky, 1990; Osman *et al,* 1992; Zhang *et al,* 1992).

2.4.2.4 Mixed trypanosome infections

Experimental trypanosome infections mainly consist of homogeneous parasite populations. In field conditions however, animal infections may be mixed whereby there are different trypanosome species involved. This implies that the infecting species relate with each other in a manner that allows them to co-exist, with each species maintaining its own identity. A different kind of mixed infection is the concurrent presence of trypanosomes of the same species but different serological identities or drug sensitivities. In such mixed infections, it has been observed that there exists a competition between stocks that are different either serologically (Seed, 1978; Barry *et al,* 1979) or regarding their drug sensitivities (Laveran and Rondsky, 1912; Oehler, 1914; Teichman, 1918; Cantrell, 1956). When comparing two trypanosome stocks, trypanosomes which grow faster develop infections earlier; a situation that may result in a selecting-out of the slower-growing trypanosomes. In other studies of mixed infections resulting from subsequent trypanosome inoculations, different trypanosome populations may not act independently but interact with each other in a manner which alters the course of their development. Herbert (1975) investigating two antigenically different strains of *T. rhodesiense* noticed that if mice were already chronically infected with one strain, the pre-patent period of a second strain introduced was extended by as long as 14 days. In a similar experiment with *T. congolense*, the establishment of infection of one stock in rabbits and goats interfered with that of a second antigenically different stock (Luckins and Gray, 1983; Dwinger *et al,* 1986). Sones *et al,* (1989) also investigated interference *T. congolense* but used two strains which were different in respect of their sensitivity to Samorin. These researchers observed that once a drug sensitive strain had established an infection, it interfered with an attempt for a resistant one to do likewise. The opposite was, however, not the case for the establishment of a drug resistant infection did not prevent a drug sensitive strain to establish itself. Such a phenomenon may be important in the distribution of drug resistant strains for if their development is interfered with in mixed infections, their spread may be influenced due to a selective mechanism.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1. TRYPANOSOME IDENTITY AND HISTORY

Forty one of the stocks used in this study were from natural infections of Somali x Rendille camels from seven different areas of Kenya (see Figure 3.1), two from camels in Sudan and one from Colombia. These stocks were cryopreserved in liquid nitrogen in the KETRI bank. The trypanosomes were originally classified as *T. evansi* on the basis of their morphology and the hosts from which they were isolated. The stocks were later confirmed as *T. evansi* by a recent molecular biology method based on kDNA analysis (Waitumbi, unpublished data). These trypanosome stocks are described below in groupings according to their origin.

3.1.1 GALANA

Seven of the 44 stocks came from camels kept at Galana (see Figure 1). These were KETRI numbers 2448, 2449, 2450, 2451, 2455, 2458 and 2489. All the stocks were isolated in 1979 except KETRI 2489 which was isolated in 1980. Camels were kept on a ranch owned by the Galana Game and Ranching company situated in the coastal hinterland of Kenya. This was a commercial ranch which kept cattle for beef production. The camels were introduced on the ranch to provide milk for the herdsmen and were kept in the drier western part of the ranch which was not suitable for cattle keeping due to the low rainfall and thus sparse vegetation. This western part, unlike the eastern part where cattle were kept, has little or no tsetse due to its aridness and unsuitable tsetse habitat (Njogu *et al*, 1985).

Camels were all ear tagged or branded for identification purposes and were kept in the traditional husbandry method. In this husbandry method, the animals were released from the night shelter ("boma"), allowed to roam freely in search of pasture (without herding) and were only rounded up at the end of the day. The herdsmen were from traditional camel-keeping communities who readily recognised a sick camel and promptly informed the ranch management. Veterinary input in this camel herd was provided by the ranch and KETRI staff and consisted of treatment at the indication of

trypanosomiasis or any other disease. Where trypanosome infection was diagnosed by the wet blood film technique or the micro-haematocrit centrifugation technique (see 3.3.4), suramin was administered. Drug availability was thus regular and professionally supervised.

3.1.2 GARISSA

Three of the isolates, KETRI numbers 1188, 1377 and 2416, were isolated from camels in Garissa in 1968 (see Figure 1). The camels in Garissa are kept by Somali tribesmen who are the original camel keepers in Kenya and have a very good knowledge of camels and their diseases. Camel husbandry is traditional whereby the camels are moved from area to area in search of pasture and water. The camel owners and herdsmen know each camel by "name" and can readily identify them. Supervised veterinary attention in this area was not regularly available in 1968 due to the nomadic nature of this community, the difficulty of accessibility to this remote area and the security situation in the then Northern Frontier District. The use of trypanocides, however, may have been greater than would be expected due to the easy availability of drugs from unofficial sources in this area of close association with Somalia. The confidence with which these camel keepers believe they can diagnose and treat camel disease also aggravates the problem of unsupervised drug use. The belief that trypanocides were overused in this area is supported by the observation that more trypanocides were reported to have been used than were supplied by the official veterinary department outlets (Mahaga and Rottcher 1982).

3.1.3 MARSABIT

Twenty one of the *T. evansi* stocks were isolated in Marsabit (see Figure 1). Eighteen of these were isolated in 1979:- KETRI numbers 2439, 2440, 2441, 2442, 2443, 2444, 2446, 2453, 2454, 2456, 2457, 2465, 2466, 2467, 2468, 2469, 2470 and 2471. The other three, KETRI 2447, 2477 and 2481, were isolated in 1980.

The Marsabit area was included in a UNESCO Integrated Project in Arid Lands (IPAL) during this period; a project which among other objectives aimed at the improvement of the Rendille peoples' livestock. This improvement was achieved by regular and properly supervised use of trypanocides (suramin in camels). The camels were either owned by the Rendille and kept by their traditional methods or were owned by the project and kept under ranch conditions. In order to allow the project staff regular access to the animals for monitoring purposes, the movement of the camels involved in the study was less than would normally be the case of traditionally

managed Rendille camels. All the camels were identified by either ear tagging or branding. Since the animals were treated free of charge, these camels were certainly treated more often than normal in a traditional Rendille herd where the camel owner would have to seek veterinary attention for his animals and pay for the necessary drugs.

3.1.4 NGURUNIT

Three stocks were isolated from this area. KETRI 2462 was isolated in 1976 while KETRI 2476 and 2480 were isolated in 1980. This area is about 100 km from Marsabit (see Figure 1) and neighbouring the area involved in the UNESCO project. The camels here were owned and herded by the Rendille in the traditional husbandry system where animals are known by name and are allowed to move from one area to the other in search of water and pasture. Other than the occasional treatments provided by the project staff, drug use was neither regular, nor properly supervised, and depended on indication of disease by camel owners and the availability of drugs from both official and unofficial government veterinary sources.

3.1.5 OL MAISOR

Ol Maisor is a ranch owned by camel enthusiast and promoter Jasper Evans. Camels are kept on this ranch to utilise its drier parts in a manner that does not degrade it environmentally. Camel milk provides food for the workers on the ranch. The camel on this ranch is also used for tourist "safaris" as well development of cheese and other products from camels milk.

The camels on Ol Maisor are kept in a semi-traditional manner by herdsmen who are traditional camel-keepers and move the animals within the ranch. All the camels are ear tagged or branded for identification and a sick camel noticed by the herdsmen is quickly reported to the ranch management. Regular and supervised use of acaricides, anthelmintics and any other drugs when needed is practised on this ranch. Suramin was the trypanocide used against camel trypanosomiasis on this ranch. Two stocks - KETRI 2485 and 2488 - were isolated from this ranch in 1980.

3.1.6 RUMURUTI

One stock - KETRI 2552 - was isolated from Rumuruti in a ranch owned by camel enthusiast Guilford Powys in 1980. Although this is a cattle ranch, camels are kept here due to the personal interest of the ranch owner.

3.1.7 UKUNDA

Ukunda is the southern-most area from which stocks were acquired. Camels were kept in this area as part of an animal production project on the utilisation of the drier parts of the coastal area and the introduction of the camel to people who are not traditional camel-keepers. The management of these camels was semi-traditional in a ranch setting where the camels, which were branded for identification, roamed freely with little herding. Very little information is available on the veterinary practice and the use of drugs on this camel herd.

Three of the stocks - KETRI 2424, 2426 and 2429 - were isolated in 1978 while the fourth - KETRI 2417 - was isolated in 1979. The reasons for the isolation of these stocks is not known although the acquisition of material for epidemiological study may be the case.

3.1.8 SUDAN

Two stocks - KETRI 2472 and 3136 - were isolated from Sudan in 1977. Information concerning the later stock indicates that it was isolated from a camel in Kassala in the eastern part of Sudan (Boid *et al,* 1979) as part of a trypanosomiasis survey. Information pertaining to the origin of the earlier isolate is scanty and only indicates that this stock was received in KETRI from Khartoum and may have been isolated on suspicion of trypanocide resistance. These two Sudanese isolates are probably the same isolate as indicated by the year of origin and their suramin sensitivities.

3.1.8 SOUTH AMERICA

Stock KETRI 2540 was isolated from a horse in Colombia, South America in 1973. No further information on this isolate is available.

3.2 EXPERIMENTAL ANIMALS

3.2.1 RODENTS

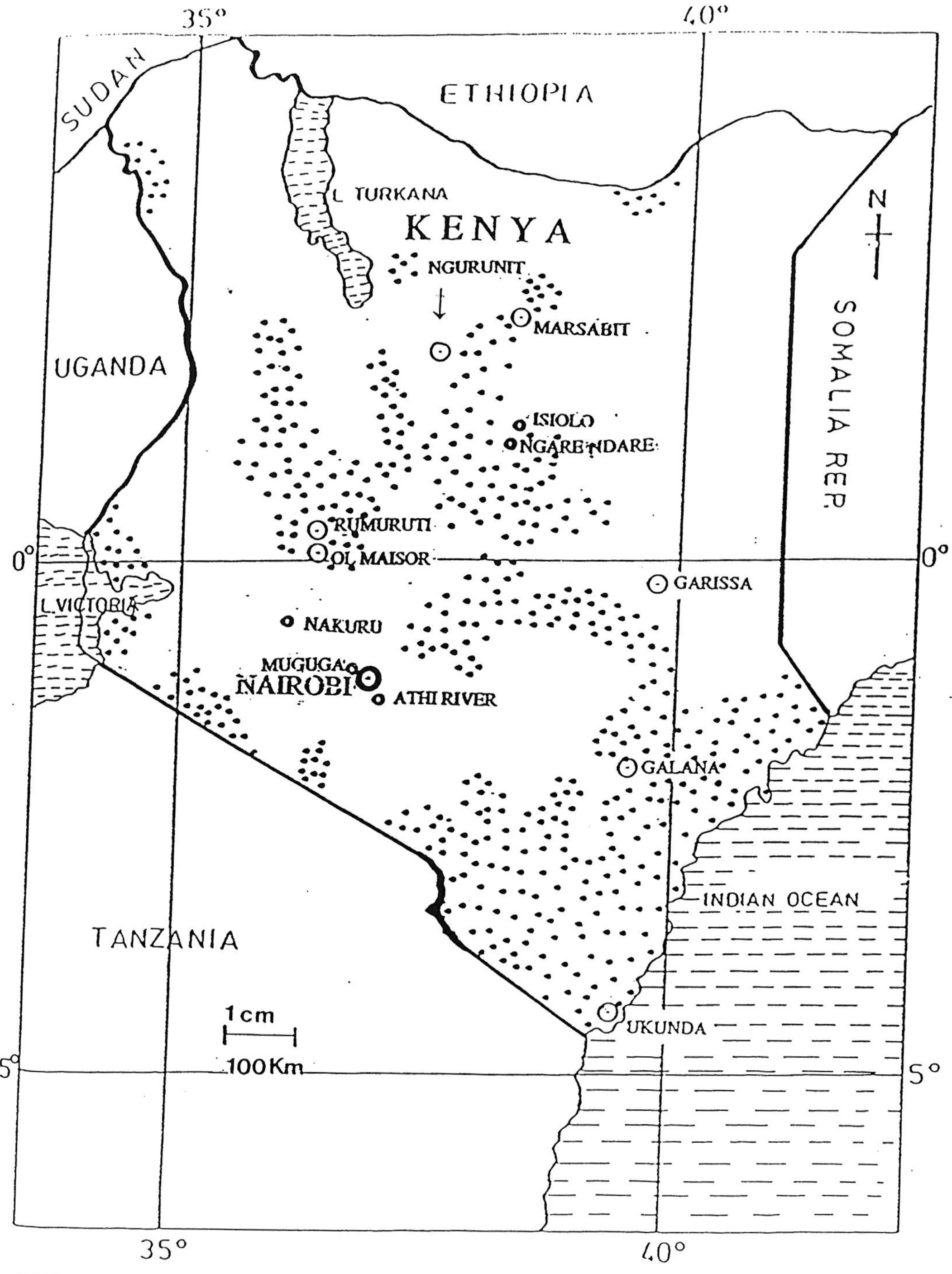
Unless otherwise indicated, the mice used throughout this work were male Swiss white albino from the KETRI breeding unit. The mice, weighed 20-25 g. The rats used were male Wistar and weighed 150-200 g. The rodents used throughout this work were transferred from the breeding unit to the fly-proof experimental small

animal facility and given food and water *ad libitum*. At least three days of acclimatisation were allowed before any experimental procedures were started.

3.2.2 CAMELS

The camels used were of the Somali x Rendille crossbreed from the KETRI breeding herd at Athi-River (see Figure 1). These camels came to Athi River in 1987 from Ngare-Ndare near Isiolo. Originally, they had been purchased from nomadic pastoralists in the North-Eastern province of Kenya. At both Ngare-Ndare and the traditional camel-keeping areas in the North-Eastern Province, camels are kept in arid areas of sparse vegetation which are habitats neither suitable for tsetse nor cattle keeping. All the camels used were immature males less than one-year old weighing between 90 and 250 kg. Although there is trypanosomiasis in the Athi River herd, the camels chosen for transfer to KETRI were parasitologically negative for one month prior to movement to KETRI. The Athi River camel herd was monitored for trypanosome infection twice a month using both the micro-haematocrit centrifugation technique and by mouse sub-inoculation (see section 3.3.4.1). Selected camels were transported to a fly-proof barn at KETRI where they were given food, water and mineral lick *ad libitum*. On arrival in the barn, pre-experimental procedures involved weighing, ear tagging and acaricide treatment with Ectopor^R (Ciba Geigy). They were also drenched with Valbazen^R (Smith Kline and Beecham) and treated with Trypacide Sulphate^R (Rhone Poulenc) at 5 mg/kg in case of any trypanosome infection.

Camels were allowed one month of acclimatisation in the barn before the start of any experimental procedures and they were monitored weekly by the micro-haematocrit centrifugation technique (see section 3.3.4) to ascertain that they were free of trypanosome infection and to obtain pre-infection values of PCV and weight.



Water mass - lake or ocean

Tsetse infested areas

Areas where stocks were isolated

Other areas referred to in the study

Figure 3.1 The map of Kenya showing areas from which *T. evansi* stocks were isolated and other areas referred to in the study.

3.3 EXPERIMENTAL PROCEDURES

Routine methods and procedures used to prepare material needed for the experiments undertaken during this work are described below.

3.3.1 TRYPANOSOME SEPARATION

All the trypanosome stocks used in this study were isolated from natural infections in blood, passaged in mice and the infected mouse blood cryopreserved (see section 3.3.2). In order to expand the stocks for use in experiments, two mice were inoculated with trypanosomes. These mice were monitored daily until they developed parasitaemia of at least 40 trypanosomes per field (x 400 magnification) when they were killed and infected blood collected by cardiac puncture. The blood was mixed with phosphate saline glucose buffer pH 8.0 (PSG, see appendix 1), in a 1:3 ratio. Trypanosomes in mouse blood were then separated from erythrocytes using the ion-exchange chromatography method of Lanham and Godfrey (1970). Diethylamino-ethyl cellulose (DE 52 cellulose, Whatman Biochemicals Kent, England) slurry was prepared in PSG according to the manufacturer's instructions. Five or ten ml syringes with a piece of cotton wool placed at the bottom were packed 3/4 full with DE 52 for use as columns. The DE 52 slurry was allowed to settle in the columns and was washed with three column volumes of PSG. Infected mouse blood previously diluted with PSG was then applied to the top of the column and allowed to run into the cellulose. Approximately 10 g of wet DE 52 was used for each 1 ml of infected blood and this ratio determined the size and number of columns used. Trypanosomes were eluted from the column with several column volumes of PSG which was collected into a centrifuge tube cooled in ice.

The trypanosome concentration in the eluate was determined using the matching technique of Herbert and Lumsden (1976). A concentration of $1x10^9$ trypanosomes per ml was then obtained either by dilution with PSG or concentration by centrifugation. This final trypanosome suspension was either further diluted with PSG to the concentration required by specific experimental protocols or used to prepare stabilate material for cryopreservation in liquid N_2 (see section 3.3.2).

3.3.2 CRYOPRESERVATION OF TRYPANOSOME STOCKS

Stabilates were prepared to store viable trypanosomes for later use as a source of trypanosomes for infecting animals. The stabilates were prepared from trypanosome-infected mouse blood (at least 40 trypanosomes per field) collected into

heparinised tubes. Trypanosomes in blood at 4^oC were mixed in a 1:1 ratio with 20% v/v glycerol and drawn into capillary tubes until the tubes were 3/4 full. The tubes were sealed at one end using either heat or plugs of Cristaseal (Hawksley, England), placed in the vapour phase of a liquid N_2 tank overnight and then immersed into the liquid (East African Oxygen, Kenya limited) for storage. Column-separated trypanosomes prepared as described in section 3.3.1 at a concentration of $1x10^9$ trypanosomes per ml were cryopreserved as described above for trypanosome-infected mouse blood.

3.3.3 INFECTION

Column-separated trypanosome suspensions produced as described in section 3.3.1 were first diluted in PSG to a concentration of $4x10^6$ trypanosomes per ml. The trypanosome concentration was determined using a haemocytometer as described in section 3.3.3.1. This trypanosome concentration was chosen to standardise the inoculum at a reasonable size for the animals used.

Except where otherwise indicated in specific chapters, each rodent was infected with 0.25 ml of the trypanosome suspension which corresponds to $1x10^6$ trypanosomes. Where the experimental protocol required a lower trypanosome inoculum, the column-separated trypanosomes were appropriately diluted in order to maintain the 0.25 ml inoculum volume for each animal.

For infection of camels, intravenous inoculations were made into the jugular vein. Camels received inoculum volumes of 15 ml of the standard trypanosome stock ($4x10^6$ trypanosomes per ml); a volume corresponding to an inoculum dose of $6x10^7$ trypanosomes.

3.3.3.1 Haemocytometry

The concentration of the trypanosomes used for infection was determined by counting the parasites in a haemocytometer. Ten µl of PSG was put into the cavity of a haemocytometer slide (Neubauer, Improved Double Ruling Superior, West Germany) to maintain a moist environment and thus prevent the trypanosome suspension from drying. Five µl of a trypanosome suspension in PSG was then carefully transferred by touching a pipette tip onto the coverslip edge. For a successful transfer, the sample advanced evenly without any spills into the moat. Both cells on either sides of the haemocytometer were used as duplicates when counting. Using a microscope (x 400 magnification, Leitz Wetzlar SM-Lux, Germany) the number of trypanosomes in 16 small squares or one large square were counted. All trypanosomes

touching the left and upper borders were included. The total number of trypanosomes in the four squares were determined and the mean calculated.

The number of trypanosomes per ml was determined using the following formula:

= mean no. of tryps. x 10 x 1000 x dilution factor

Appropriate dilutions of the trypanosome suspension were made and the counting procedure repeated until the required trypanosome concentration in PSG was achieved.

3.3.4 MONITORING OF INFECTION

3.3.4.1 Parasitological monitoring

In order to monitor the course of infection, samples were collected regularly from animals. Blood was examined microscopically either as a wet blood smear on a slide (Baker 1970) or using the microhaematocrit centrifugation technique (Woo, 1970) as described below. Blood was collected daily from mice, rats and camels. In mice and rats a drop of tail blood was collected onto a slide or in a heparinised capillary tube. In camels, blood was collected from the jugular vein into 5 ml heparinised vacutainers.

Wet blood films were prepared by placing a small drop of blood on a clean, grease-free glass slide (75 x 25 mm) and overlaid with a coverslip (6 x 22 mm). The slide was then examined for the presence of trypanosomes in 20 fields at x 400 magnification. The condensor top was removed and the diaphragm closed to increase diffraction thus increasing contrast and thereby make it easier to detect parasites.

For the microhaematocrit centrifugation technique, heparinised glass capillary tubes were 3/4 filled with blood, sealed with Cristaseal at one end and centrifuged at 12,000 x g at room temperature for 5 minutes in a micro-haematocrit centrifuge (Hawksley, England). After centrifugation, the packed cell volume (PCV) of camel blood was determined by the use of a PCV reader (Hawksley, England). Then, using a diamond pencil, each capillary tube was cut at the buffy coat area where the leukocytes and the plasma interface. The cut ends of the capillary tube were then touched on a microscope glass slide to expel the contents of the buffy coat as well as the plasma and erythrocyte areas adjacent to the buffy coat. The resulting material was then overlaid with a coverslip and examined for trypanosomes in 20 fields at x 400 magnification in a microscope whose condensor top was out and the diaphragm closed.

If trypanosomes were not detected microscopically in camel wet blood films, 0.25 ml of blood was inoculated intraperitoneally into two mice. The mice were then microscopically examined for up to 60 days post-inoculation using the wet blood film technique.

3.3.4.2 Serological monitoring

Infection in camels was monitored serologically using an enzyme linked immunosorbent assay (ELISA) to detect trypanosomal antigens in serum. Blood was collected from the jugular vein into clean universal bottles and allowed to clot and separate overnight at room temperature. The separated serum was then decanted, aliquoted into 5 ml centrifuge tubes and centrifuged at 500 x g (Cryofuge 6.6 Heraeus, Christ, Germany) at 4°C for 10 minutes. The sera was then stored at -20^{0}C in 5 ml bijoux bottles.

The antigen ELISA test used was a modification of that used by Olaho-Mukani (1989) and Luckins (1991). In this method, ELISA plates (Dynatech Immulon 1, USA) were pre-sensitised by adding 100 μl of 1% v/v gluteraldehyde (see appendix 3) into each well and leaving it at room temperature for one hour. The wells were emptied by flipping the plate and then rinsed by flushing them three times with distilled water. Wells were then coated with a rabbit anti-*T. evansi* antibody diluted 1:3000 in carbonate-bicarbonate buffer pH 9.6 (appendix 4) by putting 100 μl per well and leaving it at 4°C overnight. The following morning, the plates were emptied and washed three times with PBS-Tween 20 washing buffer, pH 7.2 (see appendix 5). Plates were washed by filling the wells while taking care to avoid any bubbles, leaving them for 5 minutes and emptying them by flipping the plates over onto tissue until the wells were dry. Uncoated sites in the wells were blocked by filling the wells with 100 μl per well of the blocking buffer containing 1% v/v bovine serum albumin (BSA) in PBS-Tween-20 (appendix 6) and leaving them at 37^{0}C for half an hour. The plates were emptied and washed once with the washing buffer before 50 μl of diluting buffer (see appendix 5) was aliquoted into each of the wells. The first two wells of the first row (A1 and B1) were used as positive controls and received 50 μl of known positive serum. Wells C1 and D1 acted as negative control wells and received 50 μl of known negative control serum. All the other wells received 50 μl of undiluted test sera in duplicate. The plates were briefly shaken on a microshaker (Dynatech, U.S.A.), incubated at 37^{0}C for 30 minutes, flipped empty and washed three times with the washing buffer to remove unbound serum protein. Aliquots of 100 μl of the IgG fraction of rabbit anti-*T. evansi* antibody conjugated to horseradish peroxidase

(RATE/IgG/HRPO) diluted 1/100 with the diluting buffer was put in each well and incubated at 37^0C for 30 minutes. The plate was washed three times with the washing buffer. Finally, 100 µl of substrate solution was aliquoted into each of the wells and incubated at 37^0C for 15 minutes. The substrate used was 3, 3, 5, 5-tetramethylbenzidine (TMB) containing 3% v/v hydrogen peroxide (H_20_2) in substrate buffer (Na-acetate/citric acid pH 6.0) prepared as indicated in appendix 7. The reaction was stopped by adding to each well 50 µl of 2M sulphuric acid and the absorbance of the resulting colour reaction read on a micro ELISA plate reader. (Dynatech MR 580 USA) at a wavelength of 450 nm.

3.3.5 TRYPANOCIDAL DRUGS

Experimental infections were treated using suramin (Naganol^R Bayer, Leverkusen, Germany) Batch 7920 Z in all the rodents; suramin and Cymelarsan^R (Rhone Merieux, Lyon, France) Batch 2249 in camels. The drugs, which were in powder form, were diluted in sterile distilled water so that mice received the drug dose in a volume of 0.25 ml. For treating camels, the drugs were diluted according to the manufacturers recommendations of 10% w/v in sterile distilled water.

Suramin was administered intraperitoneally in mice and rats and intravenously in camels. The solutions were always prepared fresh. Mice and rats were treated 24 hours post-infection unless otherwise indicated in specific experimental protocols. Before each treatment, all animals were weighed to determine how much of the drug was to be given.

3.4 STATISTICS

The statistical tests used in the various experiments are described below.

3.4.1 RANKIT TEST

The Rankit test was used to test whether data is normally distributed and thus determine whether to use parametric or non-parametric statistical tests for analysing it. To perform this test, the data values were ranked from lowest to highest and tabular rankit values assigned to each rank. When observations were tied, their mean rankit value was determined and assigned to them. The rankit values were then plotted on the vertical axis (y) against the observed values in the horizontal axis (x). If the points were in a straight line, then the data was normally distributed and indicated the use of

parametric statistics. If the data points were not in a straight line, then the data was not normally distributed and only non-parametric statistics could be used.

3.4.2 KRUSKAL-WALLIS TEST

This test is used to compare data found in more than two independent groups which is not normally distributed to find out if variations between the groups are due to chance. The Kruskal-Wallis test measures the H value whereby:

$$H = \frac{[\ 12\ ]}{(\Sigma N(\Sigma N+1)} \frac{[(R_1{}^2) + ... + (R_k{}^2)]}{(N_2) \qquad (N_2)} - 3(\Sigma N+1)$$

A correction factor D was used when there were ties in the ranking.

$$D = 1 - \frac{[\Sigma V]}{[\Sigma N^3 - \Sigma N]}$$

where $\Sigma V = V_1 + V_2 + ...V_k$

$V_1 = (t_1{}^3 - t_1)$ etc.

t_1 = number of tied variates in the first set

The corrected H value when the variates were tied was thus calculated as

$$\text{corrected } H = \frac{H}{D}$$

where R is the ranks in each sample

N is the number of observations in each group

t_1, t_2 are the number of tied variates in the first and second ties respectively

The calculated H value is compared to the tabular value at 5 and 1 % - at the number of groups k and the number of observations per group n - and if it is larger, then the difference is significant.

3.4.3 MANN-WHITNEY U TEST

This test compares only two groups of data that is not normally distributed to determine whether the variation between the groups is due to chance or is significant. A significant difference is when the calculated U value is less than the tabulated value at 5% at n_1 and n_2 the number of observations in the smaller and larger sample sizes respectively.

The Mann-Whitney test determines U value where;

U is either U_1 when $U_1 < U_2$ or U_2 when $U_2 < U_1$

$$U_1 = n_1 . n_2 + \frac{n_1 . (n_1 + 1)}{2} - \Sigma R_1$$

$$U_2 = n_1 . n_2 + \frac{n_2 . (n_1 + 1)}{2} - \Sigma R_2$$

where n_1 = the smaller sample size

n_2 = the larger sample size

R_1 = sum of the ranks of the smaller sample

R_2 = sum of the ranks of the larger sample

3.4.4 CHI-SQUARE TEST

The chi-square test is a non-parametric test for determining whether the dispersion of data that is not normally distributed is due to chance and thus not significant. If the calculated value is more than the critical tabulated value of 5% at n-1 degrees of freedom, then the difference is significant. In this test, the chi-square value, x^2, was calculated where:

$$x^2 = \Sigma \frac{(O-E)^2}{E}$$

Where O is the observed value

E is the expected value

3.4.5 ONE-WAY ANALYSIS OF VARIANCE

The one-way analysis of variance compares two or more independent groups with normally distributed data. For each group, the sum, mean, and sums of mean squares were calculated as well as

the;

$$T\Sigma X = \Sigma x_1 + ... + \Sigma x_y$$

$$TN = k_1 + ... + k_y$$

$$T\Sigma X^2 = \Sigma x_1^2 + ... + \Sigma x_y^2$$

a correction factor:

$$C = \frac{T\Sigma X^2}{TN}$$

sums of squares:

$$SS = T\Sigma X^2 - C$$

Between groups sums of squares:

$$SS = \frac{(\Sigma x_1)^2}{k_1} + ... + \frac{(\Sigma x_y)^2}{k_y} - C$$

Within sums of squares (error) SS = Total SS - Between SS

Within groups degrees of freedom = (TN-1) - (y-1) where y is the number of groups.

$$\text{Between groups variance } s^2 = \frac{\text{Between groups SS}}{y - 1}$$

$$\text{Within groups variance} = s^2 \frac{\text{Within SS}}{\text{Within d.f.}}$$

The variance ratio F was then calculated:

$$F = \frac{s^2}{s^2}$$

Where;

ΣX is the total of the data in each group.
$T\Sigma X$ is the total ΣX values in all the groups.
k is the number of cases in each group.
TN is the sum of k values in all the groups.
ΣX^2 is the sums of squares.
$T\Sigma X^2$ is the total of ΣX^2 values in all the groups.
y is the number of groups.

If the calculated F value is greater than the tabular value at 5% and y-1 degrees of freedom, then the difference was significant.

3.4.6 SPEARMAN'S RANK CORRELATION

The Spearman's rank correlation test was used in data that is not normally distributed to determine whether one set of variables was dependent on another set of fixed variables. If the calculated r value was more than the tabulated one at 5%, then the rank correlation was significant meaning that the set of variables y were dependent on the fixed variables x.

$$r_s = \frac{(N^3-N) - (6)(\Sigma d^2) - (T_x+T_y)}{\gamma\,(N^3-N) - (T_x+T_y)(N^3-N) + T_x . T_y}$$

where:

N is the number of observations
Σd^2 is the sum of squares of the differences between the two rankings x and y
T_x is the value of ties among the x values - fixed independent variables
= (number of values tied)^2 - number of values tied x number of times tied
T_y is the value of ties among the y values - dependent variables
= (number of values tied)^2 - number of values tied y number of times tied

CHAPTER FOUR

STOCK CHARACTERISATION

4.1 INTRODUCTION

4.1.1 CHARACTERISATION

The importance of characterising trypanosomes is to identify the particular species involved in an infection in order to decide on appropriate control measures. To achieve this characterisation, various features either singly or more usually in combination have been used to identify trypanosomes. For example, *T. evansi* can be distinguished from *T. congolense*, *T. vivax* and *T. simiae* by the size and position of the kinetoplast and the presence of a conspicuous undulating membrane and free flagellum. The three tsetse-transmitted *Trypanozoon* species have been shown to contain two morphologically distinct trypanosome populations, namely short stumpy and long slender forms and are referred to as pleomorphic (Hoare, 1972). *T. evansi* does not normally exhibit pleomorphism and is described as being monomorphic with a mean length of 26 µm (Hoare, 1972). There is a wide range of length from 15 to 35 µm, but this is due to individual trypanosome variation and not the presence of distinct populations. These simple criteria for differentiation cannot be used distinguish reliably *T. evansi* from other members of the sub-genus *Trypanozoon*.

A more technical method used to distinguish *T. evansi* from the other members of the *Trypanozoon* sub-genus is isoenzyme analysis. Gibson *et al*, (1980, 1983) showed that certain isoenzyme patterns are distinctive for *T. evansi* and can therefore be used to differentiate it from other group members. More specific methods for identifying *T. evansi* are based on DNA analysis. One of the methods is based on the observation that *T. evansi* and *T. equiperdum* have only one class of homogeneous DNA in their kinetoplast (kDNA) namely the 1 kb mini circles. Other members of the *Trypanozoon* sub-genus have both heterogeneous 1 kb mini circles and larger kDNA fractions, the maxi circles (Borst and Hoeijmakers, 1979; Borst *et al*, 1987). One method of identifying *T. evansi* involves extracting kDNA and subsequently examining

it for the absence of maxi-circles and homogeneity of the 1 kb minicircles (Borst and Hoeijmakers, 1979; Borst *et al,* 1987). Two recent techniques applied to the characterisation of *T. evansi* involve the use of *T. evansi*-specific DNA sequences (Masiga and Gibson 1990; Masiga *et al,* 1992).

4.1.2 TRYPANOCIDE RESISTANCE

Characteristics such as trypanosome length, isoenzyme patterns and kDNA analysis are important in determining morphological, biological, genetic and epidemiological features. A characteristic of greater practical importance however, is the drug-sensitivity of trypanosome isolates. Despite the fact that a trypanocidal drug may be active against a particular trypanosome species, different stocks of that species may have varying sensitivities to the drug (Lumsden and Ketteridge, 1979). In the most sensitive stocks, all the trypanosomes are destroyed by the drug. In the less sensitive stocks, however, only a proportion of the trypanosomes are destroyed, thus leaving a population of organisms unaffected by the drug.

4.1.3 TESTING FOR RESISTANCE

Initial indication of trypanocide resistance in the field comes when animals do not respond to treatment. In such cases, the treated animals may have either persistent parasitaemia or clinical signs of infection. In extreme cases, the animals may die. The usual method of determining trypanocide resistance has been by testing drugs against trypanosome isolates in laboratory rodents (Godfrey and Killick-Kendrick, 1962). Rodents are inoculated with trypanosomes in the range of $1x10^5$ to $1x10^6$ (Abebe *et al,* 1983; Schillinger *et al,* 1985b; Peregrine *et al,* 1990; Zhang *et al,* 1992) and the trypanocide administered intraperitoneally at times such as 4, 6, 24 and 48 hours post-infection (Jones and Folkers, 1966; Abebe *et al,* 1983; Schillinger *et al,* 1985b; Peregrine *et al,* 1990; Zhang *et al,* 1992). The rodents are then examined regularly for parasitaemia using the wet-blood smear or the haematocrit centrifugation technique (Woo, 1970; Murray *et al,* 1977). Any persistent parasitaemia or relapse after treatment is taken as an indication of resistance to the trypanocide used. These methods have shown that there is trypanocide resistance in cameline *T. evansi* in Kenya (Gitatha, 1979; Rottcher and Schillinger, 1985) but the actual extent of the problem is not known.

Although rodents have been widely used in testing for trypanocide resistance, a major disadvantage of this traditional method is the neccessity of passaging the trypanosomes in rodents; a process which may result in differences between the original field and the laboratory passaged populations. There are also variations in the methodologies used and their possible effects on the success of treatment have not been adequately investigated. The number of trypanosomes exposed to the drug may be important especially in parasites of the *Trypanozoon* sub-genus which are able to invade the CNS (Innes and Saunders, 1962; Seiler *et al*, 1981). Once the trypanosomes invade the CNS, they are not accessible to drugs such as Berenil, Samorin, quinapyramine and suramin which cannot cross the blood-brain barrier. This is probably because these drugs are large water or lipid-soluble molecules which need active-transport to cross the blood-brain barrier into the CNS.

Although studies on drug resistance are usually carried out in experimental animals, there is an indication that biochemical techniques also might enable the identification of drug resistance. One such method is the identification of a specific isoenzyme marker which would discriminate between populations which are resistant to a particular drug and those that are sensitive to its action. Such a method would be superior to the rodent model because it would directly analyse the parasite avoiding any host effect. Boid *et al*, (1989) suggested that there was a linkage between the presence of a particular *T. evansi* malic enzyme pattern and suramin resistance. Such a linkage could be important in the development of a marker for resistance to suramin.

It was the purpose of this study to characterise a group of 44 *T. evansi* stocks in terms of their length and to determine whether they fitted the classical description of *T. evansi* (Hoare, 1972). Another aspect of the study looked at the spectrum of drug sensitivity against four trypanocidal drugs used in Kenya in circulating *T. evansi* populations. The potential of the malic enzyme as a marker for suramin resistance in *T. evansi* was evaluated. The effect of the number of trypanosomes inoculated into an animal on the pre-patent periods was investigated and the effect of trypanosome inoculum, dose rate and the timing of treatment to cures determined.

4.2 MATERIALS AND METHODS

4.2.1 PRE-PATENT PERIOD

The pre-patent period of each of the 44 isolates was determined in groups of five mice. Each mouse was inoculated intraperitoneally with 0.25 ml of column-separated trypanosomes diluted to a concentration of $4x10^6$ trypanosomes per ml using PSG (Appendix 1). Mice were checked daily until parasites were detected (section 3.3.4). For each isolate, the mean number of days until parasites were detected in the blood was recorded as the pre-patent period.

4.2.2 MEASUREMENT OF TRYPANOSOME LENGTH

When the mice became parasitaemic, a Giemsa's stained smear was prepared from blood obtained at the rising, the peak and the falling parasitaemic periods of the first wave. A thin-blood smear of the parasitaemic blood was made by placing a drop of blood on a clean grease-free slide and spreading it with a second slide placed at an angle of $30\text{-}45^o$. The slide was air-dried, fixed by briefly immersing it in methanol and stained by immersing it for 60 minutes in 10% v/v Giemsa's stain in Giemsa's buffer (see Appendix 8 and 9). After the slide was air-dried, the length of each trypanosome was measured by the eye-piece graticule method adapted after Opiyo (1984). In this method an ocular micrometer was calibrated by matching it to a stage micrometer to determine the length of each subdivision of the ocular micrometer (x 1000 magnification, Leitz Wetzlar, SM-Lux, Germany). A drop of immersion oil was placed on the slide and the stained thin-blood smear observed at x 1000 magnification. The total length of each trypanosome was determined by measuring a series of straight lines as illustrated in Figure 4.1. Starting from the posterior end of each trypanosome, the calibrated line of the ocular micrometer was aligned with the trypanosome at a point where the trypanosome curved away from the straight line. This length was recorded. The micrometer was next aligned to the trypanosome at the point at which the trypanosome curved away from the previous straight line to the next curvature and this length recorded. The process was repeated until the entire trypanosome, including the flagellum, was measured thus enabling its total length to be determined. For each trypanosome stock, the mean length of 300 parasites was determined; 100 trypanosomes each from the rising, the peak and the falling parasitaemic periods.

4.2.3 DETERMINATION OF DRUG SENSITIVITY

For determination of drug sensitivity, mice were inoculated with $1x10^6$ column-separated trypanosomes as described in section 3.3.3 and divided into four groups of six mice each. Five mice in each cage were treated with a trypanocidal drug 24 hours after infection (see 3.3.5). The sixth mouse remained as an untreated control. The dose rates of the drugs used shown in Table 4.1 were chosen because they are recommended by the respective manufacturers for the treatment of *T. evansi* infections in large animals. Where a group of mice was not cured at a dose rate of 5 mg/kg of suramin, the experiment was repeated using suramin at progressively higher dose rates of 10, 20, 40, 80 and 160 mg/kg. Mice were examined daily by wet film and weekly by haematocrit centrifugation technique over a period of 60 days for evidence of trypanosome infection (section 3.3.4).

Table 4.1 Trypanocides and doses used in determining the sensitivity of the 44 *T. evansi* stocks investigated.

Trypanocide	Dose in mg/kg body weight
Suramin^R	5, 10, 20, 40, 80, 160
Trypacide^R	5
Samorin^R	1
Berenil^R	7

4.2.4 MALIC ENZYME PATTERNS

4.2.4.1 Lysate preparation

Soluble trypanosomal lysates were prepared for isoenzyme electrophoresis work using a method modified after Wraxall and Culliford (1968), Gibson *et al,* (1980) and Boid (1988). For each stock, two rats were infected with stabilate material containing $1x10^6$ trypanosomes and the rats screened daily until a parasitaemia of 40 parasites per field (x 400 magnification) was observed. The trypanosomes were harvested and separated from blood elements by anion-exchange chromatography (Lanham and Godfrey, 1970). The column eluate was then pelleted by centrifugation for 10 minutes at 1000 x g (MSE Chilspin 2, Fisons, England). Trypanosomes were lysed using an equal volume of a lysing cocktail (appendix 10) and the resulting lysate was freeze-thawed (-80^{0}C / room temperature) three times and then homogenised inside the barrel of a Pasteur pipette. The homogenate was centrifuged at 34,000 x g

(Beckman L8 -70m) and the supernatant formed into beads by dropping 10 µl amounts in liquid nitrogen. Trypanosomal lysate beads were kept at -196^{0}C until required for electrophoresis.

4.2.4.2 Electrophoresis

The malic enzyme banding patterns of all trypanosome stocks were determined by electrophoresis of lysate material using a method adapted after Gibson *et al,* (1980) and Boid (1988). An 8.3% w/v starch gel in 0.016M phosphate buffer pH 7.4 (Appendix 12) was made by slowly heating the starch-buffer solution with regular swirling in a side-arm flask. The solution gradually thickened, became opaque and then became clear. The solution was then de-aerated by plugging the neck of the flask, attaching its side-arm to a vacuum pump (Speedivac ES 35 Edwards high vacuum Ltd, England) and degassed until only large bubbles formed in the solution. The solution was then poured onto a glass plate (22 cm x 15 cm) edged with perspex strips (5 x 0.8 mm) and allowed to cool and set for 10 minutes at room temperature and for 60 minutes at 4^{0}C. A 10-slot sample-comb was then pressed into the gel 7 cm from the cathodal end of the starch gel to make sample wells. The beaded lysate samples were removed from -196^{0}C and placed in microtitre plate wells (Dynatech Immulon USA) to thaw at 4^{0}C. Cotton threads, cut to 5 mm lengths, were placed in the wells (two per well) and left for 5 minutes to absorb the lysate, after which they were carefully loaded into the wells of the gel. The plate was then covered with a similar glass plate in order to sandwich the starch gel. An electrophoresis tank with 0.2M phosphate buffer pH 7.4 (Appendix 11) was set up as illustrated in Figure 4.2. Spontex wicks were placed onto a cooling plate with the wick ends hanging into the tank buffer. A glass plate was then placed on the wicks to hold them in place and the gel which was held between the two glass plates placed on top with the wells close to the cathode end. Electrophoresis was performed at a constant 300 Volts for 3 hours while maintaining the temperature of the gel at 8^{0}C with a cooling unit (Grant Camlab Ltd Cambridge, England). Malic acid developer solution (appendix 13) was added to 10 ml of 1.2% warm agar solution and used to overlay the starch gel which was then incubated at 37^{0}C (Microtitre incubator, Dynatech) in the dark. The overlaid starch gel was checked regularly until the enzyme bands were judged to be optimally developed. The enzyme reaction proceeded according to the following equation:

$$\text{L-Malate} + \text{NADP} = \text{pyruvate} + CO_2 + \text{NADPH} + H^+$$

A colour reaction was observed due to the formation of a formazan dye as indicated in the staining reaction illustrated in Figure 2.9. The presence of enzymic activity was

indicated by the appearance of dark-blue bands against a yellow background. Isoenzyme banding patterns were classified according to the criteria of Gibson *et al,* (1980) and Godfrey *et al,* (1990). The gel bands were photographed using a Kodak yellow filter (Wratten 15, Sigma Chemical Company).

4.2.5 COMPARISON OF TREATMENT REGIMES

Three *T. evansi* stocks each with different sensitivities to suramin were used in this experiment. KETRI 2454 was sensitive to suramin at dose rates as low as 0.01 mg/kg (see section 6.2.1), and represented a stock which is very sensitive to suramin. KETRI 2455 resistant to suramin at a dose rate of 10 mg/kg represented a stock of medium sensitivity and KETRI 3136 resistant to suramin at a dose rate of 160 mg/kg represented a stock of high resistance. For each of the three stocks, groups of ten mice were inoculated intraperitoneally with $1x10^3$, $1x10^4$ and $1x10^6$ trypanosomes per mouse. For each group of mice, drug-treatment was administered at five different times:- 0, 24 and 48 hours post infection, at the onset of parasitaemia as detected by the microhaematocrit centrifugation technique and at the onset of parasitaemia as detected by the examination of wet blood films (section 3.3.4).

Mice infected with KETRI 2454, were treated with suramin at dose rates of 0.05, 0.1, 1 and 5 mg/kg. Mice infected with KETRI 2455 were treated with suramin at dose rates of 1, 5, 10 and 20 mg/kg. Mice infected with KETRI 3136 were treated with suramin at dose rates of 5, 10, 20, 40 and 80 mg/kg. For KETRI 3136, 50 mice were used for each treatment regimen and suramin dose level. A total of 750 mice were thus used for the three trypanosome inoculum doses as shown in Figure 4.3. For KETRI 2454 and 2455, a total of 600 mice were used per stock since only four suramin dose rates were used. In every cage, there were ten mice which had the same trypanosome inoculum, suramin dose and treatment regimen.

In order to determine cures, all mice were screened daily for parasites by the wet blood film technique and three times a week by the haematocrit centrifugation technique for 60 days post treatment. Activity indices, defined as the number of mice out of ten which did not develop parasitaemia during the 60 day observation period, were used to represent the suramin sensitivities of the three stocks. For each stock, activity indices were determined for each combination of trypanosome inoculum, suramin dose and time of treatment.

4.2.6 STATISTICAL PROCEDURES

4.2.6.1 Pre-patent periods, trypanosome lengths and malic enzyme patterns

The modes, means and ranges of the pre-patent periods and trypanosome lengths were calculated as well as their geographical distributions. The geographical distribution of the malic enzyme patterns of the 44 isolates was also determined.

The Rankit test was used to determine if the pre-patent periods and the mean trypanosome lengths were normally distributed (section 3.4.1). To find out if the mean pre-patent periods and mean trypanosome lengths were related to the origins of the 44 stocks, the Kruskal-Wallis test and the one-way analysis of variance respectively were used. These three tests are described in 3.4.1, 3.4.2 and 3.4.5 respectively.

4.2.6.2 Comparison of treatment regimes

The Rankit test was used to determine if the activity indices were normally distributed in order to decide if parametric or non-parametric statistical procedures were appropriate. The Kruskal-Wallis test (section 3.4.2) was then used to determine whether there was a significant difference in the activity indices between the treatment times and the trypanosome inoculum dose. Where a significant difference was indicated by the Kruskal-Wallis test, the Mann-Whitney test (section 3.4.3) was used to identify where the difference was by comparing any two groups of mice which either received the same number of trypanosomes or were treated at the same time. In order to determine whether there was a difference in the pre-patent periods of the three stocks at the three trypanosome inocula using the two methods of parasite detection, the chi-square test (section 3.4.4) was used.

The Spearman's rank correlation test (section 3.4.6) was used to determine if there was any relationship between the number of trypanosomes inoculated and the pre-patent periods. For each of the three stocks, trypanosome inoculum dose was also plotted against the median values in order to graphically present this relationship.

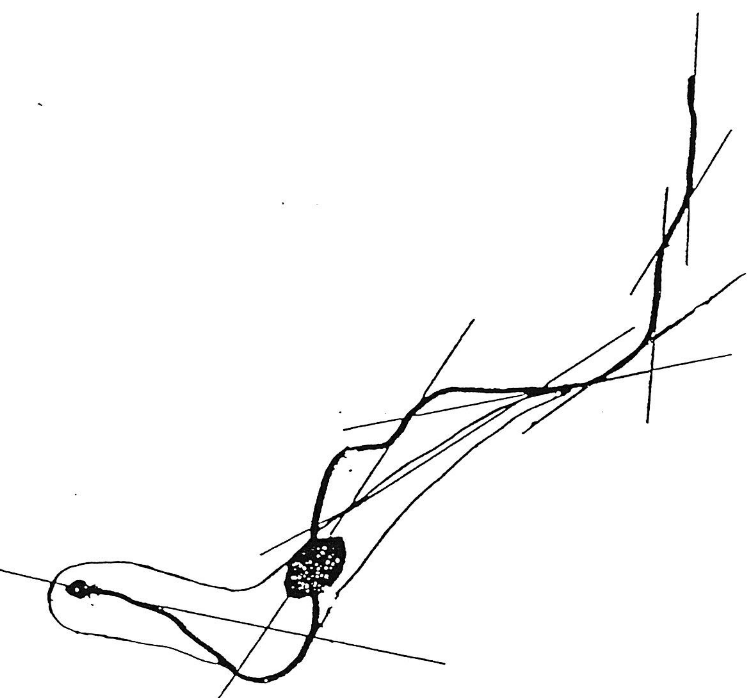


Figure 4.1 Illustration of how trypanosome length was measured.

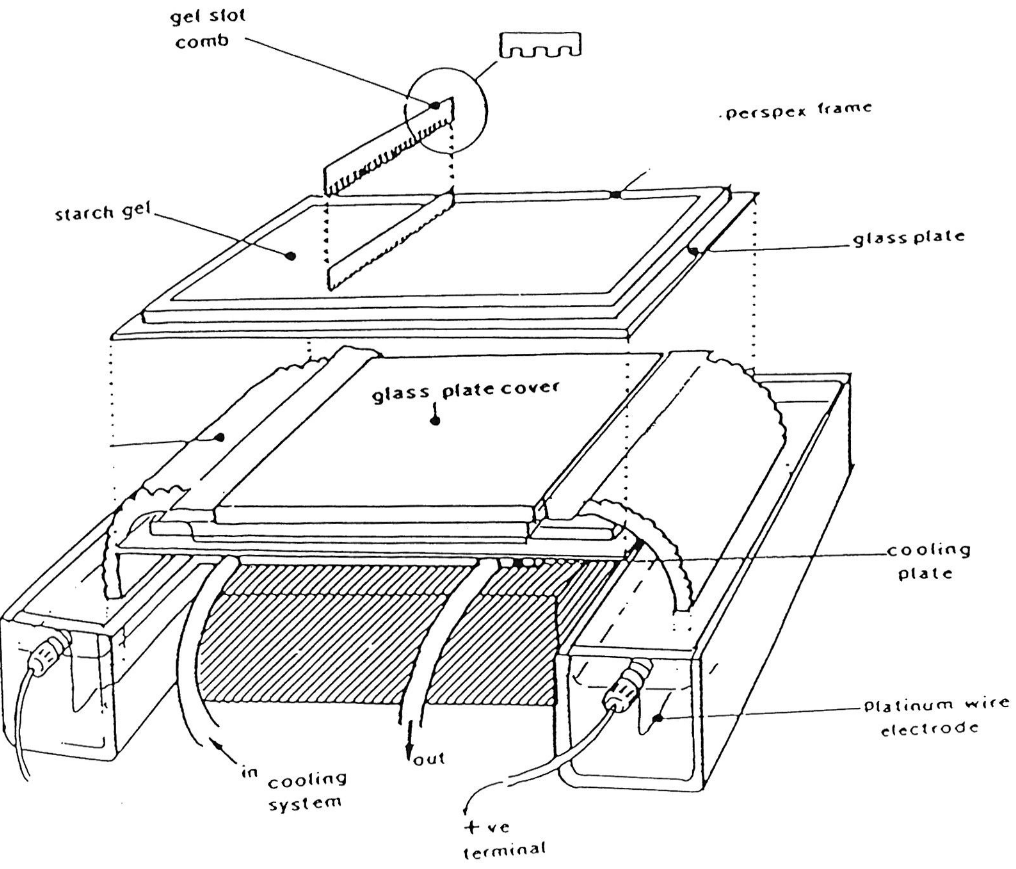
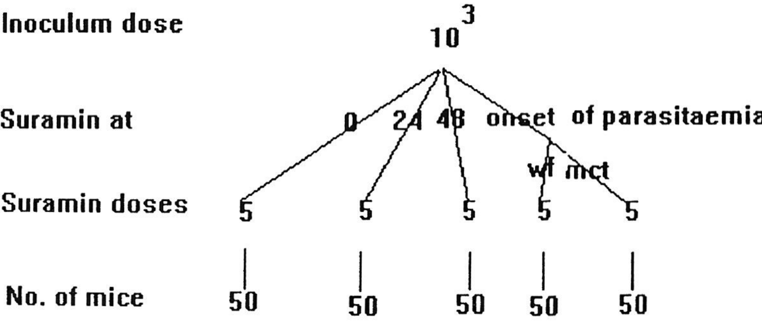


Figure 4.2 The set-up for isoenzyme electrophoresis.



Legend: 0, 24, 48 - hours after infection when suramin was administered.
5 - number of suramin dose rates in mg/kg body weight.
wf - detection of parasitaemia by wet blood film technique.
mct - detection of parasitaemia by microhaematocrit centrifugation technique.

Figure 4.3 Experimental protocol for comparison of treatment regimes in *T. evansi* KETRI 3136.

4.3 RESULTS

Table 4.2 Mean pre-patent periods, mean lengths and malic enzyme patterns of 44 *T. evansi* stocks in the KETRI bank.

KETRI No.	Origin of isolate	Mean pre-patent period, standard deviation and (range) in days	Mean length in μm and SD	Malic enzyme pattern
2448	Galana	8	25.2 ± 0.8	X
2449	Galana	6	29 ± 0.6	X
2450	Galana	5 ± 3.16 (2 - 10)	23.9 ± 1.2	X
2451	Galana	6	27.7 ± 0.7	II
2455	Galana	6 ± 2.45 (2 - 8)	27.4 ± 1.1	II
2458	Galana	5	22.1 ± 1.2	II
2489	Galana	5 ± 0.71 (4 - 6)	27.7 ± 1.4	II
1188	Garissa	6	26.0 ± 0.9	IV
1377	Garissa	2	30.0 ± 0.7	IV
2416	Garissa	4	33.1 ± 0.8	IV
2439	Marsabit	3	25.9 ± 1.2	X
2440	Marsabit	6	27.3 ± 0.9	II
2441	Marsabit	3	22.0 ± 1.8	II
2442	Marsabit	20 ± 2.55 (17 - 24)	26.2 ± 1.1	II
2443	Marsabit	4	25.4 ± 1.5	X
2444	Marsabit	7	27.0 ± 0.4	II
2446	Marsabit	4	22.0 ± 2.1	X
2447	Marsabit	6	27.8 ± 0.7	II
2453	Marsabit	3	23.3 ± 2.3	X
2454	Marsabit	3	26.1 ± 0.6	II
2456	Marsabit	5	20.0 ± 2.4	II

2457	Marsabit	4	25.2 ± 0.8	II
2465	Marsabit	3	22.4 ± 1.1	II
2466	Marsabit	4	23.0 ± 1.2	II
2467	Marsabit	4	25.4 ± 0.6	II
2468	Marsabit	4	24.6 ± 1.0	II
2469	Marsabit	2	26.0 ± 1.0	II
2470	Marsabit	2	22.0 ± 1.5	II
2471	Marsabit	2	24.3 ± 0.8	II
2477	Marsabit	2	24.0 ± 1.1	II
2481	Marsabit	4	23.8 ± 1.1	II
2462	Ngurunit	4 ± 1.41 (2 - 6)	24.2 ± 1.5	II
2476	Ngurunit	3.8 ± 1.10 (2 - 5)	22.9 ± 0.8	II
2480	Ngurunit	2	26.1 ± 0.9	II
2485	Ol Maisor	5	26.4 ± 1.4	X
2488	Ol Maisor	2	26.0 ± 1.5	II
2552	Rumuruti	3	27.0 ± 0.7	II
2415	Ukunda	16	30.1 ± 1.5	II
2424	Ukunda	7 ± 1.0 (6 - 8)	28.0 ± 1.2	X
2626	Ukunda	4 ± 0.7 (3 - 5)	26.2 ± 1.7	X
2429	Ukunda	2	27.0 ± 1.8	II
2472	Khartoum	2	28.0 ± 0.9	VII
3136	Kassala	5	29.4 ± 1.1	VII
2540	Colombia	4	19.4 ± 0.8	II

Note: The standard deviation (SD) of the pre-patent periods is 0 unless otherwise indicated.

4.3.1 PRE-PATENT PERIODS

The mean pre-patent periods of the 44 *T. evansi* stocks examined varied from 2 to 20 days with an overall mean of 4.7 ± 3.3 days (Table 4.3). With most isolates the pre-patent period was 4 days, as found in 13 stocks from Garissa, Ukunda, Marsabit and Ngurunit as well as in the stock from South America (KETRI 2540). The three longest pre-patent periods - 8, 16 and 20 days - were found in stocks from Galana, Ukunda and Marsabit (KETRI 2448, 2415 and 2442 respectively). There was no significant difference ($p > 0.05$) between the pre-patent periods of stocks from various parts of Kenya or between the stocks from Kenya and those from other geographical areas.

The distribution of the pre-patent periods shows that the most frequent pre-patent period is 4 days found in 12 of the 44 stocks(Figure 4.4). A pre-patent period of 2 days was observed in 9 stocks while pre-patent periods of 3, 5 and 6 days were found in six stocks each. Two stocks took 7 days to be patent and one stock each took 8, 16 and 20 days. The frequencies of the pre-patent periods observed is skewed to the left showing that the majority of stocks had pre-patent periods of less than six days.

Table 4.3 Summary statistics of pre-patent periods of the 44 *T. evansi* stocks

	Mean	S.D.	Median	Mode	Range in days
Pre-patent period in days	4.7	3.4	4	4	2-20

4.3.2 TRYPANOSOME LENGTHS

All of the stocks examined were monomorphic with lengths ranging from 19 to 33 µm and a mean length of 25.4 ± 2.8 µm (Table 4.4). The stock with the longest trypanosomes (33 µm) came from Garissa (KETRI 2416) while the other two stocks from this area had lengths of 26 and 30 µm. The stock with the shortest trypanosome population (19 µm) was KETRI 2540, the stock from Colombia. The mean length of most stocks was 26 µm as observed in stocks from Marsabit (4 stocks), Ol Maisor (both stocks), Garissa, Ukunda and Ngurunit (one stock each). Of the 21 stocks from Marsabit, the frequency of trypanosomes with a mean length of 22, 25 and 26 µm was four; the frequency of 24 µm, three; the frequency of 23 and 27 µm, two; and the frequency of 20 and 28 µm, one. Two stocks from Galana had a mean length of 28 µm while the other five stocks ranged between 22 and 29 µm. The four stocks from Ukunda had mean lengths ranging from 26 to 30 µm while both stocks from Ol Maisor

had mean lengths of 26 μm. The mean lengths of the three stocks from Ngurunit were 23, 24, 26 and the stock from Rumuruti had a mean length of 27 μm. KETRI 2472 and 3136, the two stocks from Sudan, had mean lengths of 28 and 29 μm respectively.

If the high frequency of 22 μm length is ignored, the mean lengths of the Kenyan stocks are normally distributed (Figure 4.5). Using the Kruskal-Wallis test, no significant difference ($p > 0.05$) was found between the mean lengths of the stocks from different areas in Kenya and there was no significant difference between the stocks from Kenya and those from other geographical regions.

Table 4.4 Summary statistics of trypanosome lengths (μm) of the 44 *T. evansi* stocks

	Mean	S.D.	Median	Mode	Range
Length in μm	25.5	2.8	26	26	19-33

4.3.3 MALIC ENZYME PATTERNS

Four distinct isoenzyme patterns of malic enzyme were found among the 44 trypanosome stocks examined (Figure 4.6). All of these patterns had a characteristic large i band with pattern IV having two additional large bands h and g (Figure 4.7). In addition to the large bands, patterns II, IV and VII also had small bands designated as a b or c. Pattern X was the only one without a small band.

Pattern II, consisting of a large band i and three small bands a, b and c, was the most common being found in 29 of the stocks from all areas in Kenya except Garissa, and in the South American stock (Table 4.5). Seventeen stocks from Marsabit had this pattern, four from Galana, three from Ngurunit, two from Ukunda and one stock each from Ol Maisor, Rumuruti and Colombia. Pattern X, consisting of a single large band i, was the second most common pattern found in 10 stocks from Marsabit (four stocks), Galana (three stocks), Ukunda (two stocks) and Ol Maisor (one stock). Malic isoenzyme patterns II and X were found together in all the areas in Kenya except Garissa and Rumuruti. Only the three stocks from Garissa had three large bands g, h and i and a small band c which make up malic enzyme IV pattern. Pattern VII consisting of one large i band and a small band a was found only in the two stocks from Sudan. Table 4.5 shows the distribution of these patterns in the different stocks.

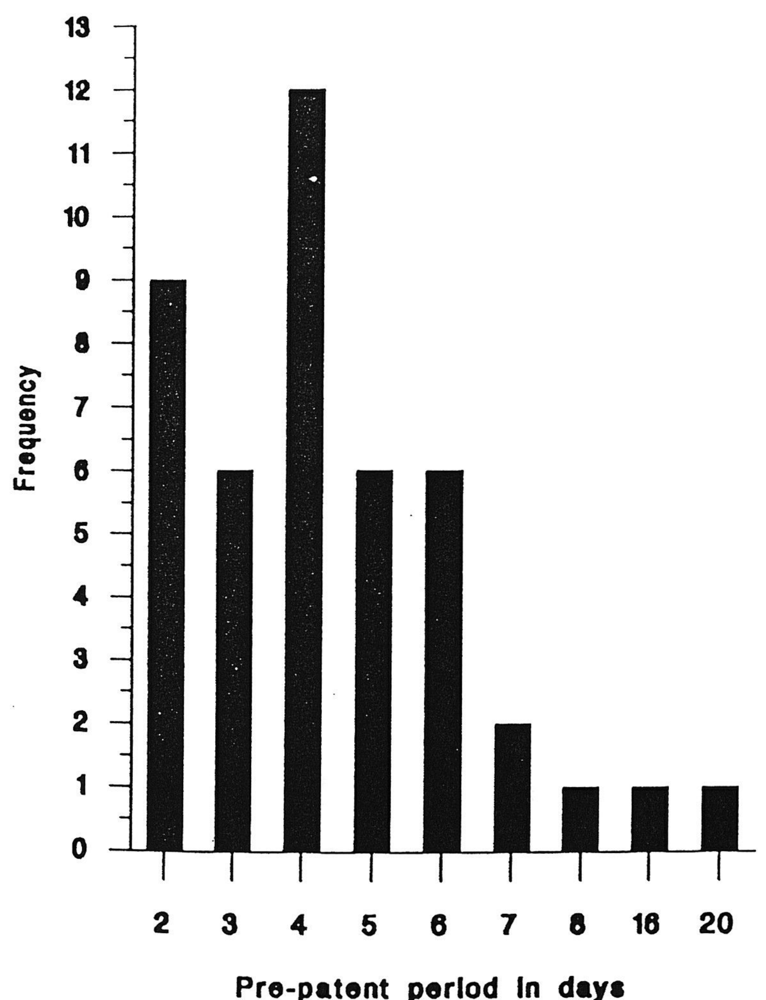


Figure 4.4 Frequencies of pre-patent periods in 44 stocks of *T. evansi*.

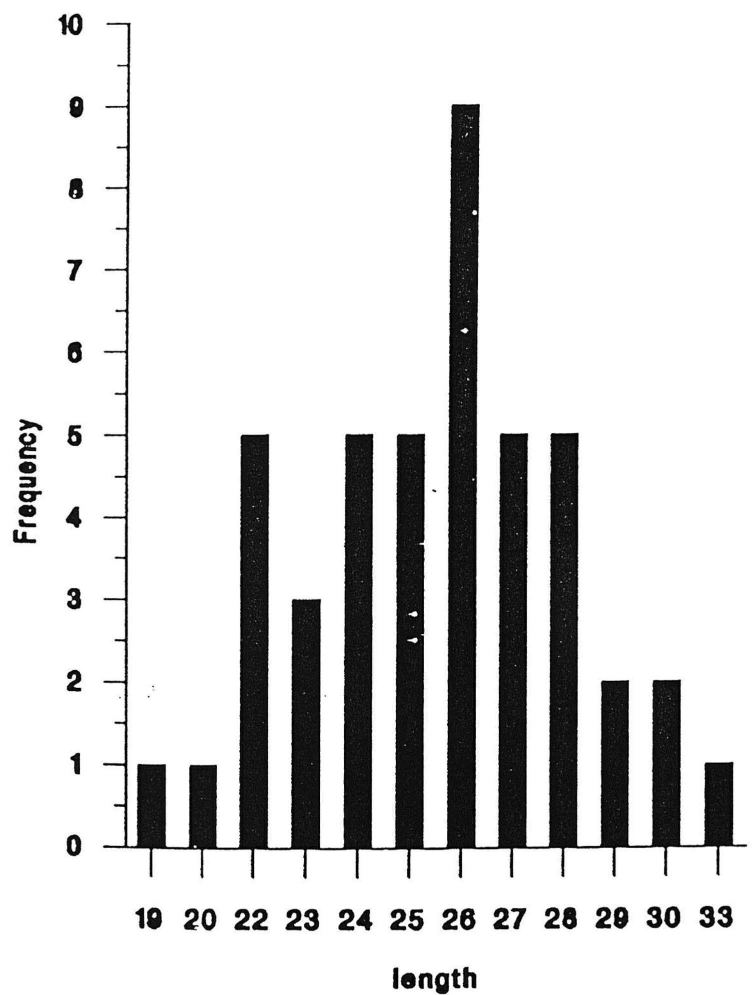
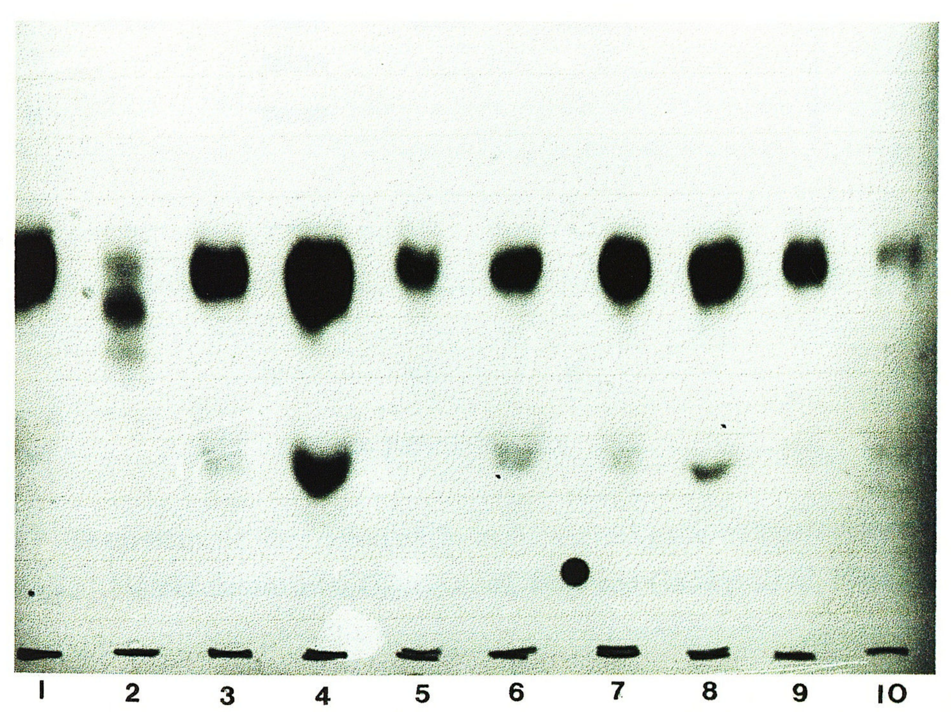


Figure 4.5 Frequencies of trypanosome lengths in 44 stocks of *T. evansi*

Figure 4.6 Photographs of the malic enzyme patterns seen in the 44 *T. evansi* stocks. Pattern II lanes 1, 11, 13, 14 and 16; Pattern IV lane 12; Pattern VII lanes 3, 4, 5, 6, 7 and 8, Pattern X lane 15.


1
2
3
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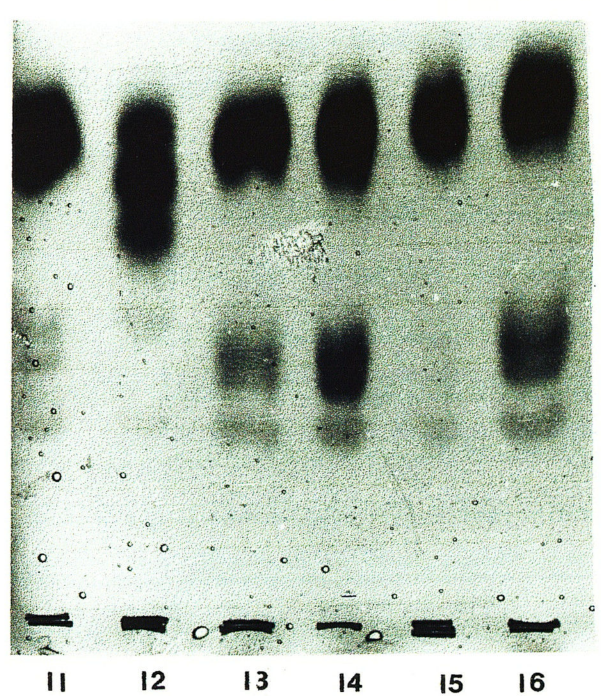
10


11
12
13
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15
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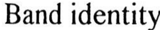


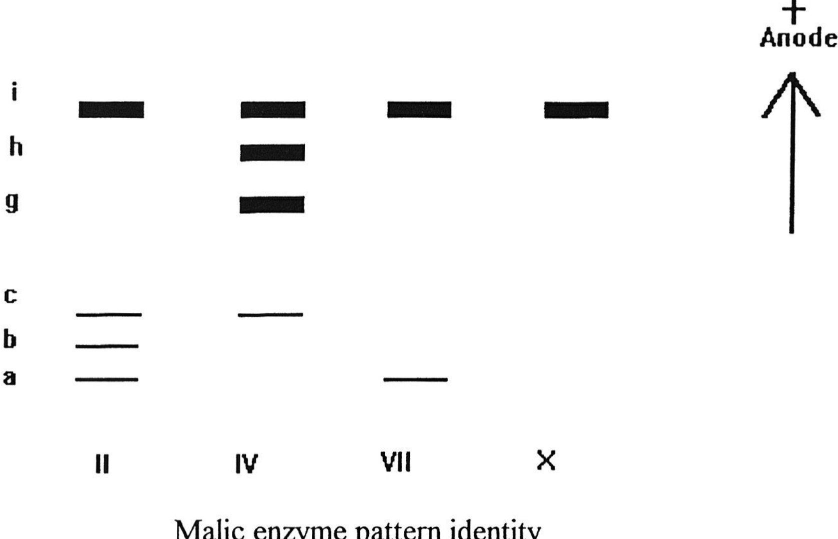


Figure 4.7 Diagrammatic illustration of the malic enzyme patterns seen in the 44 *T. evansi* stocks. Nomenclature after Gibson *et al*, 1980 and Godfrey *et al*, 1990

Table 4.5 The distribution of the four malic enzyme patterns in the various areas

	Frequency - number of stocks with			
	Pattern II	Pattern IV	Pattern VII	Pattern X
Area of origin				
Galana	4			3
Garissa		3		
Marsabit	17			4
Ngurunit	3			
Ol Maisor	1			1
Rumuruti	1			
Ukunda	2			2
Sudan			2	
Colombia S. America	1			
Total	29	3	2	10

4.3.4 TRYPANOCIDE SENSITIVITY

Twelve of the *T. evansi* stocks examined (Table 4.6 and Figure 4.8) were sensitive to the action of the four trypanocides tested at the stated dose rates; Berenil at 7 mg/kg, suramin at 5 mg/kg, Samorin at 1 mg/kg and Trypacide at 5 mg/kg. These stocks originated from Marsabit (four), Garissa, Galana (two stocks from each area), Ngurunit, Ol Maisor, Rumuruti, and Colombia (one stock from each area). The proportion of the Kenyan sensitive stocks in respect to the total number of stocks from each area is shown in Figure 4.8 Garrisa, Ol Maisor and Rumuruti had the highest proportion of stocks sensitive to all trypanocides tested. There were, however, only three, two and one stock isolated from the three areas respectively. One of the three stocks from Rumuruti was sensitive to the trypanocides used. Almost 30% of the stocks isolated from Galana were sensitive to the four drugs compared to 20% of stocks from Marsabit. The other 32 stocks (Table 4.7) were resistant to at least one of the trypanocides tested.

When considering individual trypanocides (Table 4.8), 25 stocks (57%) were resistant to 7 mg/kg of Berenil; 17 were from Marsabit, three from Ukunda, two from Ngurunit and one each from Galana, Garissa and Ol Maisor. Six (14%) and four stocks (9%) were resistant to suramin at 5 and 10 mg/kg respectively. Three stocks from Galana, and one each from Marsabit, Ol Maisor and Ukunda were resistant to a suramin dose rate of 5 mg/kg. Two stocks resistant to suramin at 10 mg/kg were from the Sudan and the other two stocks came from Galana. As shown in Table 4.9, the maximum suramin resistance levels of the two stocks from Galana were shown to be 10 and 15 mg/kg for KETRI 2455 and 2451 respectively while the two stocks from Sudan were resistant at 160 mg/kg. Of the three stocks (7%) resistant to Samorin at 1 mg/kg, one was from Garissa and two from Ukunda. There were 8 stocks (18%) resistant to Trypacide at 5 mg/kg body weight; six from Marsabit, and one each from Ol Maisor and Ukunda.

Twelve stocks were resistant to more than one trypanocide. Two stocks (KETRI 2426, and 2451 from Ukunda and Galana respectively) were resistant to Berenil and suramin. KETRI 1188 from Garissa, and KETRI 2424 from Ukunda were resistant to both Berenil and Samorin. Five stocks from Marsabit (KETRI 2442, 2453, 2454, 2469 and 2477) and one from Ukunda (KETRI 2429) were resistant to Berenil and Trypacide. No stocks were resistant to both suramin and Trypacide or combinations of Samorin with either suramin or Trypacide. Two stocks, KETRI 2465 and 2488 from Marsabit and Ol Maisor respectively, were resistant to Berenil, suramin and Trypacide. None of the stocks was resistant to all four trypanocides. Figure 4.9 shows the distribution of trypanocide resistance in the stocks from Kenya.

Table 4.6 Twelve *T. evansi* stocks which were sensitive to Trypacide, suramin, Samorin and Berenil.

Isolate KETRI no.	Origin of isolate
2448	Galana
2489	Galana
1377	Garissa
2416	Garissa
2439	Marsabit
2444	Marsabit
2446	Marsabit
2467	Marsabit
2462	Ngurunit
2485	Ol Maisor
2552	Rumuruti
2540	Colombia

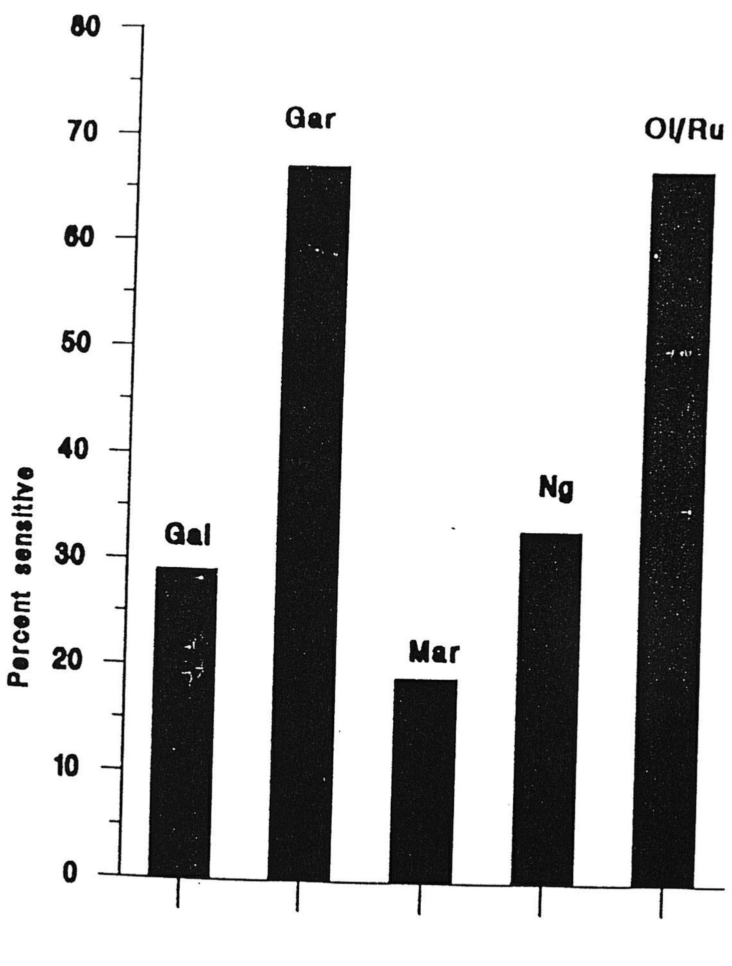


Figure 4.8 The distribution of Kenyan *T. evansi* stocks sensitive to all four trypanocides; Berenil, Samorin, suramin and Trypacide.

Table 4.7 Sensitivities of 32 *T. evansi* stocks resistant to at least one trypanocide.

Isolate KETRI no.	Origin of isolate	Trypacide 5 mg/kg	Suramin 5 mg/kg	Suramin 10 mg/kg	Samorin 1 mg/kg	Berenil 7 mg/kg
2449	Galana	S	R	S	S	S
2450	Galana	S	R	S	S	S
2451	Galana	S	-	R	S	R
2455	Galana	S	-	R	S	S
2458	Galana	S	R	S	S	S
1188	Garissa	S	S		R	R
2440	Marsabit	S	S		S	R
2441	Marsabit	S	S		S	R
2442	Marsabit	R	S		S	R
2443	Marsabit	S	S		S	R
2447	Marsabit	S	S		S	R
2453	Marsabit	R	S		S	R
2454	Marsabit	R	S		S	R
2456	Marsabit	S	S		S	R
2457	Marsabit	S	S		S	R
2465	Marsabit	R	R	S	S	R
2466	Marsabit	S	S		S	R
2468	Marsabit	S	S		S	R
2469	Marsabit	R	S		S	R
2470	Marsabit	S	S		S	R
2471	Marsabit	S	S		S	R
2477	Marsabit	R	S		S	R

2481	Marsabit	S	S		S	R
2476	Ngurunit	S	S		S	R
2480	Ngurunit	S	S		S	R
2488	Ol Maisor	R	R	S	S	R
2415	Ukunda	S	S		R	S
2424	Ukunda	S	S		R	R
2426	Ukunda	S	R	S	S	R
2429	Ukunda	R	S		S	R
2472	Khartoum	S	-	R	S	S
3136	Kassala	S	-	R	S	S
Total resistant		8	6	4	3	25

Legend: S - Sensitive

R - Resistant

\- Resistant to suramin at this level and at the higher level

Table 4.8 The numbers and proportions of *T. evansi* stocks resistant to the trypanocidal drugs used.

Trypanocide	Dose in mg/kg	No. of stocks resistant	% of stocks resistant
Suramin	5	6	13.6
	10	4	9.1
Trypacide	5	8	18.2
Samorin	1	3	6.8
Berenil	7	25	56.8

Table 4.9 Sensitivities of *T. evansi* stocks resistant to suramin at 10 mg/kg

KETRI no.	Suramin resistance in mg/kg
2451	15
2455	10
2472	160
3133	160

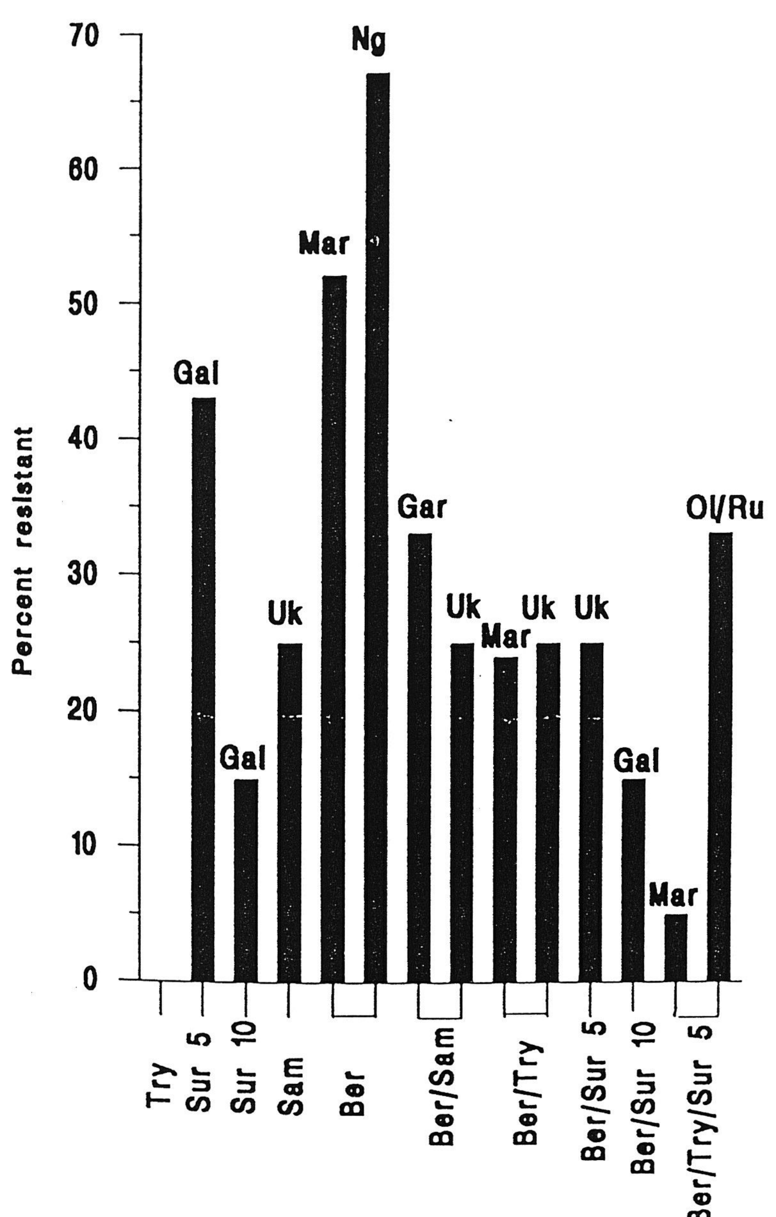


Figure 4.9 The distribution of trypanocide resistance in *T. evansi* stocks from Kenya.

4.3.5 DURATION OF PRE-PATENT PERIODS IN RELATION TO *T. EVANSI* INOCULUM

The mean pre-patent periods of the three stocks KETRI 2454, 2455 and 3136 are shown in Table 4.10. The Rankit test showed that pre-patent periods were not normally distributed and subsequent statistical analysis was based on non-parametric methods.

When using KETRI 2454, the onset of parasitaemia was detected earlier as the size of the inoculum increased. The relationship between pre-patent period and inoculum (Figure 4.10) was a negative correlation which was statistically highly significant ($p < 0.001$; Table 4.11). At an inoculum of $1x10^4$, trypanosomes were detected earlier when using the micro-haematocrit centrifugation than when using the wet film technique. At the other trypanosome inoculum doses, there was no difference in the time parasites were detected when using the wet film technique as compared to the microhaematocrit centrifugation. These differences in time of detection of parasitaemia were not significant by the chi-square test ($p > 0.05$).

Regardless of the size of inoculum, parasites were detected earlier when using the micro-haematocrit centrifugation compared with the wet film technique in KETRI 2455. There was, however, no significant difference ($p > 0.05$) in the pre-patent period as detected by the two methods when compared by the chi-square test. As with KETRI 2454, the pre-patent period decreased with increased trypanosome inoculum in a highly significant ($p < 0.001$) negative correlation (see Table 4.11). The correlation between size of inoculum and pre-patent period is shown in Figure 4.11.

In KETRI 3136, when $1x10^3$ or $1x10^4$ trypanosomes were inoculated, parasitaemia was detected earlier by microhaematocrit centrifugation than by the wet film technique. This difference however was not significant ($p > 0.05$) when compared by the chi-square test. The mean pre-patent periods were identical using the two methods of parasite detection when $1x10^6$ trypanosomes were inoculated. As with the other two stocks, the relationship between size of inoculum and pre-patent period was highly significant ($p < 0.001$ see Table 4.11). This correlation is shown in Figure 4.12.

KETRI 2454 had the shortest mean pre-patent period while KETRI 2455 had the longest one. The longest pre-patent period was detected by wet blood smear 42 days after a mouse was inoculated with KETRI 2455. These differences between pre-patent periods of the three stocks were not significant ($p > 0.05$). In KETRI 2454 and 3136, the correlation (r_s) between the size of inoculum and pre-patent period was

lower when the onset of parasitaemia was detected by the microhaematocrit centrifugation technique compared with the wet film technique. For KETRI 2455, the reverse was the case and the correlation was higher when the onset of parasitaemia was detected by the microhaematocrit technique. In spite of the smaller difference in trypanosome numbers between the two lower inocula $1x10^3$ and $1x10^4$, the slope between them is steeper than the slope between $1x10^4$ and $1x10^6$ (see Figure 4.10, 4.11 and 4.12). This suggests that the effect of the trypanosome inoculum on pre-patent period is greater at lower inoculum doses.

Table 4.10 Mean pre-patent periods and ranges (in days) as determined by microscopic examination of wet blood films or micro-haematocrit centrifugation technique of three isolates of *T. evansi* using different trypanosome inocula.

Stock	Detection	Trypanosome inoculum					
KETRI no.	method	$1x10^3$		$1x10^4$		$1x10^6$	
		Mean	(Range)	Mean	(Range)	Mean	(Range)
2454	omct	11.5	(7 - 22)	5.5	(4 - 11)	3.0	(2 - 6)
	owf	11.0	(5 - 23)	5.8	(4 - 9)	2.8	(2 - 6)
2455	omct	8.7	(5 - 17)	7.3	(2 - 14)	5.6	(3 - 8)
	owf	10.0	(4 - 42)	8.5	(4 - 13)	6.4	(3 -10)
3136	omct	7.9	(5 - 21)	4.5	(3 - 9)	4.8	(2 - 8)
	owf	16.2	(8 - 25)	6.4	(4 - 14)	4.8	(2 - 7)

Legend: omct - onset of parasitaemia as detected by the micro-haematocrit centrifugation technique.

owf - onset of parasitaemia as detected by the examination of wet blood film.

Table 4.11 Correlation between size of trypanosome inoculum and pre-patent period using Spearman›s coefficient.

Isolate KETRI no.	Onset detected by	r_S value	Difference
2454	omct	-0.89	* *
	owf	-0.95	* *
2455	omct	-0.83	* *
	owf	-0.59	* *
3136	omct	-0.67	* *
	owf	-0.83	* *

Legend: omct - onset of parasitaemia by microhaematocrit centrifugation technique.

owf - onset of parasitaemia by wet film technique.

* * - highly significant. The calculated value is more than the tabulated value at 1%.

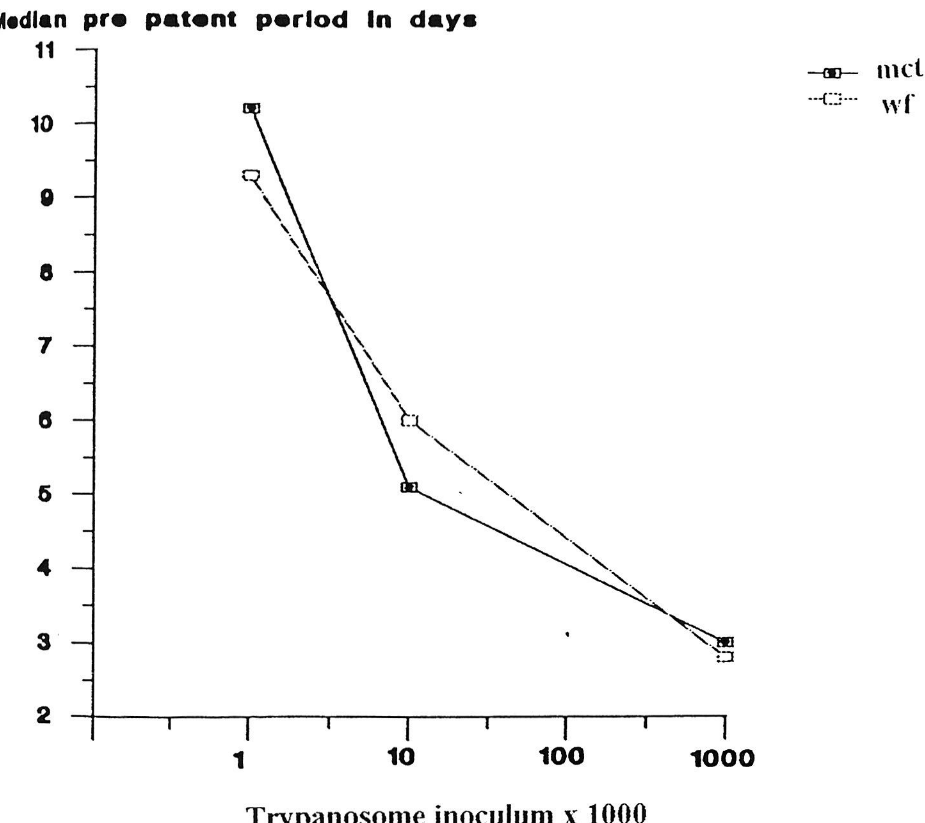


Figure 4.10 The relationship between size of trypanosome inoculum and pre-patent period in *T. evansi* KETRI 2454.

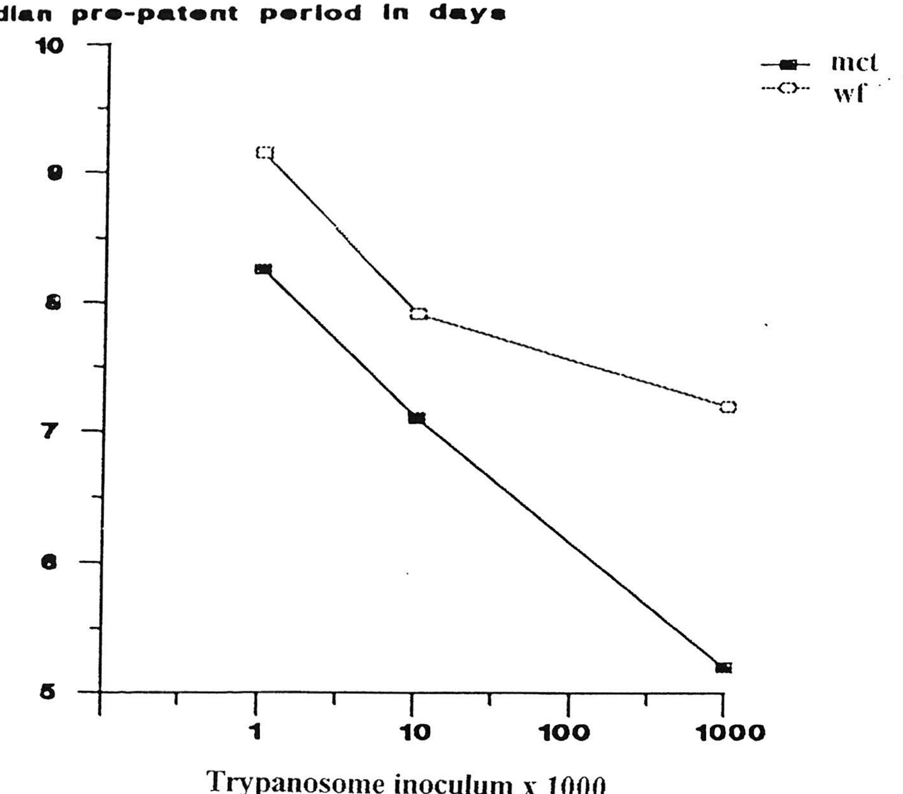


Figure 4.11 The relationship between size of trypanosome inoculum and pre-patent period in *T. evansi* KETRI 2455.

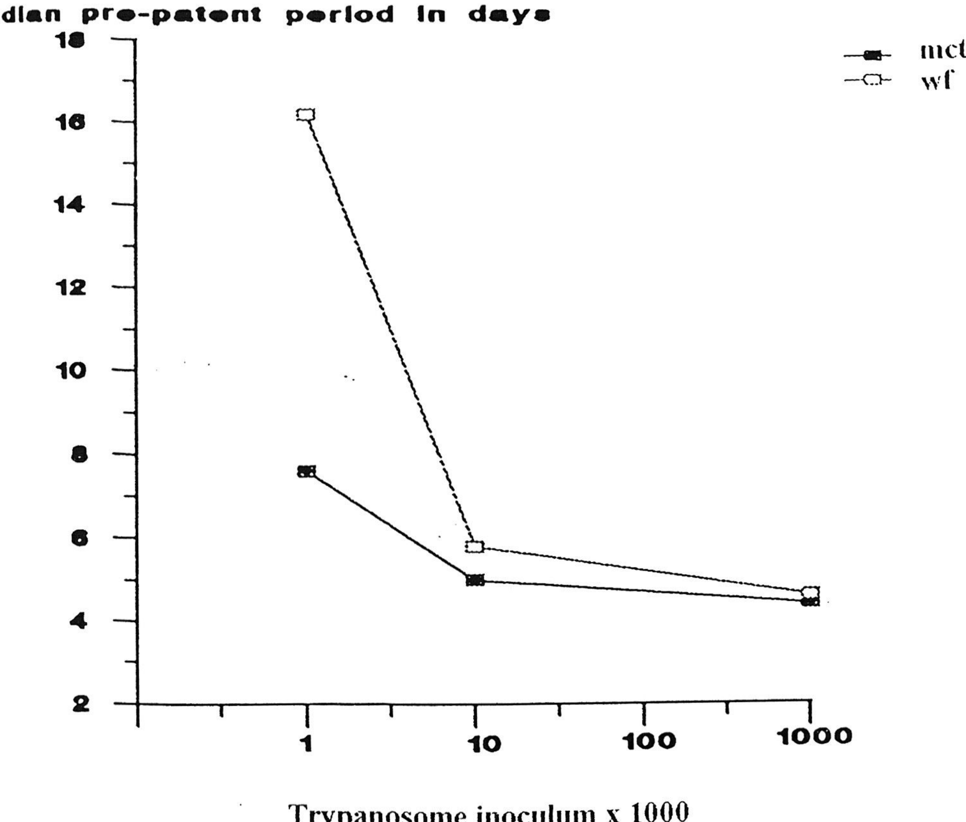


Figure 4.12 The relationship between size of trypanosome inoculum and pre-patent period in *T. evansi* KETRI 3136.

4.3.6 COMPARISON OF TREATMENT REGIMES

The effects of different treatment regimes were compared in three ways. To investigate the effect of timing of treatment, results were compared from mice infected with the same number of trypanosomes but treated at different times with suramin at different dose levels. To investigate the effect of trypanosome inoculum, results were compared from mice which received treatment at the same time but were inoculated with different numbers of trypanosome and treated with suramin at different dose rates. Finally, results were compared from groups of mice which were treated with different doses of suramin.

4.3.6.1 *T. evansi* KETRI 2454

The activity indices of suramin tested in mice against KETRI 2454 are shown in Table 4.12a. All mice were cured when treated with 5 mg/kg of suramin irrespective of timing of treatment and size of inoculum. In addition, all mice treated at time of infection, or 24 hours later, were also cured irrespective of inoculum or drug dose. Marked variation occurred, however, in the response to treatment with 0.05 - 1 mg/kg of drug when treatment was administered 48 hours post infection or when parasites were first detected in the blood. Where treatment was delayed, in most cases there were fewer cures irrespective of the size of inoculum. There were, however, exceptions where either more cures were observed with delayed treatment (one instance), or no difference in cure rates (four instances). The difference in activity indices between different treatment times was significant ($p < 0.05$) only at the 1×10^6 trypanosome inoculum (Table 4.13). There was a significant difference in the response of mice which were treated at the onset of parasitaemia detected by micro-haematocrit centrifugation technique, compared with mice which were treated immediately after infection, or 24 hours post infection (omct and 0, omct and 24 hours). The difference in the activity indices of suramin in mice which were infected with 1×10^3 trypanosomes was marginally significant ($p = 0.0571$) between groups of mice which were treated immediately after infection and 48 hours later and also between mice which treated immediately and those treated when parasitaemia was detected using either of the two detection methods (0 and 48, 0 and omct, 0 and owf). A similar marginally significant difference was observed between groups of mice which were treated 24 hours post infection and when parasites were detected by either of the two detection methods (24 and omct, 24 and owf). In mice inoculated with 1×10^6 trypanosomes, a marginally significant difference was observed between groups of mice treated when parasites

were detected in blood by the micro-haematocrit centrifugation technique immediately after infection and 24 hours later (omct and 0, omct and 24).

When comparing the time of treatment irrespective of trypanosome inoculum (see Table 4.12b), in most cases, the number of animals cured decreased as the trypanosome inoculum increased. There were two instances whereby more cures were observed with increased inocula and five instances where there was no difference in the activity indices. None of these differences was statistically significant (Table 4.14).

A series of comparisons was made between the four suramin doses (0.05, 0.1, 1 and 5 mg/kg) in groups of mice inoculated with the same number of trypanosomes (Table 4.12a). With a few exceptions, there was a statistically significant ($p > 0.05$) increase in the number of mice cured when the dose of suramin was increased. These significant differences were between suramin dose rates of 0.05 and 5 mg/kg and between 0.1 and 5 mg/kg when mice were inoculated with either 1×10^3 or 1×10^4 trypanosomes respectively, and also between the 0.1 and 5 mg/kg doses when mice were inoculated with 1×10^6 trypanosomes.

Table 4.12a Activity indices for different doses of suramin tested against *T. evansi* KETRI 2454 at different trypanosome inocula and treatment times: comparisons of treatment times at the same inocula.

Trypanosome inoculum	$1x10^3$					$1x10^4$					$1x10^6$				
Treatment time	0	24	48	omct	owf	0	24	48	omct	owf	0	24	48	omct	owf
Suramin dose mg/kg															
0.05	9	10	7	7	6	9	10	8	6	6	10	10	5	4	4
0.1	9	10	10	7	6	10	8	8	5	4	10	9	6	5	4
1	10	10	10	9	7	10	10	7	6	5	10	10	7	6	8
5	10	10	10	10	10	10	10	10	10	9	10	9	10	9	10

Legend: 0 - suramin treatment administered immediately after infection.

24, 48 - suramin administered 24 or 48 hours after infection.

omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.

owf - suramin administered at the onset of parasitaemia as determined by the wet blood film technique.

Table 4.12b Activity indices for different doses of suramin tested against *T. evansi* KETRI 2454 at different trypanosome inocula and treatment
times: comparisons of trypanosome inocula at the same treatment time.

Treatment time	0			24			48			omct			owf		
Trypanosome inoculum	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$
Suramin dose mg/kg															
0.05	9	9	10	10	10	10	7	8	5	7	6	4	6	6	4
0.1	9	10	10	10	8	9	10	8	6	7	5	5	6	4	4
1	10	10	10	10	10	10	10	7	7	9	6	6	7	5	8
5	10	10	10	10	10	9	10	10	10	10	10	9	10	9	10

Legend: 0 - suramin treatment administered immediately after infection.
24, 48 - suramin administered 24 or 48 hours after infection.
omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.
owf - suramin administered at the onset of parasitaemia as determined by the wet blood film technique.

Table 4.13 *T. evansi* KETRI 2454: statistical comparison of treatment times at the same trypanosome inocula.

Tabular H value K=5 n=4 is 8.68 at 5% and 11.07 at 1%

Trypanosome inoculum	H value observed	difference
$1x10^3$	7.69	N.S
$1x10^4$	7.20	N.S
$1x10^6$	9.45	*

Legend: N.S - not significant. Calculated value less than tabulated value at the 5% critical value.

* - significant. Calculated value more than tabulated value at the 5% level.

Table 4.14 *T. evansi* KETRI 2454: statistical comparison of different trypanosome inocula at the same treatment times.

Tabular H value k=3 n=4 is 5.69 at 5% 7.65 at 1%

Treatment times	H value observed	difference
0	2.44	N.S
24	0.99.	N.S
48	2.06	N.S
omct	3.08	N.S
owf	1.23	N.S

Legend: N.S - not significant. Calculated value less than tabulated value at the 5% critical value.

4.3.6.2 *T. evansi* KETRI 2455

Comparison of the activity indices of suramin tested against a resistant stock, KETRI 2455 (Table 4.15) indicated that the response to treatment at a dose rate of 20 mg/kg remained the same at all regimes irrespective of the inoculum. At lower doses of suramin (1 and 5 mg/kg), resistance was observed at all treatment regimes and treatment times. However, at the 10 mg/kg suramin dose, the response was dependent on the timing of treatment and number of trypanosomes inoculated. This suramin dose was shown previously to be effective in curing mice infected with 1×10^6 trypanosomes (Table 4.9). In mice inoculated with the same number of trypanosomes (Table 4.15), there was a decrease in activity indices as treatment was delayed. At the 1×10^3 trypanosome inoculum dose, the variation in activity indices ranged from nine (almost complete cures) to an activity ratio of one between the earliest and the latest treatment time. These differences in cure rates were however not statistically significant ($p > 0.05$; Table 4.17). The Mann-Whitney test confirmed that there were no significant differences ($p > 0.05$) in cure rates when treatment was administered at different times, even between those groups of mice in which there were apparently large differences in cure rates.

There was no difference in the activity of suramin in mice treated at the same time after infection with dose rates of 1, 5 and 20 mg/kg (Table 4.16). Fewer cures were observed when suramin was administered at a dose rate of 10 mg/kg as the number of trypanosomes inoculated was increased. These differences were not statistically significant ($p > 0.05$ Table 4.18). For each trypanosome inoculum, as the dose of suramin was increased, there was an increase in the proportion of animals cured (Table 4.15). This difference was highly significant ($p < 0.01$).

Table 4.15 Activity indices for different doses of suramin tested against *T. evansi* KETRI 2455 at different trypanosome inocula and treatment times: comparisons of treatment times at the same trypanosome inoculum.

Trypanosome inoculum	$1x10^3$					$1x10^4$					$1x10^6$				
Treatment time	0	24	48	omct	owf	0	24	48	omct	owf	0	24	48	omct	owf
Suramin dose mg/kg															
1	0	2	0	1	0	1	0	1	1	0	0	0	0	0	0
5	1	2	1	2	1	1	1	1	0	2	1	0	0	0	0
10	9	8	7	7	1	8	8	8	2	0	3	1	0	0	0
20	10	9	10	10	9	10	10	10	9	8	10	10	10	8	9

Legend: 0 - suramin treatment administered immediately after infection.

24, 48 - suramin administered 24 or 48 hours after infection.

omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.

owf - suramin administered at the onset of parasitaemia as determined by the wet blood film technique.

Table 4.16 Activity indices for different doses of suramin tested against *T. evansi* KETRI 2455 at different trypanosome inocula and treatment
times: comparisons of trypanosome inocula at the same treatment time.

Treatment time	0			24			48			omct			owf		
Trypanosome inoculum	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$
Suramin dose mg/kg															
1	0	1	0	2	0	0	0	1	0	1	1	0	0	0	0
5	1	1	1	2	1	0	1	1	0	2	0	0	1	2	0
10	9	8	3	8	8	1	7	8	0	7	2	0	1	0	0
20	10	10	10	9	10	10	10	10	10	10	9	8	9	8	9

Legend: 0 - suramin treatment administered immediately after infection.
24, 48 - suramin administered 24 or 48 hours after infection.
omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.
owf - suramin administered at the onset of parasitaemia as determined by the wet blood film technique.

Table 4.17 *T. evansi* KETRI 2455: statistical comparison of different treatment times at the same trypanosome inocula.

k=5 n=4 tabular H is 8.68 at 5% and 11.07 at 1%

Trypanosome inoculum	H value	Difference
$1x10^3$	4.26	N.S
$1x10^4$	2.66	N.S
$1x10^6$	3.45	N.S

Legend: N.S - not significant. Calculated value less than tabulated value at the 5% critical value.

Table 4.18 *T. evansi* KETRI 2455: statistical comparison of different trypanosome inocula at the same treatment times.

k=3 n=4 tabular H is 5.69 at 5% and 7.6 at 1%

Treatment time	H value	Difference
0	1.99	N.S
24	1.36	N.S
48	1.99	N.S
omct	2.79	N.S
owf	0.28	N.S

Legend: N.S - not significant. Calculated value less than tabulated value at the 5% critical value.

4.3.6.3 KETRI 3136

The activity indices of suramin in this highly suramin resistant stock are shown in Table 4.19. There was no difference in the suramin sensitivity of this stock at all treatment regimes when $1x10^4$ and $1x10^6$ trypanosomes were inoculated. Also, mice which had been inoculated with $1x10^3$ trypanosomes did not respond to treatment when suramin was administered at the onset of parasitaemia.

At the lowest trypanosome inoculum dose, however, when treatment was given within 48 hours of infection, this stock, which was resistant to suramin at 160 mg/kg responded to suramin treatment resulting in cure. All mice in three groups treated 24 hours after infection with suramin at dose rates of 10, 40 or 80 mg/kg and in a group treated immediately after infection with suramin at a dose rate of 80 mg/kg were cleared of infection. Fewer cures were observed with delayed treatment in all mice inoculated with $1x10^3$ trypanosomes. This difference in activity indices was highly significant ($p < 0.01$) as shown in Table 4.21. These differences were observed when comparing any two times except between treatment given immediately after infection and 48 hours post infection (0 and 48), and between treatment when parasitaemia was detected by the two methods (omct and owf).

In groups of mice infected with different numbers of trypanosomes but treated at the same time (Table 4.20), fewer cures were observed as the number of trypanosomes inoculated was increased. This difference in activity indices was highly significant ($p < 0.01$) at 0, 24 and 48 hour treatment times (see Table 4.22). Statistically significant differences were identified between the lowest trypanosome inoculum and any of the other two inocula ($1x10^3$ and $1x10^4$,$1x10^3$ and $1x10^6$) when mice were treated any time before and including 48 hours after infection. There was insufficient data to enable comparisons at other treatment times.

Comparisons between groups of mice infected with the same number of trypanosomes but treated at different times (Table 4.19) showed that there was no statistically significant ($p > 0.05$) effect of suramin dose on cure rates.

Table 4.19 Activity indices for different doses of suramin tested against *T. evansi* KETRI 3136 at different trypanosome inocula and treatment times: comparisons of treatment times at the same trypanosome inoculum.

Trypanosome inoculum	$1x10^3$					$1x10^4$					$1x10^6$				
Treatment time	0	24	48	omct	owf	0	24	48	omct	owf	0	24	48	omct	owf
Suramin dose mg/kg															
5	6	6	4	0	0	2	0	0	0	0	0	0	0	0	0
10	4	10	7	0	2	0	0	0	0	0	0	0	0	0	0
20	2	6	4	0	3	0	0	0	0	0	0	0	0	0	0
40	4	10	6	0	0	0	0	0	0	0	0	0	0	0	0
80	10	10	3	0	0	3	0	0	0	0	0	0	0	0	0

Legend: 0 - suramin treatment administered immediately after infection.
24, 48 - suramin administered 24 or 48 hours after infection.
omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.
owf - suramin administered at the onset of parasitaemia as determined by the wet blood film technique.

Table 4.20 Activity indices for different doses of suramin tested against *T. evansi* KETRI 2455 at different trypanosome inocula and treatment times: comparisons of trypanosome inocula at the same treatment time.

Treatment time	0			24			48			omct			owf		
Trypanosome inoculum	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$	$1x10^3$	$1x10^4$	$1x10^6$
Suramin dose mg/kg															
5	6	2	0	6	0	0	4	0	0	0	0	0	0	0	0
10	4	0	0	10	0	0	7	0	0	0	0	0	2	0	0
20	2	0	0	6	0	0	4	0	0	0	0	0	3	0	0
40	4	0	0	10	0	0	6	0	0	0	0	0	0	0	0
80	10	3	0	10	0	0	3	0	0	0	0	0	0	0	0

Legend: 0 - suramin treatment administered immediately after infection.

24, 48 - suramin administered 24 or 48 hours after infection.

omct - suramin administered at the onset of parasitaemia as determined by the micro-haematocrit centrifugation technique.

Table 4.21 *T. evansi* KETRI 3136: statistical comparison of different treatment times at the same trypanosome inocula.

k=5 n=5 tabular H value is 8.88 at 5% and 11.57 at 1%

Trypanosome inoculum	H value observed	Difference
$1x10^3$	16.84	* *
$1x10^4$	-0.68	N.S.

Legend: N.S - not significant. Calculated value less than tabulated value at the 5% critical value.
* * - highly significant. Calculated value more than tabulated value at the 1% level.

Table 4.22 *T. evansi* KETRI 3136: statistical comparisons of different trypanosome inocula at the same treatment times.

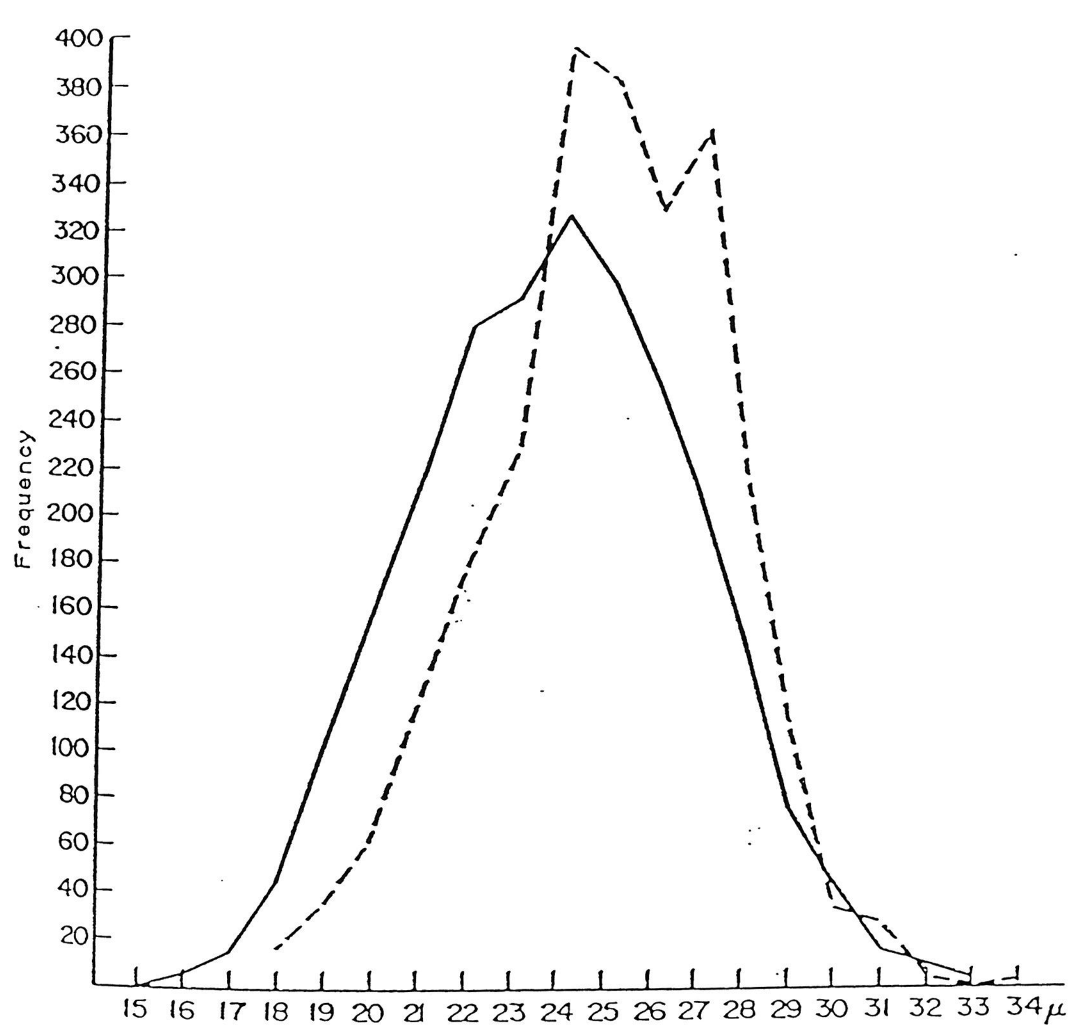
k=3 n=5 tabular H is 5.78 at 5% and 8.00 at 1%

Treatment time	H value observed	Difference
0	10.57	* *
24	13.52	* *
48	13.39	* *

Legend: * * - highly significant. Calculated value more than tabulated value at the 1% level.

4 DISCUSSION

Morphological characterisation is the most widely used parameter for identifying trypanosome species. The basis for identification was set by Hoare (1972) who determined the mean lengths and ranges of the various trypanosome species. For *T. evansi*, Hoare (1956) examined 22 populations from cameline, equine, bovine and canine hosts from Africa, Europe and Asia which had been maintained under laboratory conditions for long periods of time. To date, no study has been done to validate the results of Hoare's *T. evansi* work against more recent trypanosome isolates. In this study, the mean length of *T. evansi* parasites was determined as 25.5 μm, similar to the 24 μm recorded by Hoare (1972) for his *T. evansi* stocks. The shortest mean parasite length of the Kenyan stocks in this study was 20 μm compared to 15 μm described by Hoare. At 33 μm, the higher range of the trypanosomes in this study is close to Hoare's length of 35 μm. An earlier study by Bruce (1911) which determined mean lengths of nine populations of *T. evansi* recorded a mean of 24 μm and a range of 18-34 μm. The values determined by Bruce are closer to the values found in this study than those of Hoare. In the present study, the Colombian stock which was isolated from a horse, had the shortest mean length (19 μm) while two stocks from Garissa had the longest mean length (30 and 33 μm). The two stocks from Sudan had mean lengths at the higher range of the stocks examined in this study (mean lengths 28 and 29 μm). The mean length of trypanosomes in this study was normally distributed (Figure 4.5) suggesting that the 44 stocks were part of the same population and variation in length was due to individual variation in the length of trypanosomes. The distribution curve of the mean lengths resembled that of Hoare (1956) more than that of Bruce (1911) as shown in Figure 4.13. The case of the 22 μm length which fell outside the normal distribution curve did not indicate a peak of a separate population since the mean, median and mode (25.5, 26 and 26 respectively) of the 44 stocks did not show much variation. The possibility that mean differences in trypanosome length were due to separate populations was also discounted by statistical analysis which indicated that there was significant differences in mean length. Both Bruce (1911) and Hoare (1956) found similar distributions with single measurements which fell outside the normal distribution curve (Figure 4.13); these values were 28 and 21 μm for Bruce (1911) and Hoare (1956) respectively. Hence, cameline *T. evansi* parasites from Kenya have a mean length of 25.5 μm with a range of 20 - 33 μm and the variation in length may be attributed to heterogeneity of stocks. This



- - - - - total for 6 populations measured by Bruce.

———— total for 22 populations measured by Hoare;

Figure 4.13 Two length distributions of 2500 *T. evansi* parasites (Bruce, 1911; Hoare, 1956).

heterogeneity may be due to physiological factors such as "crops of trypanosomes of different ages" (Hoare, 1972).

There are indications in studies on other trypanosome species that length of organisms can be related to pathogenicity. In *T. congolense*, three distinct population types according to trypanosome length were identified (Godfrey, 1960). The longest trypanosome type (dimorphon) was characterised by infectivity to a wide range of laboratory animals, and resulted in high parasitaemias with high pathogenicity (Chardome and Peel, 1954; Godfrey, 1961). In comparison, the shortest type (congolense) was characterised by infectivity to only white mice, and produced infections of low parasitaemias and low pathogenicity. The pathogenicity of the type with intermediate length was not as well defined but was observed to be in between that of the other two types with infectivity limited to pigs. In *T. vivax*, a similar relationship between trypanosome length and pathogenicity has also been observed with the shortest parasite strains being more pathogenic than the longer ones (Fairbairn, 1953). This relationship between length and pathogenicity was however not as well defined as in *T. congolense.* The relationship between length and pathogenicity has not been investigated in *T. evansi*.

Individual variations found in pre-patent periods were not indicative of organisms consisting of separate populations as was discounted by statistical analysis. Differences in pre-patent periods might be due to host-related factors or be due to variation in experimental procedure. One host-related factor is the response of the host to invasion of foreign antigen. In order for trypanosomes to be detected in blood, they would have to evade the host's defence mechanisms and multiply to an extent that they are detected in peripheral blood. The immunocompetence of the mouse and the time it takes for antibodies to be produced would influence trypanosome proliferation (Barry and Turner, 1991). Although the mice used in this work were outbred, it is highly unlikely that the extent of variation in responses of individual mice account for such a wide range (2-20 days) in pre-patent periods. The most likely factor leading to such a wide variation in pre-patent period would be human error due to a variation in the number of trypanosomes inoculated, their viability or the site at which they were inoculated. Although trypanosomes were meant to be inoculated into the peritoneal cavity of the mice, there is the possibility of an error whereby parasites were inoculated into some other compartment in the abdominal cavity such as the liver, spleen or even parts of the digestive system. Inoculation into such compartments would result in trypanosomes encountering different host defence mechanisms compared to

different host defence mechanisms compared to inoculation into the peritoneal cavity. The trypanosomes might then develop parasitaemia at a different rate.

Like all organisms, trypanosomes need energy to maintain functions that are necessary for their integrity. A major source of energy is by the metabolism of glucose through reactions which are catalysed by enzymes. One of the enzymes involved is malic enzyme which catalyses a reaction associated with glycolysis whereby L-malate and NADP are converted to pyruvate, carbon dioxide and NADPH. Gibson (*et al*, 1980; 1983) showed that this enzyme was polymorphic and, along with others, could be used to distinguish *T. evansi* from other trypanosomes of the subgenus *Trypanozoon*. The present work showed that malic enzyme pattern II was predominant in the stocks of *T. evansi* examined, being found in almost two thirds of the *T. evansi* stocks. This agrees with earlier work (Gibson, 1983) carried out on Kenyan isolates. Malic enzyme pattern VII, first identified in *T. evansi* by Boid (1988), was found in the two stocks from Sudan but not in any of the Kenyan isolates. The link between suramin resistance and the possession of malic enzyme pattern VII suggested by Boid *et al*, (1989) was not observed in isolates whose suramin resistance was as high as 15 mg/kg. The association between malic enzyme pattern VII and suramin resistance in *T. evansi* may thus occur only in cases of very high resistance as shown by the two isolates from Sudan. Another possibility is that this association may have been just a chance occurrence. The present work did not show any relationship between malic enzyme patterns and resistance to any of the four trypanocidal drugs examined (suramin, Trypacide, Berenil or Samorin). In a similar study of drug resistance in *T. gambiense*, no association was found between banding patterns of ALAT, ASAT, ICD, PEP 1, PEP 2, PGM and ME and resistance to Trypamidium, Melarsoprol, Pentamidine and Berenil (Dukes, 1984).

Malic enzyme patterns IV and X were identified in *T. evansi* for the first time in the present study. Pattern IV was found only in the isolates collected from Garissa in 1968; probably a reflection of the trypanosomes circulating in this area 25 years ago. However, to confirm this, it would be necessary to obtain more recent isolates from the same area and determine their malic enzyme patterns. This isoenzyme pattern has previously been identified in isolates of *T. rhodesiense* from humans in Zambia (Gibson, 1980) but is unlikely that the stocks used in the present study are indeed human-infective *brucei* group trypanosomes. Firstly, the morphology of these parasites were not characteristic of pleomorphic *T. rhodesiense* and secondly, Garissa is outside both the identified tsetse fly belt and far from the known sleeping sickness foci in Kenya. In order to resolve this point, it would be necessary to further analyse

isoenzymes, including identification of patterns of aspartate aminotransferase (ASAT), phosphoglucomutase (PGM) and the two peptidases (PEP 1 and 2) which, along with the malic enzyme, have been shown to discriminate *T. evansi* from other *Trypanozoon* parasites (Gibson, 1983). Malic enzyme pattern X, previously identified in human *T. gambiense* isolates from Uganda (Gibson *et al,* 1980) was seen in isolates from Galana, Marsabit and Ukunda. These may actually be pattern II whose three lower bands were too faint to be visible in spite of repeated attempts. Again, analysis of other isoenzymes which distinguish *T. evansi* from other trypanosomes of the sub-genus *Trypanozoon,* would further characterise these stocks and confirm their identity.

Since most of the *T. evansi* stocks from Kenya had either patterns II or X, these parasites appear to be homogeneous in their malic enzyme patterns. If malic enzyme pattern X was actually pattern II with faint bands a, b and c (see Figure 4.7), then this would mean that 90% of the Kenyan stocks examined had only one malic enzyme pattern. This observation agrees with that of Gibson (1983) who found 92% of cameline *T. evansi* stocks from Kenya could be assigned to a group I which was characterised by the presence of a malic enzyme pattern II.

There are two major differences between the results of this study and those of Gibson (1983). In her work, one cameline stock from Rumuruti was found to have a malic enzyme pattern II which was later identified as *T. brucei* on the basis of tsetse transmissibility. Another difference is the malic enzyme pattern of KETRI 2472. Gibson (1983) identified it as pattern II while this study identified this stock as pattern VII. Unless there was a stock mix-up, this difference is difficult to explain. A possible explanation may be that this isolate is a mixed stock containing two trypanosome populations with different malic enzyme patterns. By a process of selection involving passage through rodents and cryopreservation of some ten years, it is possible that the predominant trypanosome in the mixed population altered, giving rise to the difference observed. Further investigation of this stock, involving cloning and determination of the malic enzyme patterns of the various cloned populations, would clarify this.

In Kenya, *T. evansi* is a cameline parasite which is controlled by the use of drugs. In view of the small number of drugs available, it is important to identify any development of resistance which would reduce the effectiveness of the available drugs. Although the trypanocidal sensitivities of 41 Kenyan *T. evansi* stocks are not representative of the situation in the country due to the small sample size involved and the fact that most of the stocks were from either Marsabit or Galana, the results obtained in this study serve as an indication of the possible extent of resistance. In the case of suramin, close to 20% of the stocks examined were resistant in mice at a dose

level of 5 mg/kg. This dose level is more than the 4 mg/kg determined as the MCD of suramin in mice (Abebe *et al,* 1983) but less than the 10 mg/kg recommended for the treatment of *T. evansi* infections in camels. Resistance to suramin was the second most prevalent trypanocidal resistance encountered in this study and was found in stocks isolated from five of the eight locations in Kenya. In Galana, the prevalence was especially high with 68% of the stocks being resistant to 5 mg/kg of suramin. In an earlier study, Schillinger *et al,* (1985a) also reported suramin resistance in Galana. This high prevalence of suramin resistance in Galana could be attributed either to the availability and affordability of drugs in a ranch situation or a sampling bias. The fact that suramin was used regularly on this ranch may account for the development of resistance. The other possibility of sampling bias is more likely because the reason for isolation of many stocks at Galana was due to suspected drug-resistance. The fact that suramin resistance was not found in stocks from Garissa, Ngurunit and Rumuruti is not an indication that it was not there, since only a few stocks were obtained from these areas. Since suramin has been used against cameline trypanosomiasis in Kenya since 1925 (Gitatha, 1980), this widespread pattern of resistance is expected. The fact that the level of resistance is not higher suggests that suramin resistance is hindered from higher prevalence by negative selection pressure which renders the resistant parasites disadvantaged and thus either selected out or maintained at a low frequency.

In conjuction with Berenil, Trypacide resistance was found in Marsabit and Ukunda while it was found in Ukunda and Ol Maisor in conjuction with Berenil and and suramin. The observation that resistance to Trypacide alone was not observed in any of the trypanosome stocks examined but was found with Berenil may be due to the high frequency of Berenil resistance observed. Thus, there was a high probability of observing Berenil resistance in stocks that were resistant to Trypacide. All these stocks were isolated before 1984 when Trypacide was introduced into the market for the control of cameline trypanosomiasis. Between 1950 and 1977 however, quinapyramine (Antrycide^{R} ICI), was available for the control of bovine trypanosomiasis. These results agree with those of Schillinger *et al,* (1985a) who reported Antrycide (^{R} Quinapyramine, ICI) resistance in Ol Maisor and Kisima, two ranches near Rumuruti. This situation of relatively low Trypacide resistance may be due to the short period that this trypanocidal drug has been indicated and available for the control of camel trypanosomiasis in Kenya. The development of Trypacide resistance by a cameline parasite as was observed in this study could be by cross-resistance with another drug. Since Trypacide is chemically different from the other trypanocidal drugs used in the control of camels, the first possibility is unlikely. In addition to this, no cross-resistance has been found between quinapyramine and the other trypanocides used in this study. The second possibility is that Trypacide was used to treat camels prior to 1984 when it was available though not recommended for

use in this animal species, providing an opportunity for *T. evansi* to develop resistance. This is likely in view of the confidence that camel owners believe they can diagnose and treat their animals. A third possibility is that *T. evansi* developed resistance to Trypacide in an animal species other than the camel as will be fully discussed later when considering Berenil resistance.

Three stocks of *T. evansi* from Ukunda and Garissa were resistant to Samorin; one to Samorin alone while the other two stocks were resistant to both Samorin and Berenil. The low prevalence of Samorin resistance in *T. evansi* stocks examined probably reflects the limited use of this trypanocide in the treatment of cameline trypanosomiasis in Kenya. Although Samorin is active against *T. evansi*, its use in camels has been shown to result in pain, lameness and at times fatal systemic symptoms associated with toxicity (Ballis and Richard, 1977; Schillinger and Rottcher, 1984; Schillinger *et al*, 1985b; Ali and Hassan, 1986). In Kenya, Samorin is indicated for treatment of bovine trypanosomiasis. There were, however, isolated instances when it was used to treat suramin-resistant cameline trypanosomiasis before 1984 when Trypacide became available (Schillinger and Rottcher, 1985a).

Despite the fact that Berenil is not used in camels due to its toxicity, a high proportion of trypanosome stocks studied (57%) were resistant to this drug. This situation, where a trypanocidal drug which is rarely employed for use in camels is found to be ineffective against many isolates of trypanosomes is similar to that encountered with Trypacide. There are several ways in which this might have occurred. Firstly, there might be cross-resistance between Berenil and another trypanocide that is commonly used in camels. Secondly, this trypanocide could have been misused by treating camels with it in doses that are below the recommended 7 mg/kg bodyweight. A third possibility is that *T. evansi* parasites developed resistance due to exposure to the drug in another host. The fourth reason for such a high level of Berenil resistance being observed is that this drug is not effective in curing *T. evansi* infections. Since Berenil is a diamidine and thus chemically unrelated to suramin and quinapyramine (a sulphonated naphthylamine and an aminoquinaldine respectively), cross resistance is unlikely. In view of their structurally similarities, Samorin is the only drug with which Berenil would exhibit cross-resistance. In a case of cross-resistance, one would expect that similar frequencies of resistance would be observed in both drugs involved. Samorin is however, not indicated for cameline trypanosomiasis and the association of this drug with toxicity in camels might deter its use by unqualified staff. Furthermore, the prevalence of Samorin resistance was much lower than that of Berenil resistance, making this possibility unlikely. The treatment of

trypanosomiasis in camels using Berenil in amounts that are below the recommended therapeutic doses is likely in the remote parts of Kenya. Camel owners who are desperate to treat their sick camels and yet are far from any qualified veterinary officer have been known to use any available drug without regard to the proper dosage. This usage of drugs by unqualified people has at times led to instances of camel deaths due to Berenil toxicity and has been reported in local newspapers in Kenya. As indicated by sales, Berenil was commonly used in the North-Eastern province of Kenya (Mahaga and Rottcher, 1982) and therefore the misuse of this drug in camels or the possibility of *T. evansi* exposure to this drug in other livestock are likely possibilities.

In addition to camels, goats are also preferred by livestock owners in arid and semi-arid regions of Kenya. The goat is a possible suspect as the host in which *T. evansi* parasites are exposed to Berenil leading to the development of resistance. The presence of *T. evansi* parasites in goats would provide evidence of their role as hosts and reservoirs of these parasites. No studies have shown this association and Boid *et al* (1981) looked without success for *T. evansi* in over 400 sheep and goats closely associated with camels in Sudan. More than 50% of the sheep and goats examined did, however, have antibodies against *T. evansi* implying that they had been infected with this trypanosome species. The fact that most cases of Berenil resistance were found in Galana, a ranch with qualified veterinary service, might suggest that the Berenil resistance encountered in this study did not develop from drug misuse but rather by drug/trypanosome interaction in a host other than the camel. Thus, trypanocide underdosing may not have played a principle role in the development of resistance. This possibility is further strengthened by the fact that Berenil has been widely used for a long time in controlling trypanosomiasis in both cattle and goats in this ranch. Further work is needed, however, to ascertain this suspected development of *T. evansi* resistance to Berenil, Samorin and Trypacide in hosts other than camels.

Another possibility which could explain this high frequency of Berenil resistance would be that the dose of 7 mg/kg used to treat mice in this work is not adequate. According to the conversion formula of Bushby (1963), a dose of 7 mg/kg in a 500 kg camel is equivalent to a staggering 210 mg/kg in a 20 g mouse. Using the same conversion, the 7 mg/kg used to treat mice in this study is equivalent to only 0.2 mg/kg in a camel which is certainly an under-dosage in view of the recommendations of the manufacturers. It seems that this conversion concept has not previously been considered for Gitatha (1979) used doses of 3.5, 7 and 10.5 mg/kg and Jennings *et al,* (1977 and 1979) used doses up to 40 mg/kg of Berenil and considered them to provide an indication of Berenil resistance in mice. The minimum curative dose of Berenil which could cure half of the mice infected (MCD_{50}) with *T. evansi* was determined to

be 3.4 mg/kg (Gill, 1973). The calculated dose using the conversion ratio of Bushby is 60 times more Berenil than the MCD_{50} in mice and the dose recommended by manufactures for the treatment of cattle. It may be reasoned that high frequency of Berenil resistance was due to interspecies differences between mice, cattle and camels since Berenil is not recommended for use in mice and camels. A similar discrepancy is also observed with suramin which is recommended for use in camels. When Bushby's conversion is used, the 10 mg/kg dose of suramin recommended for the treatment of *T. evansi* in camels, is equivalent to 30 mg/kg in a 20 g mouse. The minimum curative dose of suramin in mice determined at 4 mg/kg (Abebe *et al,* 1983) therefore would be 7.5 times less than the recommended dose. The difficulty of extrapolating drug sensitivity results from one animal to the other led Hawking (1963b) to conclude that "tests in mice apparently give a broad indication of the probable response of a strain in cattle". Sones *et al* (1988) working with *T. congolense* also observed that there were inconsistencies in the correlation of resistance results in mice and cattle. Authie (1984) however observed that there was a close proximity between trypanocide doses which are effective in removing trypanosomes from 75% of mice (ED_{75}) to cattle MCD values. Although it is possible that the trypanosome strains used by Gill (1973) and Abebe *et al,* (1983) were extremely sensitive and thus not representative of the sensitivity of the average trypanosome stocks, the transformation of doses from large animals to small laboratory rodents and *vice versa* needs further investigation to establish conversion ratios.

The sensitivity of *T. evansi* to Berenil in various animal species has been investigated by several workers. Dennig (1977) and Elamin *et al,* (1982) investigated the efficacy of Berenil against *T. evansi* and found that doses as high as 7 mg/kg were not enough to cure infections in mice. Bhathacharjee and Sinha (1971) determined that 16 mg/kg of Berenil cured infections in mice but not guinea pigs or dogs. In buffaloes, although 16 mg/kg cleared parasites from peripheral blood for 30 days, these workers considered that this was not a cure but the infection had taken a latent course. In trypanosome infected camels, Leach (1961) found that 3.5 mg/kg of Berenil was not enough for complete cures because relapses were observed after 60 days. All these studies indicate that the effect of Berenil is not the same in different animal species. The differences observed in the efficacy of Berenil may not just be attributed to inter-species differences but, as discussed in the previous paragraph, may be a reflection of differences in the weights of animals used. Another reason which might explain this apparent ineffectiveness of Berenil to *T. evansi* infections would be, as suggested by Bhathacharjee and Sinha (1970), Dennig (1977), Elamin *et al* (1982), Schillinger and

Rottcher (1986), that this drug has an insufficient or only moderate trypanocidal action against this trypanosome species.

There was no cross-resistance between the four trypanocidal drugs studied. Although the frequencies of suramin and Trypacide resistance were similar, resistance to the two drugs may have developed independently of each other since there were only two cases where resistance to both drugs (along with Berenil) was found in the same stock. The lack of cross-resistance between suramin, quinapyramine and diminazene was also observed by Gill (1971a) who concluded their loci of action were independent of each other. This finding is understandable in view of the different chemical structures and the mode of action of the trypanocides used (see section 2.4.2.3).

When trypanosomes are injected into an animal, they commence dividing and increasing in number until they reach a stage where they are detectable in peripheral blood. The length of time between inoculation of trypanosomes and appearance in peripheral in blood is dependent on many factors including the number of organisms initially injected into the animal. This work found a negative correlation between the number of trypanosomes inoculated into a mouse and the pre-patent period; the more trypanosomes inoculated, the shorter the pre-patent period. This inverse linear relationship was observed in all three stocks examined and was independent of the method used to detect trypanosomes. The relationship between the number of trypanosomes inoculated and pre-patent period has been investigated by other workers. Blacklock (1913) and Corson (1934) did not find a correlation between the two while working with *T. rhodesiense* in rats and neither did Opiyo (1984) with *T. simiae* in pigs. However, Baker (1960) working with *T. brucei* and *T. rhodesiense* found an inverse linear relationship between the pre-patent period and the log number of trypanosomes inoculated into mice, rats and guinea pigs. Walker (1964) working with *T. brucei* in mice and Opiyo (1984) with *T. simiae* pigs found that the number of trypanosomes inoculated influenced the virulence of infection. As would be expected, a larger number of inoculated parasites would multiply faster to a level they are detected in peripheral blood than would a smaller number. Another reason for this relationship may be that when fewer trypanosomes are inoculated into an animal, the defence mechanisms of the host have to contend with fewer parasites and thus have has time to mount an effective immune response before the trypanosomes increase sufficiently to overwhelm the defence mechanisms of the host. In these circumstances, the animal might even manage to control the infection.

The observation that the slope plotted between trypanosome inoculum and pre-patent period was steeper between $1x10^3$ and $1x10^4$ than between $1x10^4$ and $1x10^6$ indicates that the effect of large increases in trypanosome inoculum do not alter pre-patent periods as much as small increases. This suggests a threshold effect where increased trypanosome inoculum numbers decrease pre-patent periods up to a certain point after which further increases have only a minor effect on pre-patency.

In order to determine the effect of suramin treatment on different numbers of trypanosomes, suramin was administered at various times after infection to mice that had been inoculated with different numbers of trypanosomes. The results from this study indicated that suramin is more effective in curing infections where a relatively small number of trypanosomes are involved. These infections are like those found in early treatments and to a lesser extent in smaller trypanosome inocula. Hence, even with the highly resistant stock (KETRI 3136), when few trypanosomes ($1x10^3$) were inoculated and treatment administered early, (0-48 hours post-infection) the chances of successfully treating mice were increased in contrast to mice infected with $1x10^4$ or $1x10^6$ trypanosomes which could not be successfully treated at any time. In contrast, with KETRI 2454 which was susceptible to a suramin dose as low as 0.1 mg/kg when administered 24 hours post infection (Table 4.9), treatment was less successful when trypanosomes were allowed to multiply and treatment was delayed until parasites could be detected in blood. This relationship between trypanosome numbers and success or failure of treatment was not however observed in KETRI 2455, a moderately suramin-resistant stock. The reasons for this observation are not clear but may be related to the amount of suramin used; a factor shown to influence the outcome of treatment in this stock as discussed later. Unlike KETRI 2454, with KETRI 2455, the suramin dose levels used were much higher than the dose level which cures mice infected with $1x10^6$ trypanosomes of this stock. Further investigations involving higher suramin doses such as 30, 40 or 50 mg/kg might further elucidate this observation.

The number of *T. evansi* parasites mechanically inoculated by vectors at transmission is not known. The number of *T. congolense* inoculated by tsetse was, however, estimated to be 3-300 (Harley and Wilson, 1968). In another study, mean trypanosome counts of 608 *T. brucei*, 50 *T. congolense* and seven *T. vivax* were found in the salivary secretions of tsetse flies (Otieno and Darji, 1979) These studies suggest that the number of trypanosomes inoculated during experimental syringe inoculations of *T. evansi* are probably much higher than the numbers inoculated by tabanids during natural infections. In view of the results obtained in this work, this would suggest that infections in the field may be treated more successfully than experimental infections

since less trypanosomes are inoculated in the field. In making such an extrapolation, however, it is important to note that the hosts involved in the laboratory and the field are different; a factor which may influence the development of *T. evansi* infections and the success of treatment.

The reason for successful treatment when suramin is administered early may be explained by the type of infection which *T. evansi* develops in the host and the type of trypanocidal drug used. In the sub-genus *Trypanozoon*, a tissue parasite is known to invade the CNS, including that of the camel (Leese, 1927). When trypanocidal drugs which cannot cross the blood-brain barrier are used to treat such infections, only trypanosomes in the blood are affected and the trypanosomes in the brain can later re-invade systemic circulation as has been shown with *T. brucei* (Jennings *et al*, 1979). Thus successful early treatment of *T. evansi* infections may be due to parasites not having adequate time to invade extravascular sites which suramin cannot penetrate. Treatment would then destroy all parasites in circulation. Treatment administered later would kill only parasites in circulation, leaving those in tissue sites to re-invade the blood. Successful treatment, where the number of trypanosomes initially inoculated into mice were comparatively few, may be understood in view of the fact that a larger number of trypanosomes have a higher probability of invading tissue sites as compared to a smaller number. The fact that this effect, of a smaller number of trypanosomes to successful treatment, was greater in the highly resistant stock suggests that the suramin resistance observed in KETRI 3136 may be related to the ability of trypanosomes to invade tissue sites.

The role of trypanosome inoculum to the outcome of treatment was extensively investigated by Jennings *et al*, (1977) in Berenil-treated *T. brucei* infections in mice. They concluded that the occurrence of relapses was not related to the trypanosome inoculum. The trypanosome inocula used in that study were 1×10^5-1×10^8 parasites per mouse. The relapses observed in the study with *T. brucei* were explained as being due to the inaccessibility of the drug to the parasites in view of the fact that the trypanocide used does not cross the blood-brain barrier.

The role of timing of treatment to the outcome of treatment was recognised as being important in the administration of Salicyl hydroxamic acid glycerol combination (SHAM glycerol, Evans *et al*, 1977) and Berenil (Jennings *et al*, 1977, 1979) to *T. brucei* infected mice. With SHAM glycerol, cures were observed when treatment was given one day after infection but not later, when the infection was patent. The infections were always cured irrespective of the time of treatment when the infecting parasites were *T. vivax* (Evans *et al*, 1977). The work done by Jennings *et al*, (1977,

1979) showed cures when Berenil was given before seven days post-infection but not later. These two studies suggested that trypanosomes were in areas which provided an escape from chemotherapy due to inaccessibility. This would explain cures in mice infected with *T. vivax* which does not invade the central nervous system as well as cures observed in early treatments in *T. brucei* infections. In the latter situation, trypanosomes would not have had adequate time to invade inaccessible sites in the central nervous system before the drug was introduced.

As expected, suramin dosage was not important in determining cures in KETRI 3136 which was shown to be resistant to 160 mg/kg of suramin. In KETRI 2455 however, suramin dosage was an important factor which determined whether mice were cured or not as shown by a highly significant difference in cure rates of mice treated with different suramin doses. The fact that the importance of suramin dosage in treating mice infected with KETRI 2454 was not as significant as that of KETRI 2455 may be used as an indication reflecting the comparative suramin sensitivities of these two stocks.

In conclusion, investigation into the role of trypanosome burden to the outcome of treatment has shown that timing of treatment in relation to infection is crucial in the treatment of experimental *T. evansi* infections in mice. Of less importance is the number of trypanosomes inoculated into an animal. This study has also shown the importance of including known sensitive and resistant stocks as references in experimental designs for all drug resistance studies. With such a design, results obtained can then be evaluated in view of the reference stocks. Although this study investigated infections in mice, it is likely that a similar situation would be applicable in the field where early treatments of infected camels would increase the chances of curing them.

CHAPTER FIVE

INDUCTION OF SURAMIN RESISTANCE IN *T. EVANSI*

5.1 INTRODUCTION AND OBJECTIVES

Although the mechanisms underlying the development of resistance to trypanocidal drugs are not known, overwhelming evidence suggests that a change in genetic constitution, mutation, and selection of that change play a major role. Thus, "appearance of resistant mutants is a spontaneous sporadic occurrence ... exposure (to a drug) only selects mutants..." (Schnitzer and Hawking, 1966). The mutational theory for the origin of drug resistance is supported by the observation that even when a trypanosome population is derived from a single parasite by cloning, the trypanosomes comprising the population differ in their sensitivity to a particular trypanocidal drug. For example, in a strain of *T. congolense* with an overall Berenil sensitivity of 35.7 mg/kg and Samorin sensitivity of 3.6 mg/kg, clones derived from the population were shown to have sensitivities to Berenil ranging from 5.1 to 21 mg/kg and Samorin sensitivities ranging from of 1.5 to 5.1 mg/kg (Peregrine *et al*, 1990). This implies that although the original cloned organisms possess a particular level of drug sensitivity, as multiplication takes place and the population increases, mutants arise whose drug sensitivities differ from their parents. In a population of trypanosomes, resistance can be experimentally induced by taking advantage of the presence of parasites with varying sensitivities and selecting those that are resistant by applying suitable selection pressure, namely, administration of a trypanocidal drug.

It was the objective of this work to clone four *T. evansi* stocks and determine the suramin sensitivities of the derived clones. Resistance to suramin was induced using either cloned or uncloned trypanosomes in immunosuppressed or immunocompetent mice and the rate of its development investigated. The resistant trypanosomes were maintained in mice in the absence of suramin to determine if the resistance induced was stable with respect to time.

5.2 MATERIALS AND METHODS

5.2.1 DETERMINATION OF SURAMIN SENSITIVITIES

Two isolates of *T. evansi*, KETRI 2454 and 2476, previously shown to be sensitive to suramin at a dose rate of 5 mg/kg (Chapter Four), were used for experimental induction of suramin resistance. The lowest suramin dose to which these isolates were sensitive was determined by a titration experiment using suramin at dose rates of 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025 mg/kg body weight. For each isolate and drug-dose level, six mice were infected with 1×10^6 trypanosomes as described in section 3.3.3 and five of them treated with suramin 24 hours later. The sixth mouse was used as an untreated control. All mice were screened daily for parasites for a period of 60 days (section 3.3.4.1).

5.2.2 CLONE PREPARATION

An attempt was made to prepare cloned populations from four trypanosome stocks, KETRI 2454, 2455, 2476, and 3136 using a modified method of Van Meirvenne *et al*, (1975) and Gardiner *et al*, (1980). In this method, a buffer prepared by mixing PSG (Appendix 1) with Guinea pig serum in a ratio of 1:1 was used for all dilutions. Blood from a mouse with a high parasitaemia was diluted with the buffer until the parasitaemia was approximately 1 parasite in five fields (magnification x 400, Leitz Wetzlar SM-Lux, Germany). A 10 μl aliquot of buffer was put into the cavity of a haemocytometer (Neubauer, Improved Double Ruling Superior, West Germany) in order to maintain a moisturised environment. A 10 μl volume of the infected blood diluted with buffer was then put on a coverslip and the coverslip inverted over the cavity slide of the haemocytometer avoiding touching the buffer present in the cavity. The sample was then examined microscopically (x 400 magnification) and if the parasitaemia was too high, an appropriate dilution was made to decrease the number of trypanosomes per preparation to one. When a single trypanosome was observed in the sample, a second person confirmed its presence after which the drop containing the trypanosome was mixed with 0.1 ml of buffer contained in a 1 ml syringe. The trypanosome in buffer was then intraperitoneally inoculated into a mouse which had been immunosuppressed 24 hours earlier by a single intraperitoneal administration of cyclophosphamide monohydrate at 300 mg/kg body weight.

The cloning procedure was repeated until ten mice were inoculated for each isolate under investigation. The mice were monitored daily for parasitaemia by wet

blood analysis. Any development of parasitaemia in the inoculated mice represented cloned trypanosome populations. The clones were numbered as they appeared i.e. the parasites from the first mouse to develop parasitaemia in a particular isolate were numbered strain 1. Cloning success for each of the stocks was calculated as the proportion of successful infections out of all mice inoculated with a cloned parasite. If all the mice inoculated with single trypanosomes did not become parasitaemic, the experiment was repeated.

5.2.3 SPECTRUM OF RESISTANCE

The suramin sensitivity of each of the clones derived from KETRI 2454, 2455, 2476 and 3136 was investigated as described earlier (section 4.2.3) in order to determine the resistance spectrum exhibited by each stock. The suramin dose ranges used (Table 5.1) were chosen in view of the known sensitivity of the isolates to suramin as indicated in Chapter Four.

Table 5.1 Suramin doses used to determine the sensitivities of clones derived from four *T. evansi* stocks.

Stock KETRI no.	Suramin doses used in mg/kg
2454, 2476	0.005 0.01 0.02 0.05 0.1 0.2 1
2455	1 2 4 5 10 20
3136	10 20 40 60 80 100 120 140 160

5.2.4 INDUCTION OF RESISTANCE

Resistance was induced in cloned strains of KETRI 2454 and KETRI 2476, originally sensitive to suramin at a dose rate of 0.005 mg/kg. Four methods modified after Schnitzer and Grunberg (1957) which were used to induce resistance are listed below.

5.2.4.1 Relapse method

Two mice were inoculated with 1×10^6 cloned parasites and then treated 24 hours later with a suramin dose rate of 0.005 mg/kg (Figure 5.1). Mice were then

to two more mice which were then treated with the next higher suramin dose (Table 5.4). If mice were not infected after a passage attempt, then the cryopreserved material at the preceding suramin dose level was used to inoculate mice and the experiment repeated until mice were infected.

5.2.4.2 Short passage method

Two mice were infected and treated as previously described (5.2.4.1). Cryopreservation and passages were, however, made 24 hours after treatment irrespective of the presence or absence of parasitaemia (Figure 5.2).

5.2.4.3 Reduced host response method using cloned trypanosome populations

This method was similar to the relapse method described earlier except that, 24 hours prior to infection, mice were immunosuppressed by intraperitoneal administration of 300 mg/kg of cyclophosphamide monohydrate (Figure 5.3). If the passage material in this method did not infect mice after a passage attempt, then the material cryopreserved at the preceding suramin dose level was used for passage and the experiment repeated until mice were infected.

5.2.4.4 Reduced host response method using uncloned trypanosome populations

The parent stocks (KETRI 2454 and 2476) from which the clones had been obtained were also subjected to the induction procedure. This was done so as to compare the induction of resistance in homogeneous (cloned) and heterogeneous (not cloned) parasite populations. If the passage material in this method did not infect mice after a passage attempt, then the material cryopreserved at the preceding suramin dose level was used as passage material.

Suramin-sensitivity tests (section 5.2.1) were carried out at each level of suramin treatment to verify that the cryopreserved material was resistant to suramin at that level. If the sensitivity test showed that trypanosomes were not resistant to the indicated dose, it was assumed that resistance had not been induced and the experiment was repeated starting with material cryopreserved at the preceding suramin dose level.

5.2.5 STABILISING RESISTANCE

When an end-point was reached where no further resistance to suramin could be induced using either the relapse or reduced-host response methods, then a different induction method was attempted. In this method described by Peters (1987b) an attempt was made to first stabilise the induced resistance by repeating treatment with suramin at the dose rate to which resistance was expressed. If parasitaemia persisted

induction method was attempted. In this method described by Peters (1987b) an attempt was made to first stabilise the induced resistance by repeating treatment with suramin at the dose rate to which resistance was expressed. If parasitaemia persisted or there was a relapse, resistance at that dose level was assumed and the process repeated using the next higher suramin dose as illustrated in Figure 5.4.

The ease with which suramin resistance could be induced by each of the four methods, as a measure of the time taken to achieve resistance at a particular dose level, was compared using the chi-square test.

5.2.6 STABILITY OF RESISTANCE

To determine if the acquired resistance was stable with respect to time, parasites resistant to the highest dose rate of suramin produced by each induction method were passaged ten times in two immunocompetent mice without drug treatment. At the eleventh passage, mice were treated 24 hours after infection with the appropriate dose of suramin and observed for relapses for 60 days.

5.3 RESULTS

The two parent stocks, KETRI 2454 and 2476, were resistant to suramin at a dose level of 0.005 mg/kg body weight but were sensitive at dose rates of 0.01 mg/kg body weight and above.

A cloning success rate of 60 % was achieved using KETRI 2454 and KETRI 2476 but with KETRI 2455 and KETRI 3136, the success rates were much lower at 8.3% and 1.7% respectively (Table 5.2). The different clones produced from the four trypanosome stocks had suramin sensitivities ranging from 0.005 to 120 mg/kg (Table 5.3). For KETRI 2454 and 2476, one clone from each stock had a similar sensitivity to the parents (0.005 mg/kg) but the other five clones had suramin sensitivities greater than the parent stocks. The clone of KETRI 2455 had a sensitivity of 2 mg/kg which was lower than the sensitivity of the parent stock (10 mg/kg). The four clones of KETRI 3136 had suramin sensitivities ranging from 60 to 120 mg/kg which are lower than the sensitivity of the parent stock (180 mg/kg).

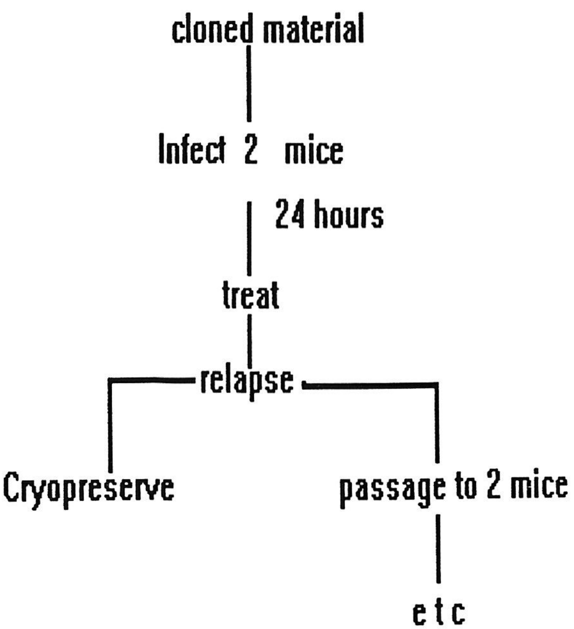


Figure 5.1 Relapse method of induction of resistance.

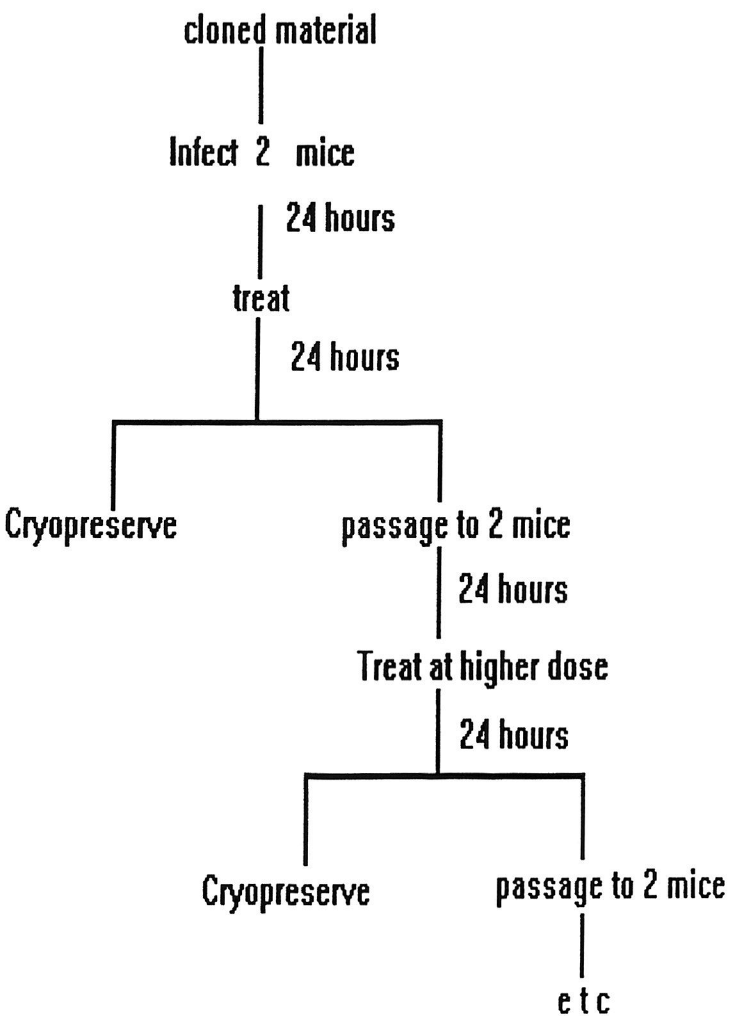


Figure 5.2 Short passage method of induction of resistance.

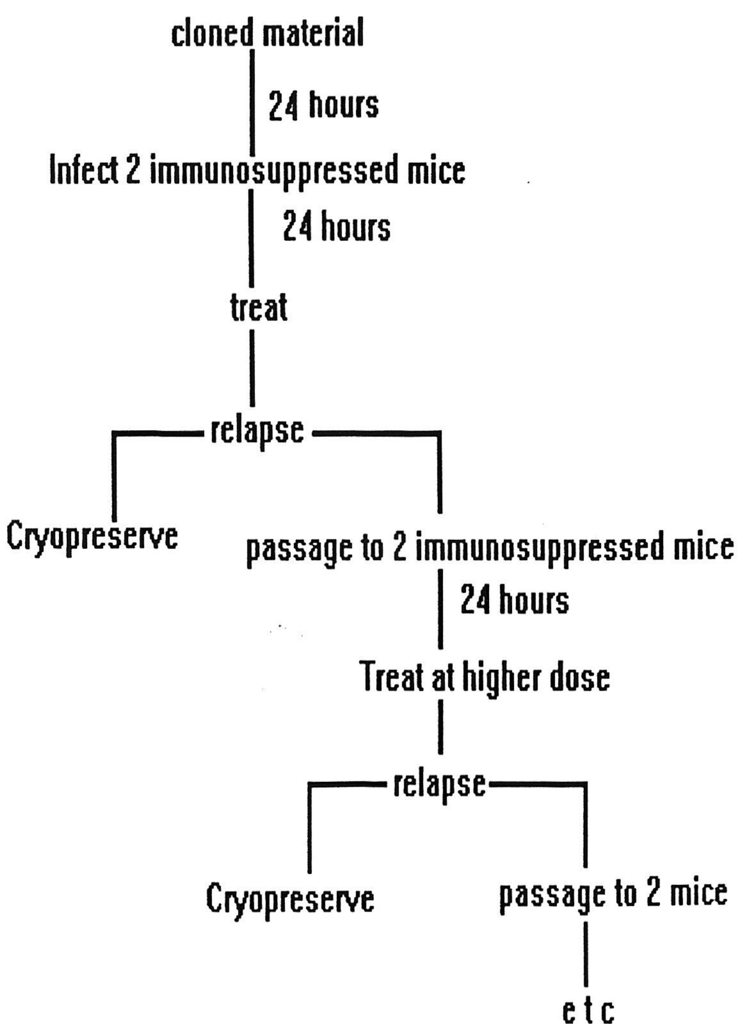


Figure 5.3 Reduced host response for induction of resistance.

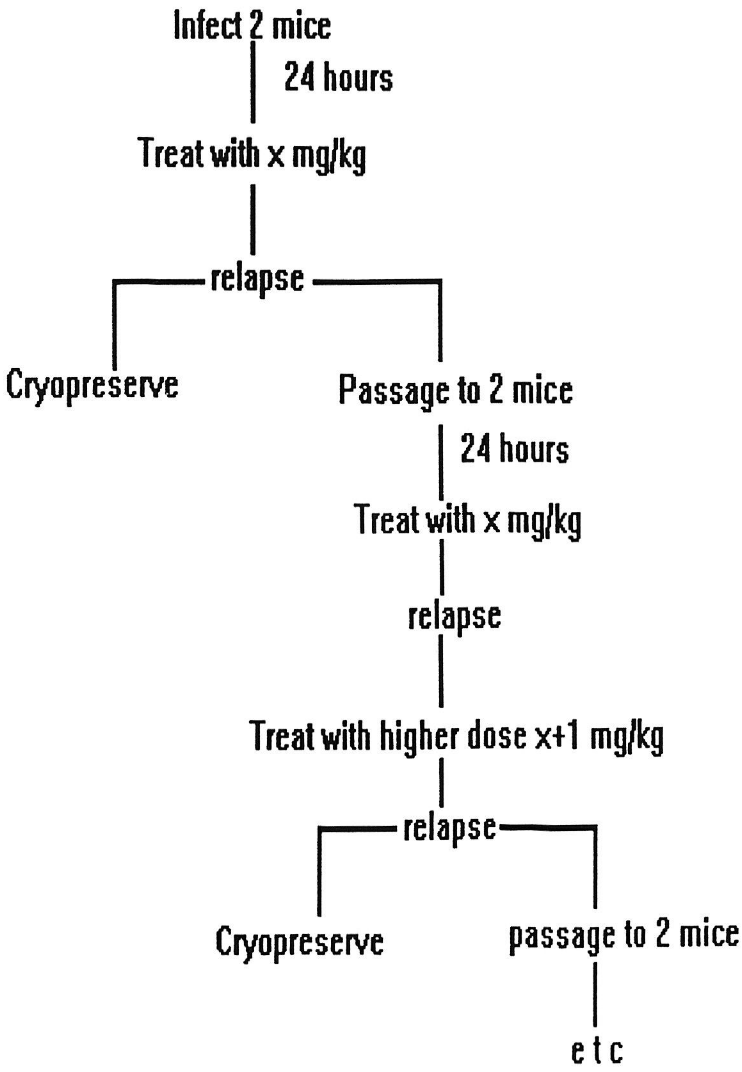


Figure 5.4 Stabilising induced suramin resistance.

Attempts to induce resistance to suramin were made using KETRI 2454 clone 6 and KETRI 2476 clone 2, which were resistant to suramin at a dose rate of 0.01 mg/kg body weight. Using the relapse and reduced-host response methods, it was possible to induce resistance in these clones by progressively increasing the doses of suramin (Table 5.4). The short-passage method of inducing resistance was abandoned when cryopreserved trypanosomes, were shown to be sensitive to suramin at all the dose levels to which they were expected to be resistant. The maximum levels of resistance achieved using the three other methods (Table 5.4) show that the most efficient method of inducing resistance, determined by the highest level of resistance attained, is by the use cloned trypanosomes in immunosuppressed mice. With this method, the highest resistance obtained was at a dose rate of 15 mg/kg of suramin compared to a dose rate of 6 mg/kg with the uncloned parent stock and 4 mg/kg with the relapse method (KETRI 2476 clone 2). Using KETRI 2454 clone 6, resistance was induced to suramin dose rates of 0.1, 2 and 6 mg/kg using the relapse method, cloned and uncloned trypanosomes in immunosuppressed mice respectively. By using cloned trypanosomes in immunocompetent mice, resistance to suramin was induced up to 0.1 mg/kg in KETRI 2454 and 4 mg/kg in KETRI 2476. Resistance levels of 2 and 15 mg/kg were attained using cloned trypanosomes of KETRI 2454 and 2476 respectively in immunosuppressed mice while 6 mg/kg was achieved when using uncloned trypanosomes of both stocks in immunosuppressed mice.

In order to compare the time it took to induce resistance using the three methods, resistance levels coded as 5, 9, 11 and 13 were used. These levels correspond to 0.1, 2, 4 and 6 mg/kg and are the maximum suramin dose rates to which resistance was induced using at least two methods. The time it took to induce resistance to these levels is shown in Table 5.5. The chi square test indicated that there was no significant difference in the time it took to induce resistance to the four levels (shown in Table 5.5) using the three methods (Table 5.6).

After ten passages in immunocompetent mice, the six strains with maximum resistance (A5, B13 and C9 in KETRI 2454 and A11, B13 and C22 in KETRI 2476) were shown to be still resistant at the doses indicated at induction.

Table 5.2 Cloning success rates of four *T. evansi* stocks, KETRI 2454, 2455, 2476 and 3136.

KETRI no	No. of clones established	No of cloning attempts	% cloning success
2454	6	10	60
2455	1	60	1.7
2476	6	10	60
3136	4	48	8.3

Table 5.3 Suramin sensitivities of clones derived from *T. evansi* stocks, KETRI 2454, 2455, 2476 and 3136.

Isolate KETRI no.	Clone no.	Suramin resistance mg/kg
2454	1	0.2
	2	0.1
	3	0.1
	4	0.05
	5	0.2
	6	0.005
2455	1	2
2476	1	0.2
	2	0.005
	3	0.05
	4	0.05
	5	0.1
	6	0.1
3136	1	80
	2	80
	3	120
	4	60

Table 5.4 Time taken to induce suramin resistant in *T. evansi* KETRI 2454 clone 6 and KETRI 2476 clone 2 using three different methods A, B and C.

Resistance level code	Equivalent suramin dose in mg/kg	Days to induction of resistance					
		KETRI 2454			KETRI 2476		
		A	B	C	A	B	C
1	0.005	5	2	2	7	2	2
2	0.01	4	2	5	5	2	5
3	0.02	7	3	2	9	3	9
4	0.05	2	7	2	3	7	2
5	0.1	2*	5	5	2	3	3
6	0.2		3	-	2	5	-
7	0.5		4	8	3	2	2
8	1		2	2*	3	2	12
9	2		9*	6*	5*	2*	5*
10	3		3*		5*	2*	3*
11	4		4*		10*	2*	7*
12	5		5*			12*	10*
13	6		9*			10*	8*
14	7						12*
15	8						14*
16	9						11*
17	10						12*
18	11						10*
19	12						11*
20	13						16*
21	14						13*
22	15						8*

Legend: A - relapse method using cloned trypanosomes in immunocompetent mice
B - reduced-host-response method using uncloned isolate material
C - reduced-host-response method using cloned trypanosomes
\- this dose level was omitted
* stabilising resistance used at this level

Table 5.5 Comparison of the time taken to induce suramin resistance to four dose levels using three methods.

Dose level	Days to induction					
	KETRI 2454			KETRI 2476		
	A	B	C	A	B	C
5	20	19	16	26	17	21
9		37	32	39	28	40
11		44		54	32	50
13		58			54	68

Legend: A - relapse method using cloned trypanosomes in immunocompetent mice
B - reduced-host-response method using uncloned isolate material
C - reduced-host-response method using cloned trypanosomes

Table 5.6 Statistical comparison of three methods A, B and C of inducing suramin resistance.

Resistance level code	Degrees of freedom	Chi square value observed	Chi square value tabular at 5%	P value	Significance
5	5	3.17	11.07	> 0.05	N.S.
9	4	2.92	9.49	> 0.05	N.S.
11	3	6.13	7.82	> 0.05	N.S.
13	1	1.73	3.84	> 0.05	N.S.

Legend: A - relapse method using cloned trypanosomes in immunocompetent mice
B - reduced-host-response method using uncloned isolate material
C - reduced-host-response method using cloned trypanosomes

5.4 DISCUSSION

There was a wide range of cloning success rates with the four *T. evansi* stocks examined. Barry *et al,* 1979 reported cloning success rates of 70% and 50% in *T. b. rhodesiense* and *T. b. brucei* respectively. For these workers, different species had different cloning success rates as compared to the results in this study where the success rates of cloning differed between different stocks of the same species. A greater success rate was obtained with the suramin-sensitive stocks of *T. evansi* (KETRI 2454 and 2476) compared with the resistant stocks (KETRI 2455 and 3136). This success, however, did not appear to be related to the degree of suramin resistance as the lowest success rate was obtained with KETRI 2455 which was resistant to suramin at a dose rate of 10 mg/kg body weight and not with KETRI 3136, resistant at a dose rate of 160 mg/kg body weight.

Cloning success is determined by the ability of a single parasite to withstand the host's defence mechanisms and establish an infection. This may be related to some innate characteristics of the drug-sensitive trypanosome, such as a fast growth rate or high turnover of VATs, which enable it to proliferate quickly and evade host defence mechanisms. Although only four trypanosome stocks were investigated, the reduced cloning success rates obtained with the suramin-resistant trypanosomes suggests that they might establish less readily than sensitive stocks in vertebrate hosts. This agrees with the observation of Cantrell (1956) who, studied the behaviour of drug-sensitive and resistant *T. equiperdum* parasites in mixed infections, and concluded that "most drug resistant strains are less well adapted to life in the absence of the drug than their unmodified parents".

Since clones from a single trypanosome stock showed a range of suramin sensitivities, it is likely that field isolates are probably mixed populations so far as suramin sensitivity is concerned. This is the first time that *T. evansi* stocks have been shown to be heterogeneous populations with respect to drug sensitivity. Similar findings have been reported in other salivarian trypanosomes such as *T. congolense* (Peregrine *et al,* 1990). In that study, nine clones of the parent isolate had a spectrum of resistance to isometamidium chloride ranging from 1.5 to 5.1 mg/kg body weight while the range of resistance to diminazene aceturate was from 5.1 to 21 mg/kg. All of the clones of KETRI 2455 and 3136 and those investigated by Peregrine *et al* (1990) had trypanocidal sensitivities which were lower than those of their parent isolates. Unlike the present observation with *T. evansi* KETRI 2455 and 3136, the study on *T. congolense* using isometamidium (Peregrine *et al,* 1990) showed that one clone was

more resistant than the parent stock. All other clones however, had sensitivities which were lower than the parents. The clones of the two *T. evansi* stocks used in this study (KETRI 2454 and 2476) differed with the two resistant stocks investigated (KETRI 2455 and 3136) in that they had sensitivities to the same or higher suramin dose levels as their parents. This may be due to the fact that their sensitivies were at very low dose levels and thus not very accurate therefore, variation in sensitivities were not significant.

Walker (1964) determined that ten resistant trypanosomes in a million are enough to give an isolate a resistant phenotype. One can thus assume that a heterogeneous stock must have at least this proportion of parasites, whose suramin sensitivity is the same as that of the parent isolate. If this assumption is correct, then there must have been at least this proportion of clones which gave identity to KETRI 2455 and 3136 and it is likely that they may have been among the ones inoculated into mice resulting in the failed cloning attempts. This would thus suggest that the resistant clones are less adapted to withstand the hostility of the host's defences than their sensitive counterparts as indicated by failure to establish infection. Hence, with *T. evansi* KETRI 2455 and 3136 and the *T. congolense* in the study of Peregrine *et al* (1990), the clones which was more resistant than the parent stocks might thus have consisted of less than 0.001 % of the total population. This argument would explain why some clones in this work and in the study of Peregrine *et al,* (1990) were of lower drug sensitivities compared to their parent clones.

Although work showed that the trypanocidal action of suramin depended on an intact host defence system (Roehl, 1926), the role of the host response in most chemotherapeutic agents against trypanosomiasis in drug resistance was not well recognised by early workers (Yorke *et al,* 1932). In contrast, in bacteriology this role was recognised indicating that the animal host did not play merely a passive role while drugs and parasites interacted (Browning, 1931). The fact that with both isolates of *T. evansi* the highest levels of resistance attained in this study were in immunosuppressed mice, confirms the importance of the host's defence mechanism in the development of resistance. The role of a reduced immune response in the induction of resistance was investigated by Von Jancso and Von Jancso (1935b) who found that *T. brucei* parasites inoculated into mice immunosuppressed by splenectomy or by administration of electro-colloidal copper, were more likely to develop resistance compared with parasites in immunocompetent mice. Schnitzer *et al,* (1946) also splenectomiced rodents before induction of p-rosaniline resistance in *T. equiperdum*. In that study, induction of resistance could be inhibited, however, by the transfer of hyper-immune sera against trypanosomes from cured animals to naive ones confirming that what was

that what was initially being blocked was the ability of the host to produce immune sera. Lately, Osman *et al,* (1992) have also demonstrated the importance of immunosuppression (by irradiation) in the induction of resistance in *T. evansi* to Mel Cy, diminazene aceturate and isometamidium chloride.

The possible role of immunosuppression in the development of resistance in trypanosomes under field conditions needs to be considered in view of conditions that may compromise the ability of animals to respond immunologically to trypanosome infection. Such conditions may occur in mixed infections when trypanosomes are found with other pathogens such as helminths and ectoparasites. Other conditions that may be important are those that are due to stress including work, malnutrition, transportation and adverse climatic conditions. These have been observed to be important in the susceptibility of buffaloes to *T. evansi* (Luckins, 1988). All these factors, together with the immunosuppressive nature of trypanosome infection (Onah, 1992) may compromise the immune system of camels in the field and thus increase the likelihood of the development of suramin resistance. These may be especially important when combined with trypanocide under-dosing; a feature which is common with nomadic camels keepers who may want to treat all their animals in a herd and yet are not able to purchase sufficient trypanocide. Under-dosing by under-estimating the weight of an animal is also possible in view of the absence of weighing apparatus in the field and the difficulties involved in handling camels for treatment procedures.

This work has shown that it is relatively easy to experimentally induce high levels of resistance to suramin in *T. evansi* using mice as was observed by Osman *et al,* (1990) working with Mel Cy, Berenil and Samorin. Yorke *et al,* (1932) and Von Jancso and Von Jancso (1934) were able to induce suramin resistance in *T. brucei* equivalent to the MTD of this drug in mice. In the present work, the maximum resistance induced was less than the MTD. The MTD which is the drug dose toxic to 80% of a particular animal species (Walker, 1964; Gill, 1971b), has been determined to be 150 mg/kg for suramin in mice (Abebe *et al,* 1983). The maximum suramin resistance of 15 mg/kg attained in this work in 20 passages within a period of 25 weeks is lower than 50 mg/kg achieved in 80 passages within 85 weeks by Gill (1971b) using the same trypanosome species. However, the resistance induced in this study was 3000-fold more than the parent clone compared to 33-fold by Gill (1971b) and 250-fold in *T. brucei* induced by Von Jancso and Von Jancso (1935b).

Experiments in this study show that the development of suramin resistance in *T. evansi* not only involves selection but also a prior mutational event. Homogeneity with respect to suramin sensitivity was established by first cloning and therefore ensuring that any selection could only have occurred after a mutation. Further evidence for a

mutational origin of resistance is supported by two other observations. Firstly, the number of days that elapsed before a relapse was observed at the various dose levels did not follow any particular pattern. Secondly, the homogeneity or heterogeneity of a population did not appear to influence the development of resistance. This is because mutation, being a spontaneous event, is unpredictable.

Chances of mutation taking place are increased by among other factors the number of organisms and the time period. Thus, more mutations will arise in a comparatively large population of organisms over a long period than in a smaller population over a shorter period. The process of first stabilising resistance by repeating suramin administration at one dose level before moving on to the next level provided more time for mutational and selective processes to occur. Thus, resistance was induced to higher levels than was previously possible. This study suggests that suramin resistance in *T. evansi* develops by an initial process of mutation as has been observed in *Plasmodium* and neoplastic cells (Diggens *et al*, 1970; Ling, 1982).

According to the prediction model of Hawking (1963b), suramin resistance should not be stable since the resistant trypanosomes multiply more slowly than their sensitive counterparts. There have been studies on the persistence of suramin resistance in the past. Morgenroth and Freud (1924) induced suramin-resistance in a strain of *T. rhodesiense* which remained stable after 28 years of sub-passage in mice (Amrein and Fulton, 1959). However, Fulton and Yorke (1941) obtained varying results with the *T. rhodesiense* stocks with which they worked. One stock lost all resistance in four years while a second lost some resistance after four years. The results obtained in this study with *T. evansi* indicate that parasites exhibited stable suramin resistance. In both this work and the work on *T. rhodesiense*, only a few stocks were investigated and it would be necessary to maintain more *T. evansi* stocks in mice without the drug for more than the ten passages used in this study in order to confirm the stability of suramin resistance.

In conclusion, this study has shown that four stocks of *T. evansi* consist of trypanosomes with sensitivities to suramin which are the same as, lower or higher than that of the parent stocks. The trypanosomes with different suramin sensitivities most probably arose by mutation and were selected in the presence of suramin resulting in a resistant phenotype of a trypanosome stock. Thus, with or without suramin, trypanosomes exhibited a range of suramin sensitivities and introduction of the drug only selected for the more resistant parasites. The rise and selection of drug-resistant mutants was not related to the method used for inducing resistance although it was enhanced by immunosuppression of the host. This supports the suggestion that mutations are events which occur at low frequencies in individual trypanosomes and

are eliminated by the immunological mechanisms of the host (Osman *et al,* 1990). In immunocompromised hosts, however, the drug-resistant mutants survive and benefit from selection by drug pressure. The success of cloning *T. evansi* in this study varied between stocks of different suramin sensitivities and may have been related to the level of suramin sensitivity exhibited by the stocks. The observation that drug resistant trypanosomes were more difficult to clone compared to drug-sensitive ones suggests that a single resistant trypanosome is unable to survive in a mouse as readily as a sensitive one. Thus, cloning may be hindered by some inability of the drug-resistant parasites to establish infections as compared with drug-sensitive organisms. Although mice were used in this study, the findings observed may have implications in the establishment and maintenance of suramin resistance in *T. evansi* infecting camels under field conditions.

Subsequent work described in Chapters six and eight was done using trypanosome populations in which suramin resistance was induced in the work described in this Chapter by the reduced host response method starting with cloned trypanosomes. These trypanosome populations are described according to their resistant level codes for instance KETRI 2476 C22 refers to a trypanosome population in which resistance to suramin was induced by the reduced host method up to a dose level of 15 mg/kg (see Table 5.4).

CHAPTER SIX

COMPARISON OF GROWTH RATES OF SURAMIN-SENSITIVE AND RESISTANT *T. EVANSI*

6.1 INTRODUCTION AND OBJECTIVE

In the presence of a trypanocidal drug, trypanosomes which have acquired resistance to that drug have an obvious selective advantage over those that are still sensitive to the drug's action. In the absence of the drug, this situation probably changes and drug-resistant parasites are selected against, otherwise resistance would spread much faster than has been observed. The behaviour of drug-resistant trypanosomes was investigated by Cantrell (1956) who worked with two stocks of *T. equiperdum*; a strain resistant to oxophenarsine and its drug-sensitive parent. In that work, Cantrell mixed the two stocks in equal proportions and inoculated them into rats. When parasites were observed in blood, they were passaged into a second rat and the first rat was then treated with oxophenarsine. This led to a temporary remission of parasitaemia indicating that the majority of trypanosomes in this population were sensitive to oxophenarsine. When a relapse was observed, he plotted the number of parasites over time. Assuming that this population consisted of drug-resistant trypanosomes, he extrapolated the plot backwards to estimate the number of resistant trypanosomes that constituted the original wave of parasitaemia prior to treatment. The number of oxophenarsine-resistant trypanosomes were thus estimated at every passage. The results obtained in that study showed that by the fifth passage, the drug-resistant strain constituted less than 1% of the total population and by the 27th passage, drug treatment resulted in complete cures indicating that trypanosomes that were resistant to oxophenarsine had been selected out. This led Cantrell to conclude that drug-resistant strains were less well adapted to survival than their drug-sensitive unmodified parents. The reason for elimination of trypanosomes resistant to oxophenarsine may have been due to their slower growth rates thus, at every passage, drug-resistant trypanosomes multiplied more slowly than the sensitive ones eventually leading to a time when populations consisted wholly of drug-sensitive trypanosomes. Hawking (1963b) in considering drug-resistance in trypanosomes observed that there

were two kinds of drug resistant parasites; those that grow as fast as or faster than the sensitive ones and those that multiply at a slower rate than the sensitive ones. He suggested that this comparative growth rate of resistant trypanosomes determined their success in the environment.

The objectives of this work was to determine and compare the multiplication rates of *T. evansi* parasites with different degrees of resistance to suramin in rats in order to investigate whether there is a relationship between drug resistance and growth rates which could influence the survival of trypanosomes in a vertebrate host.

6.2 MATERIALS AND METHODS

6.2.1 EXPERIMENT

The trypanosomes used in this experiment were either uncloned stocks or cloned lines showing different levels of resistance to suramin (Table 6.1). Three trypanosome stocks (KETRI 2454, 2476 and 3136) together with two clones from two of the stocks (KETRI 2454 and 2476) were used in this experiment. Of the two clones of each stock, one was induced-resistant to suramin and the other was its unmodified parent (see Table 5.4). Five rats were infected with each trypanosome stock as described in 3.3.1 and observed for parasitaemia daily by haematocrit centrifugation technique (3.3.4.1) until the onset of parasitaemia was detected in any rat in a group. Starting the following day at 9 a.m. which was designated hour 0, rats were bled five times daily (at 9 a.m., 11 a.m. 1 p.m. 3 p.m. and 5 p.m.) for nine days and the number of trypanosomes determined. To count trypanosomes, rats were bled from the tail using heparinised capillary tubes. For each sample, one tube-full of blood (approximately 0.1 ml) was collected and diluted in 5, 10 or 20 ml of 10% ammonium oxalate solution. This solution lysed the erythrocytes and enabled more accurate trypanosome counting. The volumes of the lysing solution were chosen because they gave blood dilutions which were optimal for counting trypanosomes in blood parasitaemias which ranged from 1 to 100 trypanosomes per microscopic field (x 400 magnification). Trypanosomes were then counted using a haemocytometer.

6.2.2 DETERMINATION OF GENERATION TIME

In order to show the trend of parasitaemia for each stock, the log of mean trypanosome counts of the five rats in each group at each sampling were plotted against time. The growth curves of trypanosomes in each rat were determined by plotting the log of trypanosome count against the time of bleeding. Using these

wave was calculated. This period was chosen because it was assumed that it would be when parasites had least interference from the host's immune response. The growth rate of trypanosomes in each rat was determined by calculating the generation time using the following formula (Topley and Wilson, 1955):-

$$G = \frac{T}{n} \quad \text{and} \quad n = \frac{\log b - \log a}{\log 2}$$

where G - generation time
T - time period
n - number of generations
a - number of organisms at the beginning of the period T
b - number of organisms at the end of the period T

The generation time for each trypanosome stock was determined by computing the mean generation times of the five rats in each group. In the determination of growth rates, a number of major assumptions were made; namely, that parasites were evenly distributed in the blood, all trypanosomes were capable of multiplying, trypanosome death rate was constant in all the stocks and errors of trypanosome counting were constant.

6.2.3 STATISTICS

The generation times of the different strains were compared using either the Kruskal-Wallis or Mann-Whitney tests.

Table 6.1 Lines of *T. evansi* used to compare growth rates of populations with different sensitivities to suramin.

KETRI no.	Homogeneity	Suramin resistance mg/kg
2454	stock	0.005
2476	stock	0.005
3136	stock	160
2454 C1	cloned line	0.005
2454 C9	cloned line	2
2476 C1	cloned line	0.005
2476 C22	cloned line	15

6.3 RESULTS

The trypanosome counts at the beginning and end of the first day of observation in each rat as well as the generation times are shown in Appendix 27. The range of generation times of three *T. evansi* stocks (KETRI 2454, 2476 and 3136) varied from 2.9 to 12.0 hours with means of 5.6, 7.8 and 5.3 respectively (Table 6.2). There was no statistically significant difference in the mean generation times of the these three stocks (comparison 1, Table 6.3) as seen by their similar growth curves during the first rise of parasitaemia (Figure 6.1a). The range of generation times of KETRI 2454 and its clones (C1 and C9) was from 3.6 to 16.2 hours with means of 5.6, 7.0 and 9.4 respectively (Table 6.2). Although the two clones had longer generation times than their parent stock, no statistical significant difference was observed between the parent stock with its clones (comparisons 2, 3, and 4) or the generation times of the two clones (comparison 5, Table 6.3). The two clones of KETRI 2454 (C1 and C22) had a similar increase of parasitaemia during the first day while the growth curve of their parent stock (KETRI 2454), though similar to those of the clones, shows a more gradual rise in parasitaemia (Figure 6.1b). KETRI 2476 and its clones (C 1 and C 22) had generation times ranging from 2.6 to 13.7 hours with means of 7.8, 5.8 and 10.4 respectively (Table 6.2) which were statistically different ($p<0.05$, comparison 6, Table 6.3). The Mann-Whitney test showed that this significant difference ($p>0.01$) was between the two clones (comparison 9, Table 6.3) and not between the parent stock with either of the two clones (comparisons 7 and 8). Figure 6.1c shows the difference in growth curves of KETRI 2476 and its clones and the similarities of the clones. Thus, trypanosomes which were resistant to suramin at a dose rate of 15 mg/kg body weight (KETRI 2476 C22) grew at a slower rate that those that were sensitive at 0.005 mg/kg (KETRI 2476). There was a rapid rise the parasitaemia of rats infected with KETRI 2476 C22 during the first day of counting while the rise of KETRI 2476 C 1 was not as rapid.

Table 6.2 The means and ranges of generation times of trypanosome strains with different sensitivities to suramin.

Stock KETRI number	Suramin resistance in mg/kg	Generation time in hours Mean and SD	Range
2454	0.005	5.6 ± 1.4	3.6 - 7.5
2454 C_1	0.005	7.0 ± 1.5	5.9 - 10.0
2454 C_9	2	9.4 ± 3.6	5.7 - 16.2
2476	0.005	7.8 ± 2.2	5.9 - 12.0
2476 C_1	0.005	5.8 ± 1.9	2.6 - 8.3
2476 C_{22}	15	10.4 ± 2.1	8.6 - 13.7
3136	160	5.3 ± 2.1	2.9 - 8.4

Table 6.3 Comparisons between generations times of three *T. evansi* stocks KETRI 2454, 2476 and 3136 and two clones each of KETRI 2454 and 2476.

Tabular H value for Kruskal Wallis test with k=3 n= 5 is 5.78

*Tabular U value for Mann-Whitney with n_1=5 n_2= 5 is 2 at 5% and 0 at 1%

Comparison no.	Comparisons	H or U* value	Difference
1	2454 / 2476 / 3136	2.58	N.S.
2	2454 / C1 / C9	4.2	N.S.
3	2454 / C1	8*	N.S.
4	2454 / C9	2.5*	N.S.
5	2454 C1 / C9	8*	N.S.
6	2476 / C1 / C22	7.9	*
7	2476 / C1	6.5*	N.S.
8	2476 / C22	4.0*	N.S.
9	2476 C1 / C22	0*	**

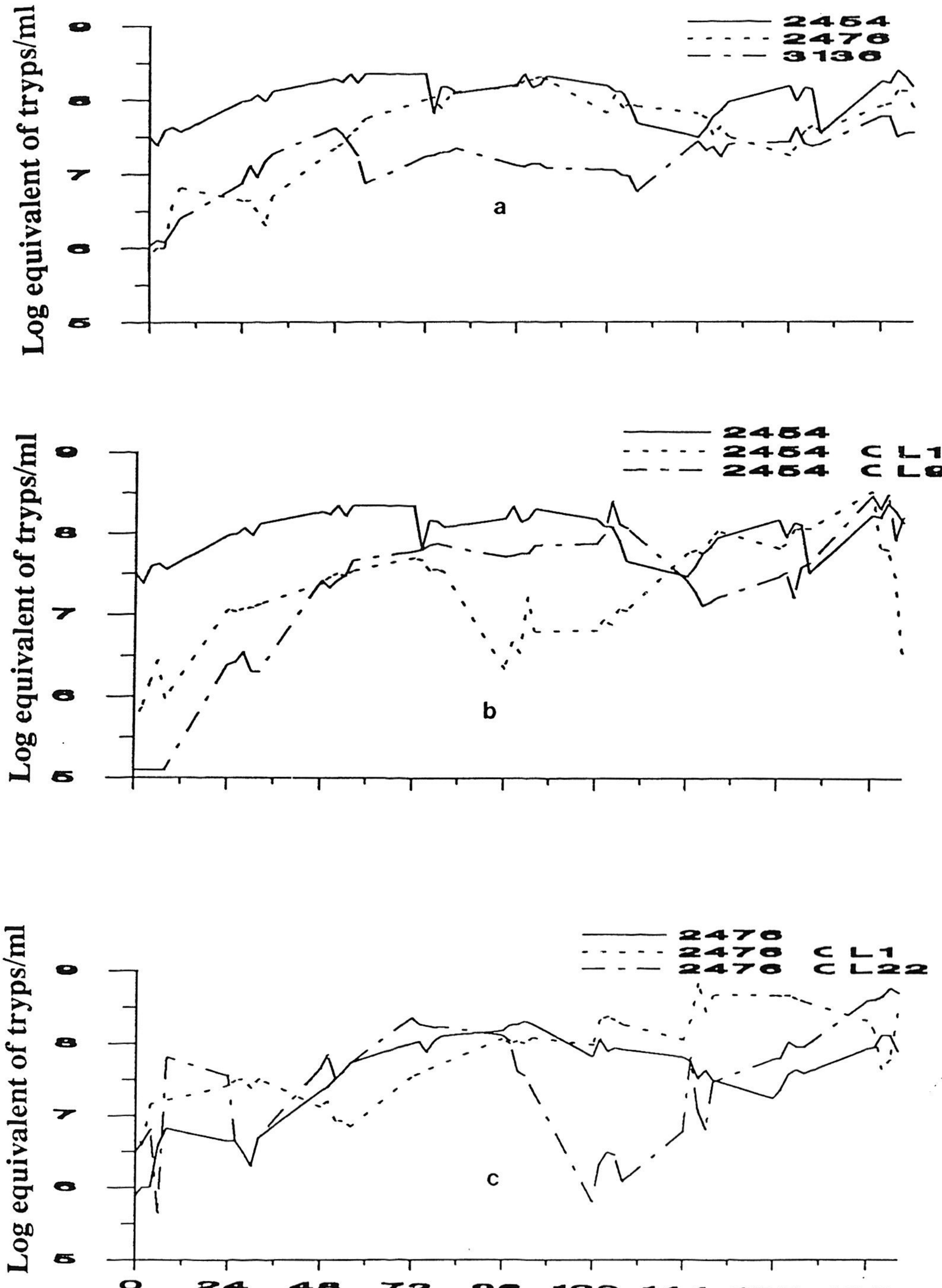
Legend:

N.S - not significant. For Kruskal Wallis; calculated value less than tabulated value at the 5% critical value, for Mann-Whitney calculated value more than tabulated value at the 5% critical value.

* - significant. Calculated value more than tabulated value for Kruskal Wallis and less than tabulated value for Mann-Whitney at the 5 % level.

** - highly significant. Calculated value more than tabulated value for Kruskal Wallis and less than tabulated value for Mann-Whitney at the 1% level.

Figure 6.1a Growth curves of *T. evansi* KETRI 2454, 2476 and 3136.
Figure 6.1b Growth curves of *T. evansi* KETRI 2454 with its two clones C1 and C9.
Figure 6.1c Growth curves of *T. evansi* KETRI 2476 with its two clones C1 and C22.



Time of counting trypanosomes in hours

6.4 DISCUSSION

Several workers have investigated growth rates of trypanosomes of different trypanocide sensitivities in mixed infections. Oehler (1914) observed that arsphenasine-resistant of *T. brucei* were displaced by their drug-sensitive parents when passaged in mice without treatment. Teichman (1918) in a similar experiment however, observed that either the drug-resistant or drug-sensitive trypanosomes were displaced. Unlike the earlier study, in the latter one, the two stocks of *T. brucei* were not related as parent and offspring. Laveran and Rondsky (1912) noticed that oxazine-resistant stocks of *T. brucei* and *T. evansi* which were acentrosomic displaced the drug-sensitive stocks in mixed infections. The results of this study were surprising in view of the well known tendency of acentrosomic trypanosomes to disappear from naturally occurring mixtures (Cantrell, 1956). This contradiction could be due to the fact that the stocks were not related. Cantrell (1956) observed that oxophenarsine-resistant *T. equiperdum* were displaced by drug-sensitive parasites in mixed infections.

The present study has shown that suramin resistant trypanosomes multiply more slowly than suramin-sensitive parent stocks from which they were derived; an association not previously observed in *T. evansi.* The relationship between growth-rate and suramin-sensitivity was only observed between parent stocks and their induced-resistant offspring and not with stocks without a parent-offspring relationship; i.e. one was not derived from the other. Hence, growth rate is a character of a particular stock irrespective of suramin sensitivity. In the present study, the relationship between resistance to suramin and growth rate was observed only between trypanosome stocks whose suramin sensitivities differed 3000 fold and not in other stocks with lower differences (400 fold) in respect to suramin sensitivity. This observation suggests that the degree of suramin resistance may be important for significant differences in growth-rate to be observed. This also suggests that differences in growth rate may be due to a progressive event such as dose effect. This would explain why the trypanosomes resistant to suramin at a dose rate of 2 mg/kg (KETRI 2454 C9), although replicating more slowly than the parent stocks (KETRI 2454 C 1), had not achieved a significant difference in growth rate as those that were resistant to suramin at a dose rate of 15 mg/kg (KETRI 2476 C22). There are other characteristics of drug-resistant parasites have been observed to have a progressive nature such as dose effect. The DNA amplification phenomenon observed in drug-resistant phenotypes has been shown to be dose related. In pyrimethamine-resistant

resistant phenotypes has been shown to be dose related. In pyrimethamine-resistant *Plasmodium* malaria, chromosome 7 determined to have the amplified gene, has an intensity dependent on the degree of resistance (Cowman, 1989). A similar dose-related effect was observed in methotrexate-resistant *Leishmania* maintained in a drug free medium. These organisms were shown to have a decline of resistance accompanied by a decrease in the copy numbers of the amplified gene (Beverley *et al,* 1984).

The practical implication of these differences in growth rates is that, after several passages in the absence of the drug in question, suramin-sensitive trypanosomes may outgrow resistant ones resulting in a situation where resistant trypanosomes finally disappear or are present at very low levels in the population as was observed by Oehler (1914) and Cantrell (1956) in oxophenarsine-resistant *T. equiperdum.* When the drug is introduced into this population, the resistant parasites are then at an advantage and might proliferate giving a re-occurrence of resistance. A similar situation was observed in serologically different clones of *T. gambiense* (Seed, 1978) and *T. rhodesiense* (Barry *et al,* 1979) where the faster growing clones gradually replaced the slower ones in a mixed infection. In view of the apparent ease of inducing resistance to suramin observed in this study and the period suramin has been used in the field, this phenomenon of slower growing resistant trypanosomes provides a possible explanation why suramin resistance or drug resistance in general is not as widespread as would be expected. It is also important to note that in the presence of a drug, this phenomenon could be reversed and resistance would spread.

Hawking (1963a) predicted that the slower-growing resistant trypanosomes would have resistance that is not stable because the resistant parasites would be outgrown by sensitive ones in mixed infections leading to their extinction. This prediction is reasonable in view of the growth rate results obtained in this study. Suramin resistance was however observed to be stable in the work described in Chapter Four, in spite of resistant trypanosomes growing more slowly than sensitive ones. Other studies both *in vivo* and *in vitro* (Morgenroth and Freud, 1924; Amrein and Fulton, 1959; Zweygarth *et al,* 1991) showed that suramin resistance persisted after maintenance without the drug. The fact that Hawking's prediction did not hold in these studies suggests that, other than growth rates, there may be other factors that determine the stability of suramin resistance. An example would be the relationship between the stocks; a factor shown to be important in the correlation of growth rate and resistance to suramin in this study. Thus, trypanosomes that are resistant to a particular drug may not multiply more slowly than drug-sensitive ones if the two

mechanisms would target different areas of suramin action and the resistant trypanosomes would thus have different characteristics such as growth rate. Such a situation has been observed in malaria whereby chloroquine resistance may develop by different mechanisms some related to the mdr phenotype while others are not (Peters, 1987a; Foote *et al*, 1989, 1990; Wellems *et al*, 1990).

This is the first time that the growth rate of trypanosomes of varying sensitivities to a trypanocide have been determined by direct counting of parasites for previous studies compared growth rates by elimination of parasites in mixed infections. Although counting of trypanosomes is laborious and may lack accuracy, it is probably a better approach of comparing the growth rates of different trypanosome stocks than elimination of one stock in a mixed infection. In the latter method, growth rate of a trypanosome stock may be influenced by other variables such as interference as will be discussed in the following Chapter. In conclusion, the work described in this chapter has shown that two *T. evansi* stocks have different growth rates. Trypanosomes which are resistant to suramin at a dose rate of 15 mg/kg grow more slowly than the parent trypanosomes which are sensitive to suramin at a dose rate of 0.005 mg/kg. This differences in growth rates is however not observed between trypanosomes which do not have a parent-offspring relationship in spite of an even larger difference (32,000 fold) in their sensitivities to suramin. Although this study was in rats infected with suramin-resistant *T. evansi*, it is possible that similar differences in growth rates are found in infected camels and may be important in limiting the spread of drug resistance under field conditions.

CHAPTER SEVEN

INTERFERENCE IN MIXED INFECTIONS

7.1 INTRODUCTION AND OBJECTIVE

It is possible for two different stocks of the same trypanosome species to be involved in a mixed infection due to concurrent or consecutive inoculations. In such situations, a mixed infection would only persist if both of them behaved independently, with neither one interfering with the other. Reports of mixed infections, however, indicate that such interference can, in fact, occur and the resulting infection is unlike that caused by either stock singly. In this interference phenomenon, one trypanosome stock modifies or even prevents another from establishing infection. Such a phenomenon was observed between serologically different strains of *T. rhodesiense* (Herbert, 1975) and *T. congolense* (Morrison *et al*, 1982; Luckins and Gray, 1983; Dwinger *et al*, 1986). Sones *et al* (1989) reported interference between strains of *T. congolense* with different sensitivities to Samorin. In Sones' study, goats were intravenously inoculated with 1 x 10^5 trypanosomes which were sensitive to Samorin at a dose rate of 0.001 mg/kg. When this infection was patent, the goats were super-infected with a similar dose of parasites resistant to Samorin at a dose rate of 2 mg/kg. The infection was, however, sensitive to treatment with Samorin at 1 mg/kg indicating that the trypanosomes which were resistant to 2 mg/kg dose rate of Samorin had not established infection. When the Samorin-resistant strain was inoculated first followed by super-infection with the sensitive strain, treatment with a dose rate of 1 mg/kg Samorin resulted in temporary remissions of parasitaemia but no cures. Hence, those trypanosomes which were sensitive to the action of Samorin at 1 mg/kg had established themselves in spite of the presence of infection with the Samorin-resistant infection. These observations led Sones *et al* to conclude that Samorin-sensitive *T. congolense* parasites interfered with the establishment of Samorin-resistant *T. congolense*. However, the reverse was not the case for the resistant strain of *T. congolense* did not prevent the establishment of the sensitive stock.

In field situations it is not uncommon to find animals harbouring mixed infections either as a result of a bite from a fly carrying more than one trypanosome species or by concurrent bites from flies carrying different trypanosome species. In the latter case, the second infection may only develop if there is no interference from the established infection. In cases where the drug sensitivities of the trypanosomes differed, they would be able to establish an infection only if they did not interfere with each other and if any drug administered did not affect either of them. In a situation where one stock was sensitive and one was resistant to the drug used, the sensitive strain would be selected out leaving only a single infection of the resistant trypanosomes. In the absence of the drug, however, drug-resistant trypanosomes do not have this advantage and such an observation led Cantrell (1956) to suggest that in the absence of the drug, most drug-resistant strains are less well adapted to survival in the naive host than their unmodified parents. Factors which might be responsible for differences in survival are differences in growth rates and VAT turnover between a drug-resistant stock of trypanosomes and a drug-sensitive stock.

Work done previously on the interference phenomenon has involved trypanosome species that are tsetse-transmitted and there have been no investigations to determine whether this phenomenon is limited to these trypanosome species or if it is also found in mechanically transmitted trypanosomes such as *T. evansi.* Previously, work has been limited to one vertebrate host and workers have not used the same trypanosome strains to investigate the interference phenomenon in the natural hosts of these trypanosome species, alongside laboratory rodents. The main objective of this work was to investigate if interference occurred between stocks of *T. evansi* with different suramin sensitivities and to compare interference in mice, rats and in the camel.

7.2 MATERIALS AND METHODS

Investigations were carried out using, mice, rats and camels. KETRI 3136, which is resistant to a suramin dose rate of 160 mg/kg body weight, was used as the suramin-resistant stock. KETRI 2454 and 2476 which are sensitive to suramin at a dose rate of 0.01 mg/kg body weight, were used as the suramin-sensitive trypanosomes stocks.

There were 8 groups of animals in each experiment (Table 7.1). In Groups A and B, both sensitive and resistant trypanosome stocks were inoculated concurrently into the animals. At the same time, as a control, the resistant stock (KETRI 3136) was

inoculated in group D and F animals. At the onset of parasitaemia in groups C and D, these animals were super-infected with respectively, a sensitive (KETRI 2454 or 2476) or a resistant (KETRI 3136) stock. Animals in group G and H were inoculated at the same time and acted as controls for the super-infections.

Animals in Group A were treated with suramin when trypanosomes were detected by microscopic examination of wet-blood film. In group B, treatment was administered only when animals in both control groups E and F were parasitaemic, indicating that both the sensitive and resistant trypanosomes had been given adequate time to establish infection. Animals in groups E and F were treated when animals in group B were treated. Animals in group C were treated when control animals in group H were parasitaemic, indicating that the secondary infection by drug-sensitive trypanosomes had sufficient time to establish. Similarly, animals in group G were treated when those in group G were parasitaemic. Animals in control groups G and H were treated at the same time that animals in groups D and C respectively, were treated. All animals were treated with suramin at a dose rate of 10 mg/kg bodyweight; rodents as a group when all were parasitaemic and camels individually as the animals became parasitaemic.

7.2.1 RODENTS

The experiments using mice and rats were carried out according to the protocol set out in Table 7.1. Animals were infected as described in section 3.3.3 and parasitaemia monitored daily by examination of tail-blood (section 3.3.4.1). A cure was recorded if at least eight of the ten mice showed no relapse in parasitaemia within 60 days after treatment. This corresponds to the minimum curative dose of 80 % (MCD_{80}). A temporary remission was recorded when trypanosomes disappeared from the blood but reappeared within 60 days while a suppression of parasitaemia was recorded when trypanosomes remained in the blood but the parasitaemia decreased. A result of "no effect" was recorded when treatment did not result in a change in the number of parasites in the blood. The time taken for *T. evansi* to kill animals in groups C (KETRI 3136 inoculated first followed by either KETRI 2454 or 2476) and D (either KETRI 2454 or 2476 inoculated first followed by 3136) was compared using the chi-square test (section 3.4.4). Two suramin-sensitive trypanosome stocks were used in mice in order to compare the results and determine if there was a difference in interference which could be attributed to the stocks. A similar experimental protocol was followed using rats except that only one suramin-sensitive stock, KETRI 2454, was used.

7.2.2 CAMELS

After pre-experimental procedures were carried out as described in 3.2.2, camels were infected, samples collected and infections monitored as described in sections 3.3.3 and 3.3.4. The cut-off point between negative and positive ELISA values was set at an absorbance rate of 0.05 which was arbitrarily determined as twice the mean reading of control sera obtained from camels known to be negative for trypanosomiasis (kindly provided by Dr. W. Olaho-Mukani). Owing to varying pre-patent periods of trypanosomes in camels within a group, animals were treated individually when they became parasitaemic. At the end of the experiment, all the camels were treated with cymelarsan (R Rhone Poulenc) at a dose rate of 0.5 mg/kg body weight.

Owing to the constraints on the numbers of camels available due to holding capacity of the animal accommodation and finance, it was only possible to use two camels per experimental group and one camel as a control. Although pre-experimental monitoring of the camels when they came to the barn at KETRI showed that they were all negative for trypanosomiasis in the field, trypanosomes were detected subsequently in some of them by micro-haematocrit centrifugation technique (mct), mouse sub-inoculation (mi) or by antigen-detecting ELISA. Thus all camels were treated with Trypacide sulphate prior to any experimental procedures (see section 3.2.2). Most of the camels lost weight during the pre-experimental monitoring period probably because of the stress of acclimatisation due to being transferred from free-range to enclosed housing conditions. Another factor which may have contributed to the weight loss observed might have been due to a change of diet whereby they were fed on hay in the barn while they fed on shrubs in the field. Their condition at arrival (day -200) and at infection (day 0) in respect to mct, mi antigen ELISA as well as weight and packed cell volumes (PCV) is shown in Table 7.2.

After treatment with Trypacide, camels remained negative for trypanosomiasis for 90 days by mct and mi before the day of infection (day 0). However, two camels (1694 and 1700) had persistent positive ELISA values for 14 and 12 days before the day of infection. All other camels were negative for trypanosome antigens for at least 60 days prior to the day of infection. Due to time and high cost it would have taken to acquire more camels, the two ELISA-positive camels were used in the experiments.

Table 7.1 Protocol for interference experiment in mice, rats and camels.

Group	Group	No. of animals		Infecting isolate		Infection day	
		Rodents	Camels	1°	2°		
Experimental	A	10	2	R + S		0	-
	B	10	2	R + S		0	-
	C	10	2	R	S	0	X
	D	10	2	S	R	0	Y
Control	E	2	1	R	-	0	-
	F	2	1	S	-	0	-
	G	2	1	R	-	Y	-
	H	2	1	S	-	X	-

Legend:

1° and 2°- primary and secondary inoculations.

R - the resistant isolate KETRI 3136

S - the sensitive isolate KETRI 2454 or 2476

X - onset of parasitaemia in group C

Y - onset of parasitaemia in group D

Table 7.2 Comparison of camel condition and trypanosomiasis infection-status at prior to infection on day -200 and day 0.

Group	Camel	Weight in kg.		PCV %		mct		mi		Ag ELISA	
	no.	-200	0	-200	0	-200	0	-200	0	-200	0
A	1687	212	182	25	27	-	-	+	-	+	-
	1696	174	159	13	22	-	-	-	-	+	-
B	1686	176	154	32	27	-	-	-	-	+	-
	1697	198	183	19	24	-	-	+	-	-	-
C	1690	235	228	32	26	+	-	+	-	-	-
	1692	239	242	18	25	-	-	+	-	-	-
D	1694	202	208	19	28	-	-	+	-	-	+
	1698	202	210	15	27	-	-	-	-	-	-
E	1695	163	151	9	20	-	-	-	-	-	-
F	1700	84	93	22	22	-	-	-	-	-	+
G	1688	202	177	18	23	-	-	+	-	-	-
H	1689	245	232	21	26	-	-	-	-	-	-

Legend:

mct - microhaematocrit centrifugation technique for the detection of trypanosomes.

mi - mouse sub-inoculation technique for the detection of trypanosomes.

Ag ELISA - Enzyme linked immunosorbent assay for the detection of trypanosomal antigens.

PCV - packed cell volume of blood.

7.3 RESULTS

7.3.1 MICE

The results of the interference experiment in mice infected with suramin-sensitive stock KETRI 2454 or KETRI 2476 are shown in Tables 7.3 and Table 7.4 respectively.

7.3.1.1 Response to treatment

Both KETRI 2454 and 2476 were sensitive to suramin at a dose rate of 10 mg/kg body weight as shown by cure of all mice in groups F and H. At this dose rate, KETRI 3136 was resistant to suramin as indicated by the absence of any effect on parasitaemia of mice in groups E and G. When both sensitive and resistant stocks were inoculated simultaneously into mice, suramin at a dose rate of 10 mg/kg had no effect on parasitaemia (Tables 7.3 and 7.4). These results were seen regardless of whether suramin was administered to mice at the onset of parasitaemia (group A) or if the drug was administered at the time that both infections were considered to have established (group B) by comparison with infections in control mice in groups E and F. There was no difference in the results obtained regardless of whether the infecting sensitive isolate was KETRI 2454 or 2476. Parasitaemia, however, developed earlier, and hence treatment was given earlier, when mice were infected with KETRI 2476.

No animals were cured in group C when the resistant stock (KETRI 3136) was inoculated first followed by subsequent infection with the sensitive stock on day 3 (KETRI 2476) or on day 7 (KETRI 2454). There was, however, some effect on parasitaemia resulting in suppression in six mice infected with KETRI 2454 and two mice infected with KETRI 2476 (Tables 7.3 and 7.4). In two mice, this suppression of parasitaemia was marked, falling from 2.5 x 10^8 to 2 x 10^5 trypanosomes per ml (Table 7.3). In eight mice infected with KETRI 2476, trypanosomes disappeared temporarily before reappearing five to eight days later (Table 7.4). Although the proportion of suppressed parasitaemias and temporary remissions was different depending on whether the sensitive isolate was KETRI 2454 or KETRI 2476, statistical analysis showed no overall differences between the two sensitive stocks as far as the response to treatment was concerned. In group D, when a sensitive stock (KETRI 2454 or 2476) was used for primary infection followed by the resistant KETRI 3136 four or five days later, treatment with suramin failed to cure any mice. There was, however, an effect on parasitaemia since in the mice infected with KETRI 2476, trypanosomes were cleared from the blood but relapses followed 4-12 days later (Table 7.4). When the KETRI 2454, drug-sensitive stock, was used, six mice had temporary remissions whereby trypanosomes could not be detected in blood for 4-10 days and in four mice, parasitaemia was suppressed, in some cases from 4 x 10^8 to 7 x 10^6 trypanosomes per ml (Table 7.3).

7.3.1.2 Virulence

When mice were infected simultaneously with both the suramin-sensitive stock, KETRI 2454, and the resistant trypanosome stocks, KETRI 3136, (groups A and B) there was no difference in the time to death (25.2 and 27.6 days Table 7.3). Similarly, when the stocks were inoculated consecutively (groups C and D), there was little difference (29.0 and 26.2). However, when the suramin-sensitive stock KETRI 2476 was used, mice inoculated simultaneously with the two isolates (groups A and B) died earlier (23 and 17.8 days) compared to mice which were inoculated consecutively (groups C and D, 53.8 and 64 days). This difference was highly significant ($P = 0.001$) using the chi-square test.

7.3.2 RATS

7.3.2.1 Response to treatment

All rats in groups F and H infected with *T. evansi* KETRI 2454 were cured by treatment with 10 mg/kg of suramin whereas KETRI 3136 was resistant to suramin as indicated by the absence of any effect on parasitaemia of infected rats in groups E and G (Table 7.5). Suramin at a dose rate of 10 mg/kg had no effect on parasitaemia when rats were inoculated simultaneously with both the suramin-sensitive and resistant stocks. This result was obtained whether the drug was administered immediately at the onset of parasitaemia (group A) or when both stocks had been given adequate opportunity to have established as judged by development of infection in the controls in groups E and F (group B).

Treatment with suramin had no effect on parasitaemia and no cures resulted in any rat in group C when the resistant stock, KETRI 3136, was inoculated initially followed, seven days later, by subsequent inoculation of the sensitive stock, KETRI 2454 (Table 7.5). Similarly, treatment had no effect on parasitaemia in six rats in group D which were inoculated initially with the suramin-sensitive trypanosomes followed by resistant ones. Unlike group C, however, the parasitaemia in four of the rats was suppressed from 4×10^8 to 1.5×10^7 trypanosomes per ml.

7.3.2.2 Virulence

When rats were inoculated simultaneously with both the suramin-resistant (KETRI 3136) and suramin-sensitive (KETRI 2454) stocks (groups A and B), there was no difference in the time to death (13.8 and 11.0 days) compared to when they were inoculated with both stocks concurrently (groups C and D, Table 7.5). The fact that the resistant isolate was inoculated initially and the sensitive one later (group D) or *vice versa* (group C) did not have any effect on the survival of the rats (day 17.8 and 18.2 respectively).

Table 7.3 Interference experiment in mice using *T. evansi* KETRI 2454 as the suramin-sensitive stock and KETRI 3136 as the suramin-resistant stock.

Group	Infecting isolate		Infection day			Treatment	Effect of treatment on parasitaemia	Mean days
	1°	2°	1°	Day patent	2°	day		to death
A	R+S	-	0	7	-	7	no effect 10 mice	25.2
B	R+S	-	0	9	-	9	no effect 10 mice	27.6
C	R	S	0	7	7	14	no effect 4 mice	29.0
							suppr. 2 mice 2.5×10^8 tryps. to 2×10^5	
							2 mice 2.5×10^8 tryps. to 3×10^7	
							2 mice 4×10^8 tryps. to 7×10^6	
D	S	R	0	5	5	9	suppr. 2 mice 4×10^8 tryps. to 2×10^7	26.2
							2 mice 4×10^8 tryps. to 1×10^8	
							temp. remission:	
							1 mice 4 days	
							3 mice 7 days	
							1 mouse 9 days	
							1 mouse 10 days	
E	R	-	0	7	-	9	no effect 2 mice	23.5
F	S	-	0	5	-	9	cure 2 mice	-
G	R	-	5	9	-	9	no effect 2 mice	25.5
H	S	-	7	14	-	14	cure 2 mice	-

Table 7.4 Interference experiment in mice using *T. evansi* KETRI 2476 as the suramin-sensitive stock and KETRI 3136 as the suramin-resistant stock.

Group	Infecting isolate		Infection day			Treatment	Effect of treatment on parasitaemia	Mean days
	1°	2°	1°	Day patent	2°	day		to death
A	R+S	-	0	3	0	3	no effect 10 mice	23.0
B	R+S	-	0	4	0	4	no effect 10 mice	17.8
C	R	S	0	3	3	9	suppr. 2 mice 1×10^{8} to 1.5×10^{7} temp. remission: 2 mice 5 days 5 mice 6 days 1 mouse 8 days	53.8
D	S	R	0	4	4	7	temp. remission: 2 mouse 4 days 2 mouse 6 days 5 mice 7 days 1 mouse 12 days	64.0
E	R	-	0	3	-	4	no effect 2 mice	20.0
F	S	-	0	4	-	4	cure 2 mice	-
G	R	-	4	7	-	7	no effect 2 mice	21.0
H	S	-	3	9	-	9	cure 2 mice	-

Table 7.5 Interference experiment in rats using *T. evansi* KETRI 2454 as the suramin-sensitive stock and KETRI 3136 as the suramin resistant stock.

Group	Infecting isolate		Infection day			Treatment day	Effect of treatment on parasitaemia	Mean days to death
	1°	2°	1°	Day patent	2°			
A	R	S	0	5	0	5	no effect 10 rats	13.8
B	R	S	0	7	0	7	no effect 10 rats	11.0
C	R	S	0	7	7	12	no effect 10 rats	17.8
D	S	R	0	5	5	12	no effect 6 rats	18.2
							suppr. 4 mice 4 x 10^8 to 1.5 x 10^7	
E	R	-	0	7	-	7	no effect 2 rats	14.0
F	S	-	0	5	-	7	cure 2 rats	-
G	R	-	5	12	-	12	no effect 2 rats	15.5
H	S	-	7	12	-	12	cure 2 rats	-

7.3.3 CAMELS

The protocol and results of the interference experiment in camels shown in Figures 1-12 are summarised in Table 7.6. The results will be presented according to the different groups in which camels were divided.

Control groups. Camel numbers 1695, 1700, 1688 and 1689.

As shown in Figure 7.1, camel 1695 in group E infected with the suramin resistant stock (KETRI 3136) developed an intermittent parasitaemia. Microscopy detected parasites on only four occasions preceding suramin treatment on day 36 and two days preceding treatment with cymelarsan on day 57. Parasites could be detected by mouse sub-inoculation from day 1 until treatment with suramin and from then, until treatment with cymelarsan. Low levels of trypanosomal antigens were detected in this camel between days 7 and 20 and its PCV did not vary much over the experimental period. In camel 1700, which was antigenaemic for 12 days before the start of the experiment, infection with the suramin-sensitive stock (KETRI 2454) was detected by all three methods used from day 1 until treatment with cymelarsan on day 43 (Figure 7.2). In spite of treatment with suramin on days 28 and 35 after infection, antigenaemia persisted until day 52. The PCV dropped from 25% at infection to 14% when the camel was treated with cymelarsan. From then, it rose to 19% at the end of the experiment. As shown in Figures 7.3 and 7.4, no trypanosomes were detected by microscopy throughout the experimental period in the two infectivity control camels 1688 and 1689 inoculated with the suramin-resistant (KETRI 3136) or sensitive (KETRI 2454) stocks respectively. Mouse sub-inoculation indicated that these camels were only positive on two occasions (days 21 and 29 for camel 1688, days 29 and 31 for camel 1689). Both camels were negative by antigen ELISA during the whole experimental period (Figure 7.3 and 7.4). The PCV values of both camels did not vary during the experiment. The weights of all camels in the control groups showed little variation during the experimental period.

Group A. Camel numbers 1687 and 1696.

These two camels were infected simultaneously with the suramin-resistant (KETRI 3136) and sensitive (KETRI 2454) stocks. Trypanosomes were detected in both camels by microscopy on day 4 and parasitaemia persisted up to days 26 and 19 after infection (camels 1687 and 1696 respectively, Figures 7.5 and 7.6). Camel 1696

further had intermittent parasitaemia on days 29, 34 and 35. Both camels were positive by mouse sub-inoculation the day after infection and trypanosomes continued to persist, even after treatment with suramin on day 11. Trypanosomes were no longer detected after the camels were treated with cymelarsan 32 (1687), 35 and 57 (1696) days after infection. Both camels were negative for trypanosomal antigens throughout the experiment although their PCV dropped and they lost weight as the infection established. In spite of treatment with cymelarsan, the camels lost condition steadily and died on day 36 (camel 1687) and day 86 (camel 1696).

Group B Camel numbers 1686 and 1697.

Camels in this group were infected simultaneously with the suramin-resistant (KETRI 3136) and sensitive (KETRI 2454) stocks. Camel 1697 had intermittent parasitaemia detected by microscopy from day 4 until treatment with suramin on day 35 (Figure 7.8). Relapse of parasitaemia was then observed in this camel on days 58 and 60 after which trypanosomes were not detected in blood after treatment with cymelarsan on day 86. Trypanosomes were detected in this camel by mouse sub-inoculation from the day after infection up to day 47. Thereafter, trypanosomes could no longer be detected until the end of the experiment. In camel 1686, infection was not detected by microscopy throughout the entire experimental period (Figure 7.7). It was positive, however, by mouse sub-inoculation the day after infection and from then intermittently until day 26 from whence it was negative, without treatment, until the end of the experiment. Although there were four occasions when antigen ELISA readings were above the antigen-positive cut-off point for camel 1686, neither camel in this group had high values associated with trypanosomiasis infection. The PCV of camel 1686 did not vary much while that of camel 1697 dropped from 25% to 20% just before treatment with cymelarsan. Both camels gained weight as the experiment progressed.

Group C Camel numbers 1690 and 1692.

Camel number 1690 in this group was inoculated with the suramin-resistant stock (KETRI 3136) on day 0 and super-infected with the sensitive stock (KETRI 2454) on day 26 (Figure 7.9). Trypanosomes were detected by microscopy in its blood from day 14 after inoculation of the primary isolate (KETRI 3136) and it continued to be parasitaemic after inoculation of the secondary stock on day 26 until day 48. This camel was positive by mouse sub-inoculation from day 1 until day 81 post-infection and was treated with suramin on day 93. In the other camel in this

group (number 1692) which was inoculated with the suramin-resistant stock (KETRI 3136) on day 0, parasitaemia was detected by microscopy only between days 75 and 77 and was thus not super-infected (Figure 7.10). This camel was positive for trypanosomiasis by mouse sub-inoculation intermittently from day 1 to day 54 after which it was positive until day 80. Trypanosomal antigens were detected in camel 1690 from day 20 until the end of the experiment. Camel 1692 was not antigenaemic throughout the experimental period. The PCV values and the weights of both camels did not vary much.

Group D Camel numbers 1694 and 1698.

Parasites were detected by microscopy in camel 1694 infected with the suramin-sensitive stock (KETRI 2454) on day 0 and the resistant stock (KETRI 3136) on 14 from day 7 to day 44 when cymelarsan was administered (Figure 7.11). This camel was positive by mouse sub-inoculation, from day 1 till day 44 when it was treated with cymelarsan. Thus, treatment with suramin on day 35 had no apparent effect on the trypanosomes. In the other camel, number 1698, infected with the suramin-sensitive stock (KETRI 2454) on day 0 and the resistant stock (KETRI 3136) on day 26, trypanosomes were detected by microscopy during five periods; days 19 to 21, 23 to 28, 43 to 47, day 83 and day 90 (Figure 7.12). This camel was positive by mouse sub-inoculation from day 1 until day 74, thereafter, parasites could no longer be detected even before treatment with suramin on day 92 and with cymelarsan on day 100. Camel 1694 was positive for trypanosomal antigens between days 1 and 9 while camel 1698 was negative throughout. The PCV of camel 1694 dropped from 28% at the start of the experiment to 19% near the end while there was little variation in the PCV of camel 1698. The weights of both camels did not vary much throughout the experimental period.

Table 7.6 Interference experiment in camels using *T. evansi* KETRI 2454 as the suramin-sensitive stock and KETRI 3136 as the resistant stock.

Group	Camel no.	Infection		Infection day		Suramin treatment		Cymelarsan treatment		Death on day
		1°	2°	1°	2°	day	result	day	result	day
A	1687	R + S	-	0	-	11	no effect	32	cure	36
	1696	R + S	-	0	-	11	no effect	35, 57	cure	86
B	1686	R + S	-	0	-	-	-	100	cure	
	1697	R + S	-	0	-	35	no effect	86	cure	
C	1690	R	S	0	26	93**	-	100	cure	
	1692	R	-	0	-	93**	-	100	cure	
D	1694	S	R	0	14	35	no effect	44	cure	
	1698	S	R	0	26	92*	-	100	cure	
E	1695	R		0	-	36	no effect	57	cure	
F	1700	S		0	-	28, 35	no effect	43	cure	
G	1688	R		14	-	-	-	100	cure	
H	1689	S		26	-	-	-	100	cure	

Legend:
- * Treated to save it although the infectivity controls negative.
- ** Treated to end the experiment because the infectivity control negative or parasitaemia very intermittent.
- \- Procedure not done.

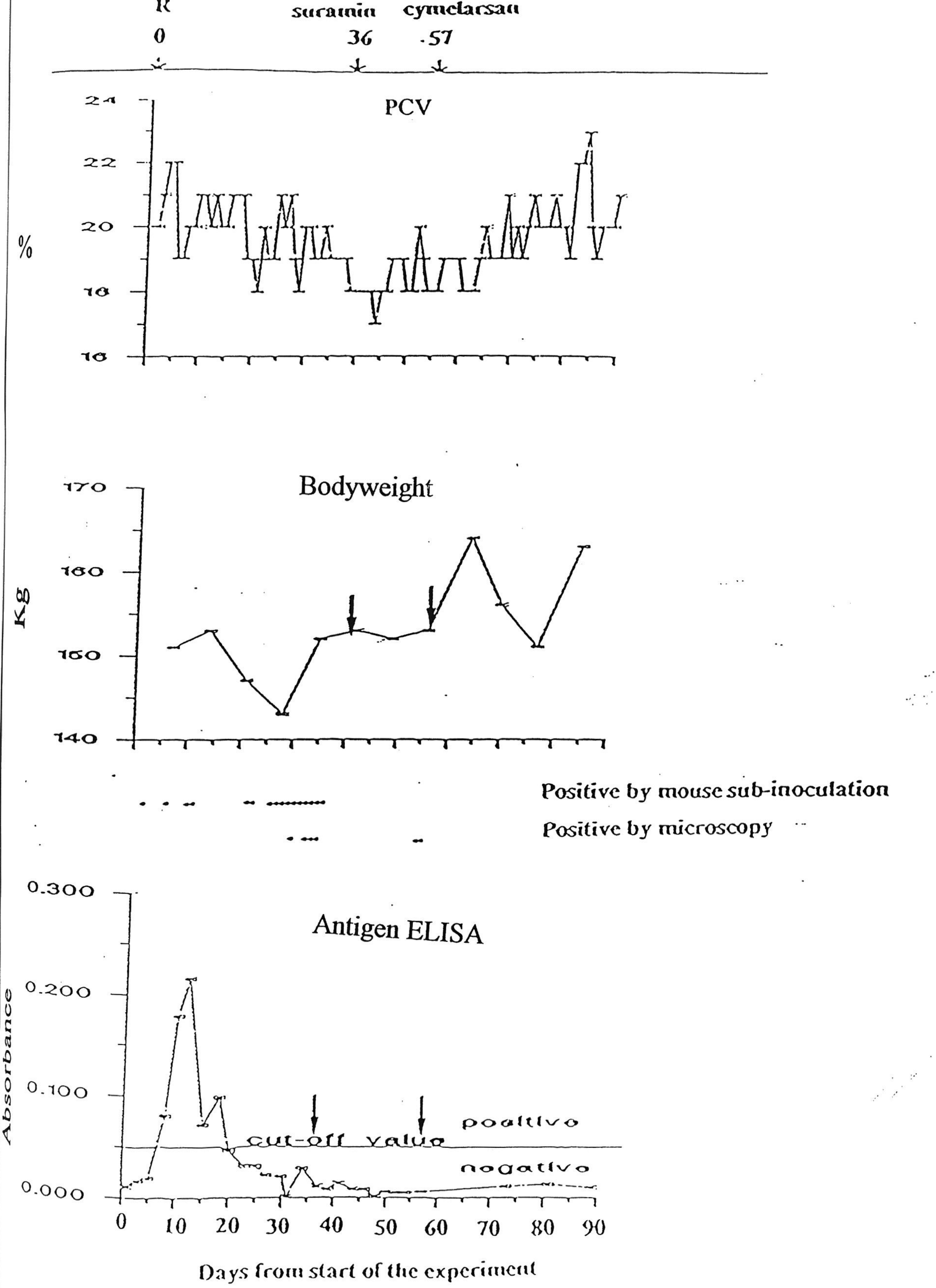


Figure 7.1 Parameters indicating *T. evansi* infection in control camel number 1695 of group E.

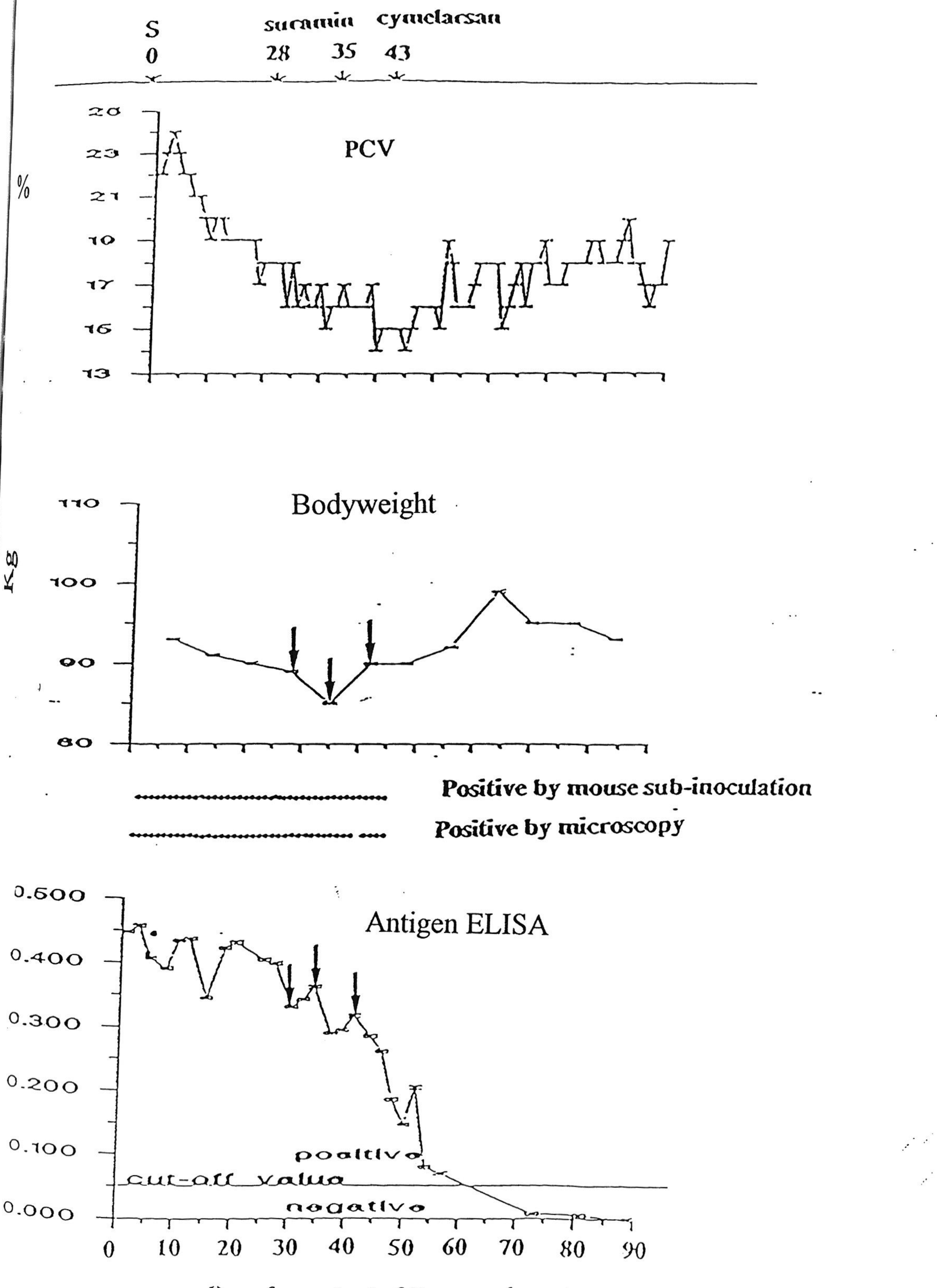


Figure 7.2 Parameters indicating *T. evansi* infection in control camel number 1700 of group F.

R
14

cymelarsan
100

PCV

%

20
23
21
10

Bodyweight

Kg

200
100
180
170

Negative by microscopy
Positive by mouse sub-inoculation

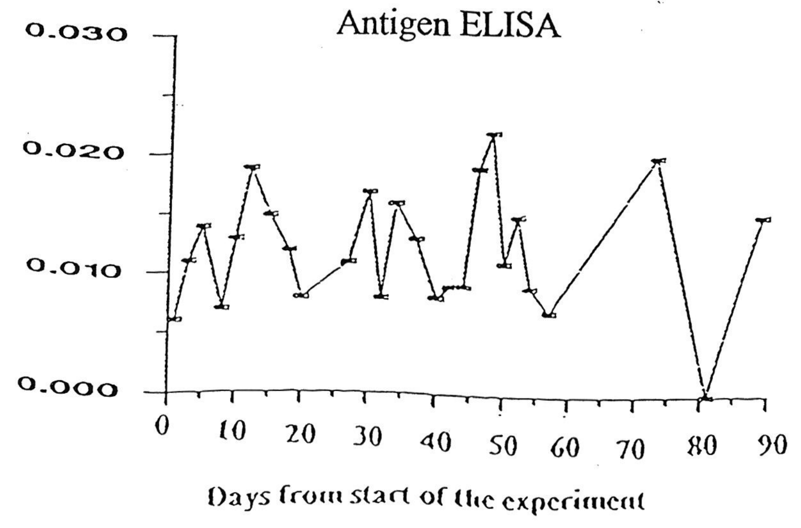


Figure 7.3 Parameters indicating *T. evansi* infection in control camel number 1688 of group G.

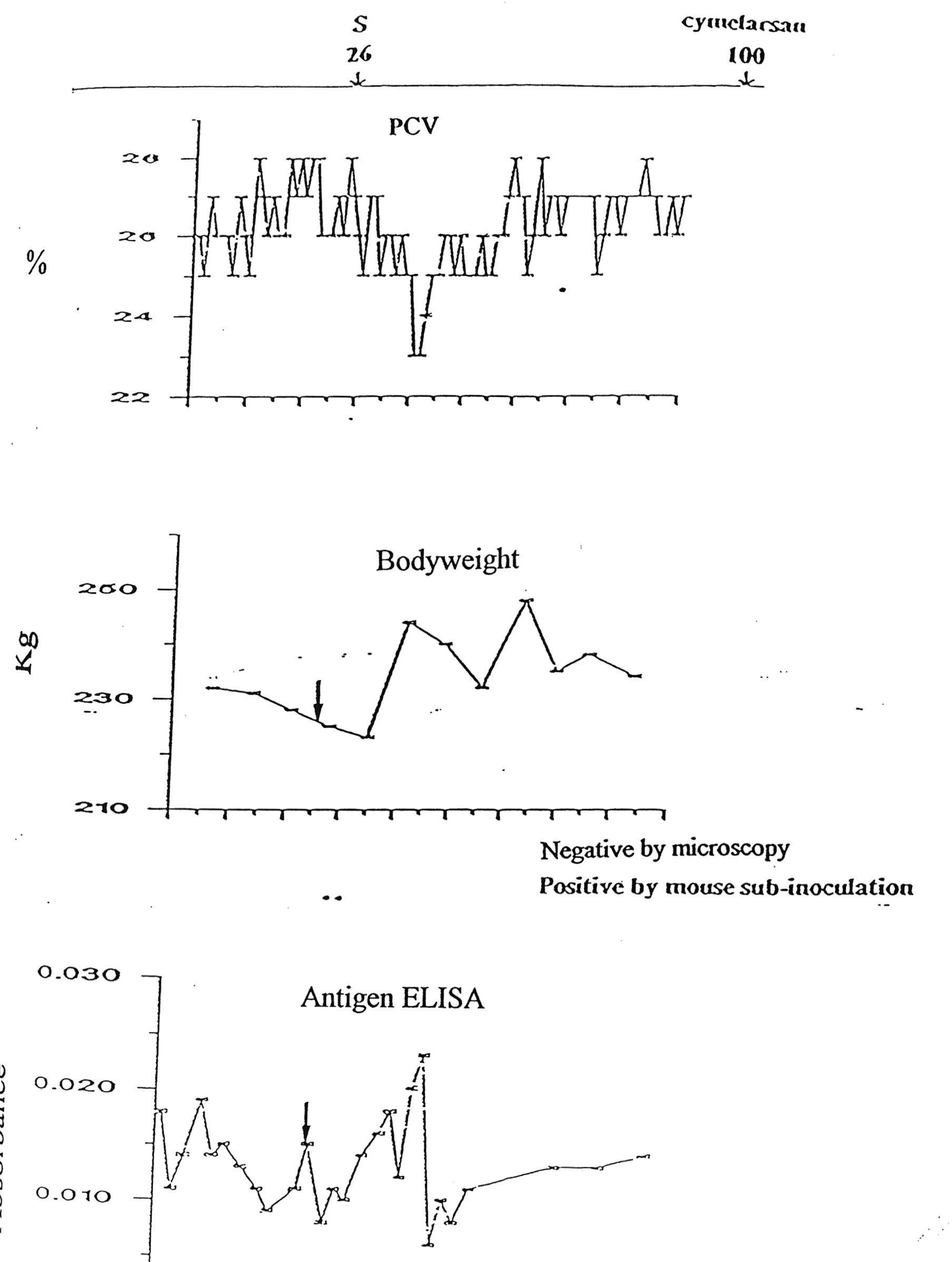


Figure 7.4 Parameters indicating *T. evansi* infection in control camel number 1689 of group H.

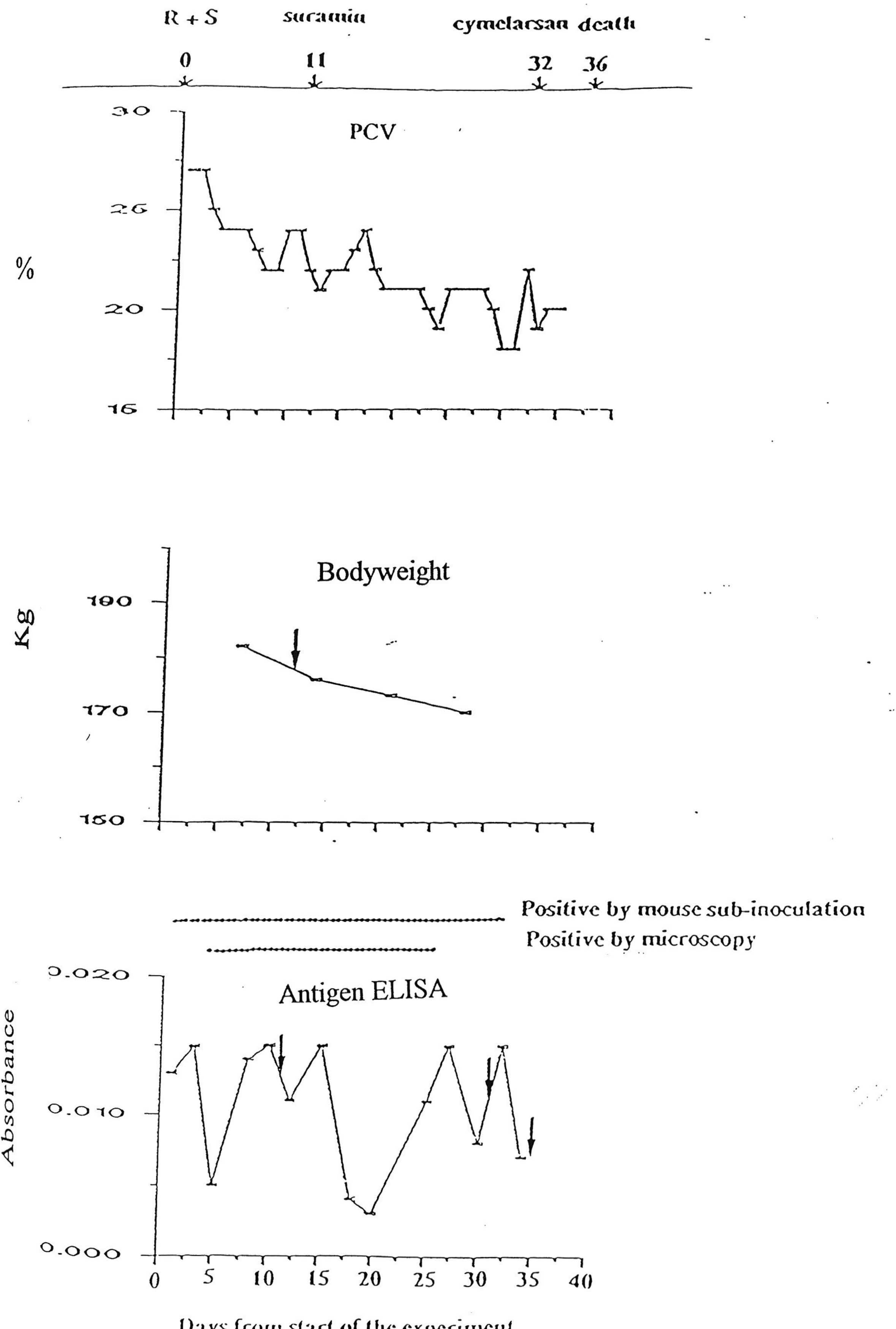


Figure 7.5 Parameters indicating *T. evansi* infection in experimental camel number 1687 of group A.

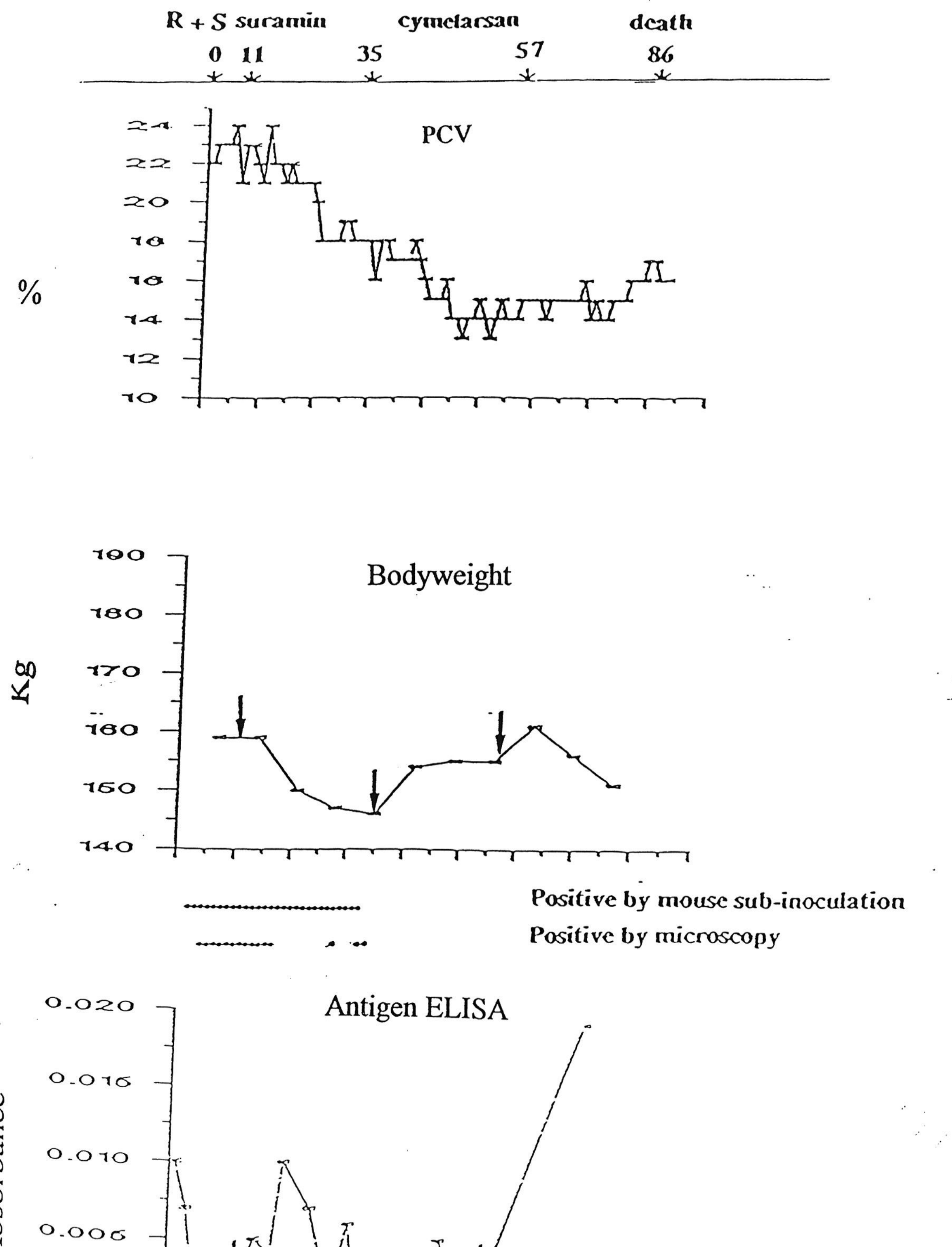


Figure 7.6 Parameters indicating *T. evansi* infection in experimental camel number 1696 of group A.

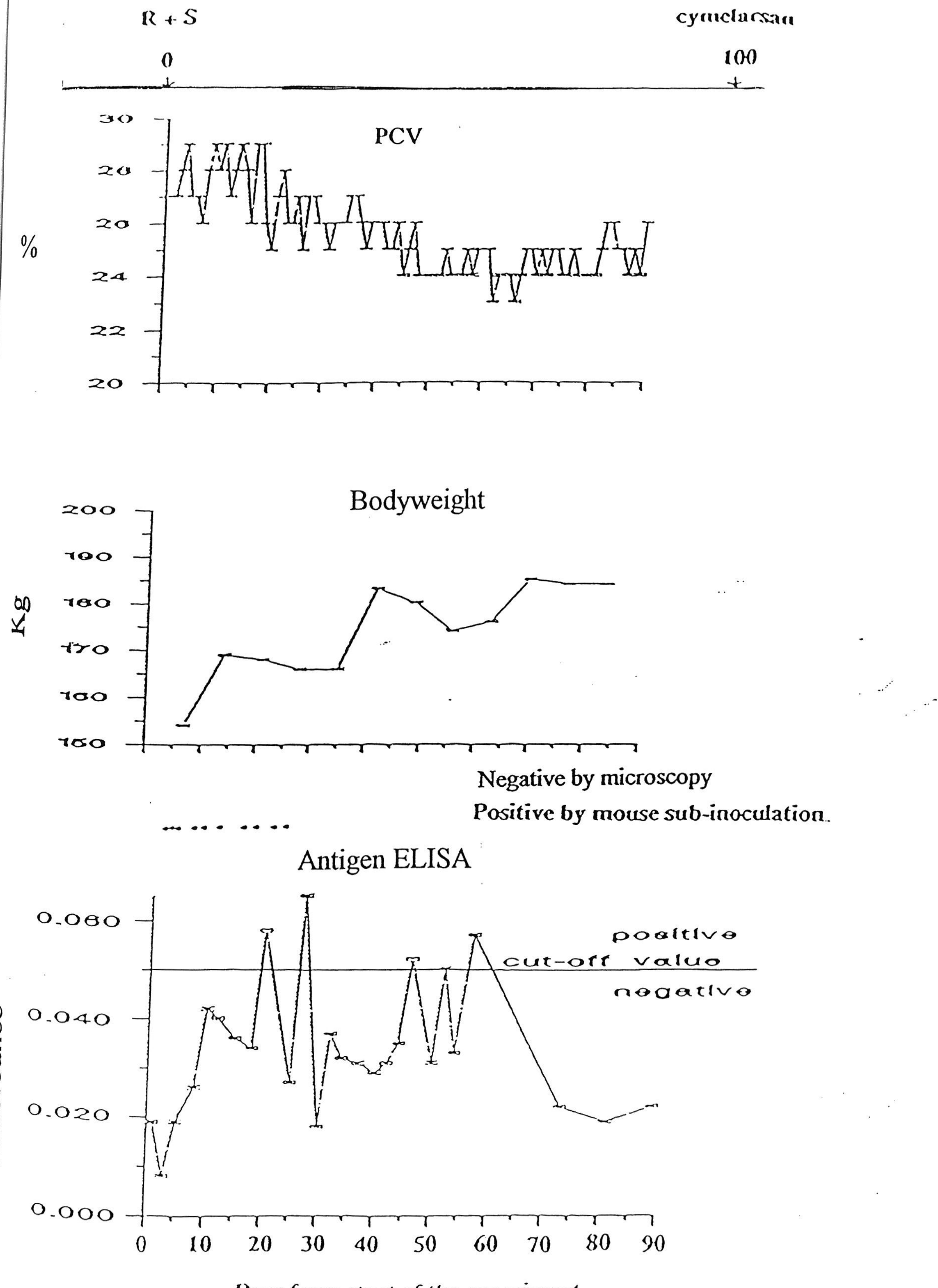


Figure 7.7 Parameters indicating *T. evansi* infection in experimental camel number 1686 of group B.

R + S   suramin   cymelarsan

0   35   86

PCV

%

26
23
21
19

Bodyweight

Kg

220
210
200
190
180

Positive by mouse sub-inoculation

Positive by microscopy

Antigen ELISA

Absorbance

0.020
0.010
0.000

0 10 20 30 40 50 60 70 80 90

Days from start of the experiment

Figure 7.8 Parameters indicating *T. evansi* infection in experimental camel number 1697 of group B.

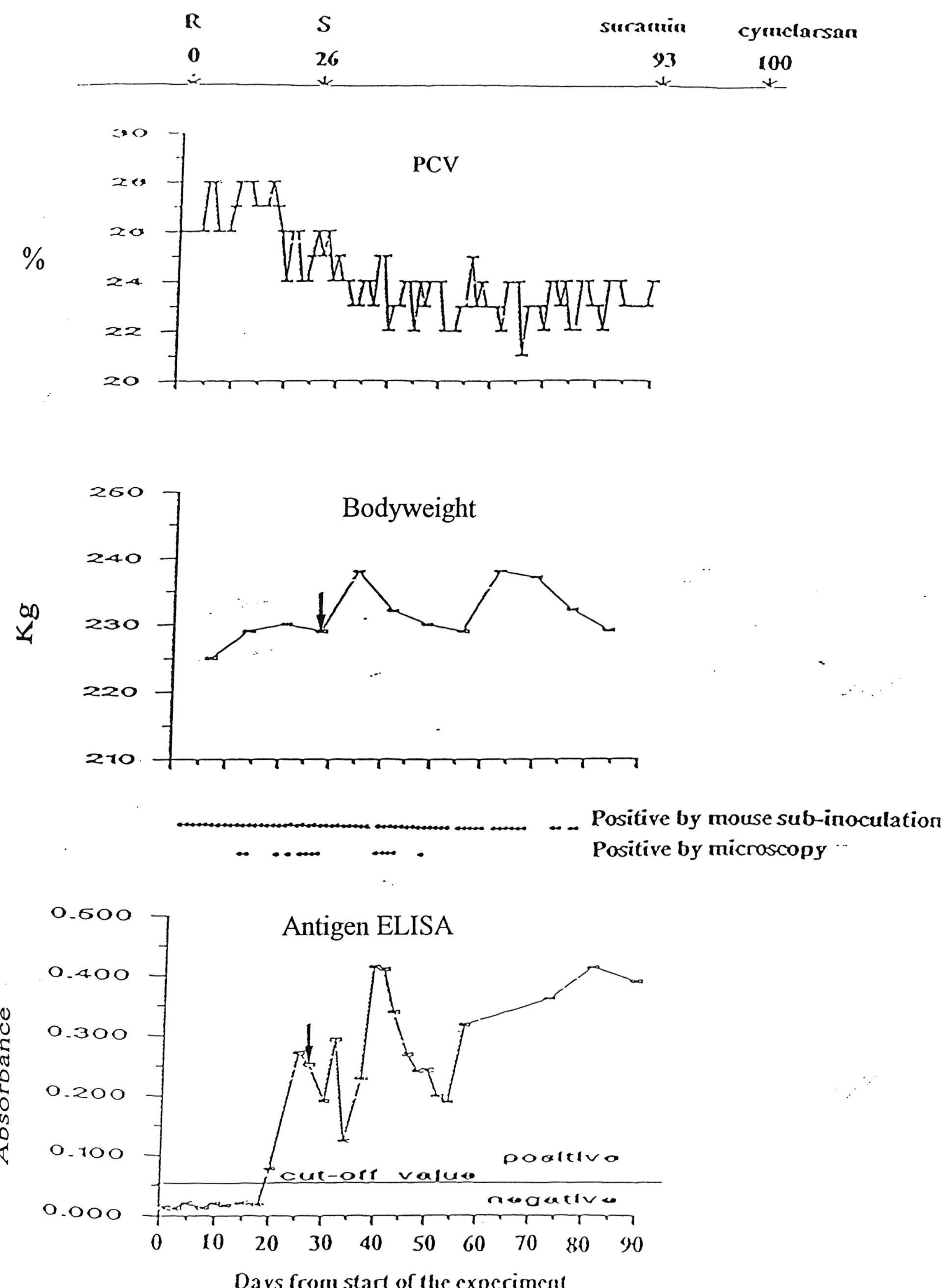


Figure 7.9 Parameters indicating *T. evansi* infection in experimental camel number 1690 of group C.

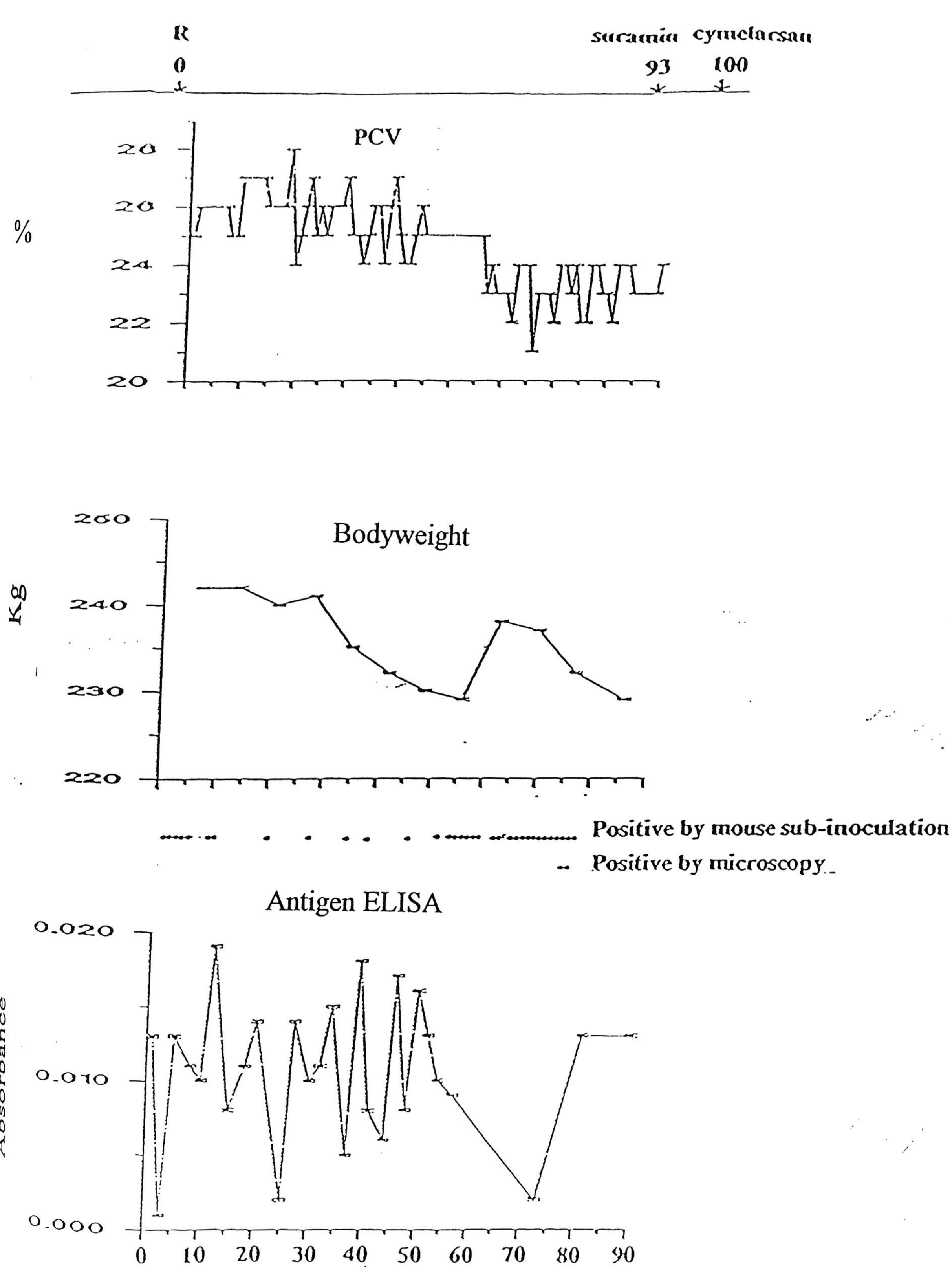


Figure 7.10 Parameters indicating *T. evansi* infection in experimental camel number 1692 of group C.

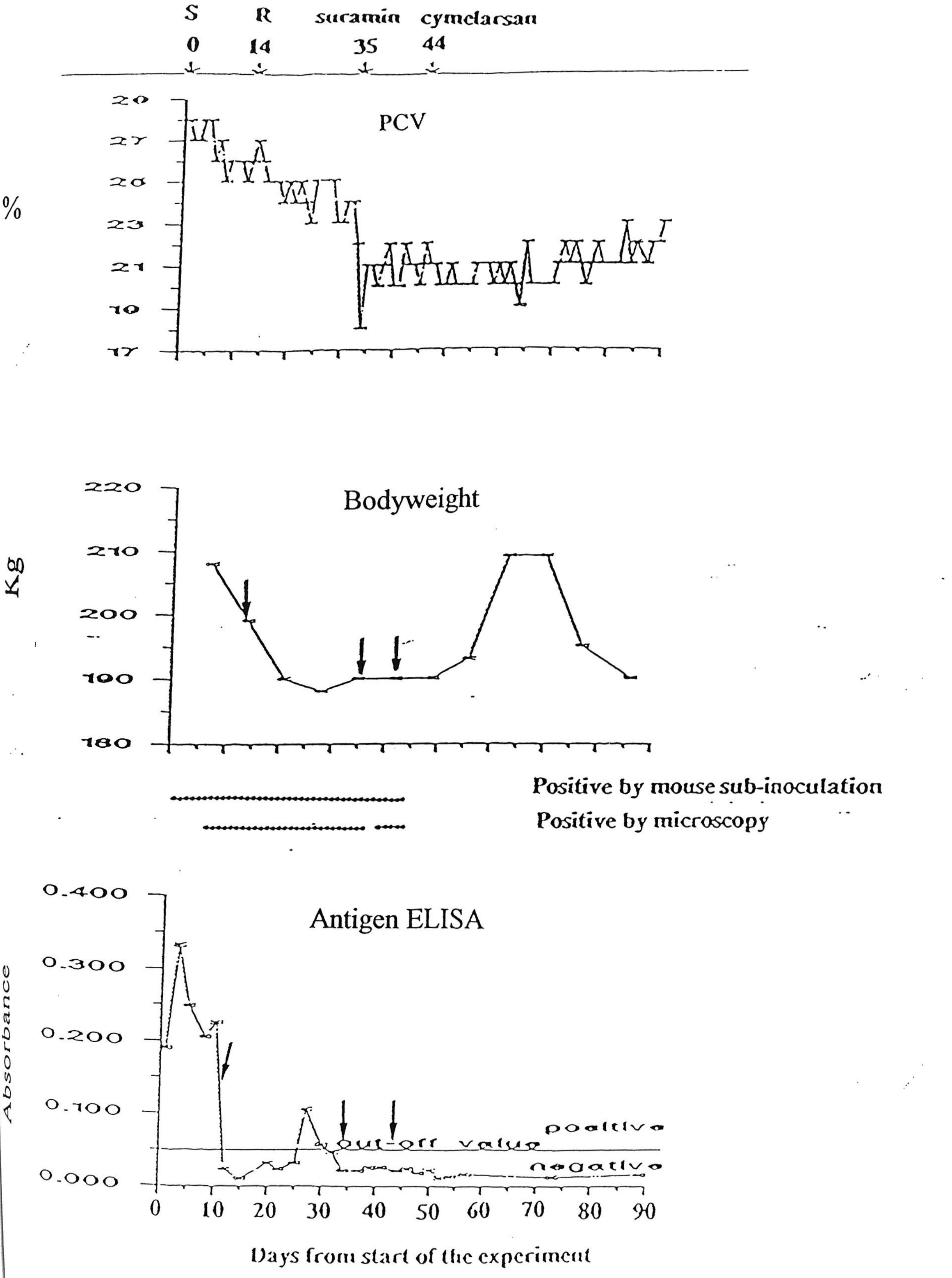


Figure 7.11 Parameters indicating *T. evansi* infection in experimental camel number 1694 of group D.

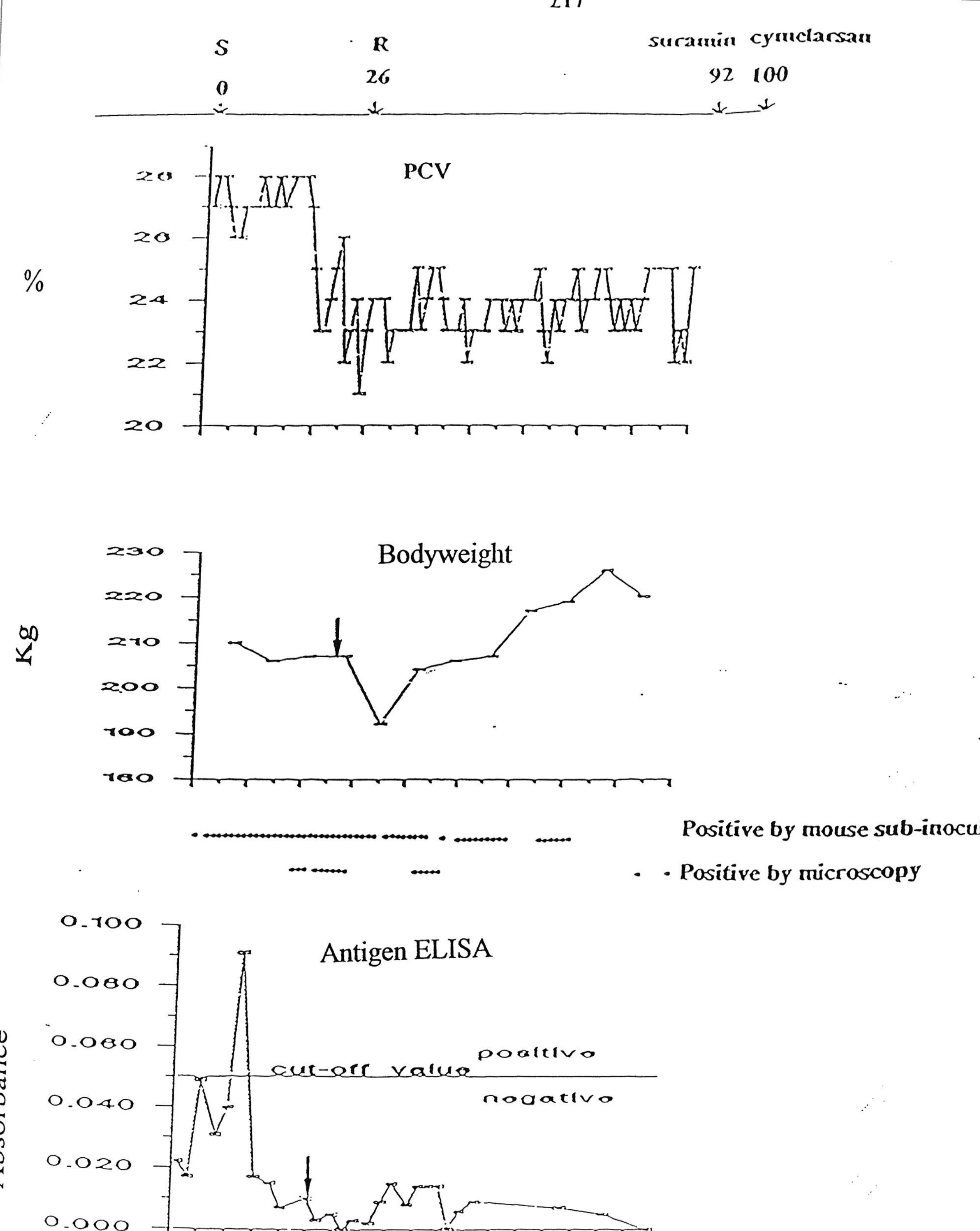


Figure 7.12 Parameters indicating *T. evansi* infection in experimental camel number 1698 of group D.

8.4 DISCUSSION

Interference between serologically different stocks of trypanosomes was investigated by Herbert (1975) who showed that mice infected with one strain of *T. rhodesiense* then super-infected with another strain, resulted in an extension of the pre-patent period (of the second strain) by up to 14 days. In comparison to single infection with the second strain, there was however no difference in the time it took for the second strain to kill mice once the second infection became patent. In *T. congolense,* a similar phenomenon was investigated by several workers using antigenically different stocks in cattle, rabbits and goats (Morrison *et al,* 1982; Luckins and Gray, 1983; Dwinger *et al,,* 1986). In these experiments, establishment of super-infections was determined by histological examination of local skin reactions (chancres) at the site of trypanosome inoculation and by the appearance of neutralising antibodies produced by the second stock. In most of these studies, infection of animals with one stock of trypanosomes resulted in unsuccessful attempts to infect them with a second stock This phenomenon was not due to host-immune responses to the first infection, for when animals were first treated before inoculation of the second stock, infections by the second stock were established. It was thus observed that interference needed an active infection of one trypanosome stock and was related to the time span between the two infections. Interference was also observed by Sones *et al* (1989) who used *T. congolense* strains which were different in respect to their sensitivity to Samorin. In this study, when goats were infected with a Samorin-sensitive strain, subsequent inoculation of the resistant stock followed by administration of Samorin did not result in cures. If the Samorin-resistant trypanosomes were first inoculated followed by the Samorin-sensitive ones, administration of the drug resulted in temporary remission of parasitaemia in all goats. Thus, establishment of Samorin-sensitive trypanosomes prevented the resistant ones from doing likewise and to a lesser extent, the Samorin-resistant trypanosomes behaved in the same way.

The reasons for this interference phenomenon were suggested as being due to sequestered trypanosomes which inhibit the growth of one type by the metabolic products of another (Herbert, 1975) and to non-specific competition between different trypanosome strains (Morrison *et al,* 1982). Another possible reason for this phenomenon is due to differences in the replication rates (Sones *et al,* 1989). Slower-replicating drug-resistant trypanosomes were thus not able to establish infection as fast as the drug-sensitive parasites. Seed (1978) working with mixed *T. gambiense* infections, however, thought that the differences in growth rate were too small to

account for interference. Dwinger *et al* (1986) suggested that a trypanosome growth-regulating factor was responsible for delays in the development of super-infections and postulated that this factor was not immunologically-mediated and could be the production of lymphokines, including interferon, during the primary infection.

In both mice and rats infected with *T. evansi*, no suppression of parasitaemia was observed after suramin treatment when both resistant and sensitive stocks were inoculated at the same time suggesting that the resistant stock had established infection. This occurred irrespective of whether suramin was administered immediately after the onset of parasitaemia or if it was given later, when observations on the control group had indicated that both trypanosome stocks had had adequate opportunity to become patent. Hence, there was no interference in the establishment of infection of the resistant stock by the sensitive one or *vice versa*.

When the resistant stock (KETRI 3136) was first allowed to establish infection before inoculation of the sensitive one (KETRI 2454), two kinds of responses were observed. No effect on parasitaemia was observed upon treatment of four mice and all the rats. This could due to the fact that the suramin-sensitive stock was unable to establish infection and thus drug-sensitive parasites contributed a negligible proportion of the total parasite population at treatment resulting in no drop of parasitaemia. This suggests that the resistant parasites interfered with the growth of the sensitive parasites.

In the second type of response where parasitaemia was suppressed in six mice, there appeared to be no interference in establishment of the suramin-sensitive trypanosomes. In mice in which such a mixed (resistant-sensitive) infection was present, treatment destroyed only the suramin-sensitive population resulting in temporary suppression of parasitaemia. This observation suggests that the suramin-sensitive trypanosomes, in spite of the shorter duration of infection in the rodents, had proliferated sufficiently to constitute a sizeable proportion so that when treated, a detectable drop in parasitaemia was observed.

When the sensitive stock was inoculated first and allowed to establish patent infection before the inoculation of the resistant one, three types of responses were observed after treatment. In the first kind seen in six rats, there was no effect on parasitaemia suggesting that the establishment of resistant parasites was not affected in spite of concurrent infection with a suramin-sensitive stock of trypanosomes. In the second type of response seen in four mice and four rats, suppression in parasitaemia was observed. The population probably consisted of a mixed (sensitive-resistant) infection at treatment and this suggests that the sensitive parasites did not prevent the

resistant ones from establishing infection. In the third type of response, a temporary remission was observed in six mice. Initially, the predominant population at treatment consisted of the sensitive parasites which were destroyed by treatment leaving the resistant ones to develop a patent infection. This suggests that sensitive parasites prevented the resistant ones from proliferating to a degree that they could be detected by microscopy or mouse sub-inoculation although they did not eliminate them.

The time taken for the suramin-resistant trypanosomes to kill mice and rats also gives an indication of whether there was interference between the sensitive and resistant parasites. Mice and rats with super-infections survived for longer periods than those that had both stocks inoculated together (irrespective of whether it was the resistant or the sensitive parasites which were inoculated first) or those inoculated with the resistant parasites alone. This suggests that once suramin sensitive trypanosomes had established, they altered the virulence of resistant ones. There was no effect on virulence when the two stocks were inoculated concurrently as seen by the time infected animals died.

Treatment produced more intermediate results in mice compared to rats with suppression and temporary remissions being observed more often in mice than in rats. Such variations in response to treatment may be due to factors which were not investigated in this study such as differences between mice and rats in respect to pre-patent periods, virulence and growth rates of the stocks used as well as the ability the parasites to invade the CNS.

In conclusion, once the suramin-resistant trypanosomes established infection in rats, they prevented the drug-sensitive ones from doing likewise. When the suramin-sensitive or resistant trypanosomes first established infection, they sometimes interfered with the establishment of infection by the other trypanosomes in mice. In rats however, super-infections with the suramin-resistant stock did not lead to interference of its establishing infection. The interference phenomenon encountered in this work using mice is similar to that observed by Sones *et al* (1989) in goats infected with *T. congolense* where super-infections with a sensitive strain did not result in cures. Unlike Sones *et al* who observed total interference whereby resistant parasites were not being able to establish an infection even after the sensitive ones were eliminated, the interference in this study was partial; although resistant trypanosomes did not proliferate, they were not destroyed either. Such partial interference was also observed by Herbert (1975) since the primary stock only modified a characteristic of the secondary one resulting in a longer pre-patent period. Another difference between the two studies is the interference of drug-resistant trypanosomes on the sensitive ones

observed in the present study. The differences observed between this study and that of Sones *et al* (1989) may be attributed to the use of different trypanosome species; a blood parasite by Sones and a parasite which invades tissues in the present study. Thus, if animals were treated after *T. evansi* had invaded the CNS, no cures could be observed irrespective of whether the parasites were sensitive or resistant to suramin. In a previous study (Jennings *et al,* 1977, 1979), failure of treatment with Berenil, Ethidium and Samorin was observed after seven days of *T. brucei* infection in mice suggesting that at this time, trypanosomes had invaded inaccessible compartments. It is not known when *T. evansi* invades tissue compartments in which suramin cannot penetrate but from the study on *T. brucei,* it is likely that by the time suramin was administered in super-infected rodents in this study, parasites had already invaded the CNS in some animals, resulting in variable results of treatment. This may thus explain partial interference for although trypanosomes were prevented from establishing infection, they were not eliminated either for they evaded the drug. A similar situation may have been responsible for the partial interference observed by Herbert (1975) with *T. rhodesiense*, another species which invades the CNS. For the same reason, complete interference was reported by Morrison *et al* (1982), Luckins and Gray (1983) as well as Dwinger *et al,* (1986) working with different stocks of *T. congolense*, a parasite which does not invade tissues.

The interference experiment in camels did not work as expected since infections were not established in some camels, thus they were not treated or super-infected as required by the experimental protocol. However, the apparent differences in the susceptibilities of individual camels to *T. evansi* infections and their ability to self-cure was an important observation. Thus camel number 1686 was apparently able to self-cure infection resulting from inoculation with both suramin-sensitive and resistant parasites. Non-patent infections, which could not be detected microscopically but could be detected by mice sub-inoculation, were observed in some camels irrespective of whether the camels were infected with the suramin-sensitive or resistant stock. In the three camels which had cryptic infections, self-cures (determined by negative observations by the three methods for at least 60 days) were observed in spite of camels not being treated with a trypanocide. These self-cures were only observed in camels which had not developed antigenaemia. This observation highlights the value of the antigen ELISA test in detecting active infections. There were however, two instances where camels (1687 and 1696), which were parasitologically positive but serologically negative, died even after treatment with both suramin and cymerlasan. Such false negative serological findings could lead to situations where camels infected

with *T. evansi* are deprived of treatment and may result in deaths. It is thus important to combine both parasitological tests and the antigen ELISA test in order to decide on camels which need treatment.

Suramin-resistant trypanosomes were able to establish infection in spite of the presence of sensitive parasites and suramin did not cure camels in which both stocks had been inoculated concurrently. In camel 1694 infected with the sensitive parasites followed by infection with the resistant trypanosomes 14 days later, administration of suramin did not clear trypanosomes from blood. This indicates that suramin-sensitive trypanosomes did not prevent resistant ones from establishing infection suggesting that there was no interference. Unfortunately, this camel was antigenaemic prior to the start of the experiment and it is possible that it was naturally infected with trypanosomes of unknown suramin-sensitivity prior to infection; a complication which invalidates the results. The case of camel 1700 which was not cured of infection with the suramin-sensitive isolate can be explained by the fact that it too had a persistent antigenaemia before the start of the experiment and thus could had an infection which was resistant to suramin. Although infection in this camel was not detected earlier by parasitological methods, it developed to patency after the experiment began resulting in failed therapy. The possibility of the "sensitive isolate" (KETRI 2454) being sensitive in mice but resistant in camels could be another reason for failed therapy in this camel; a possibility which cannot be, however, confirmed due to the antigenaemia found in this camel prior to experimental infection. Another observation from this work was the efficacy of cymelarsan at a dose rate of 0.5 mg/kg in curing *T. evansi* infections in camels; including those which were resistant to suramin. Hence, this observation indicates that there is no cross-resistance between the two drugs. It is important to note that there were three instances where mice sub-inoculations were negative although trypanosomes were detected in camel blood by microscopy. In two of these, camels had been treated with suramin 9 and 23 days earlier (1695 and 1697 respectively) suggesting that although trypanosomes were present in blood, they were not infective, probably due to the action of suramin. Such a possibility agrees with work where it was observed that suramin works by first reducing the infectivity of trypanosomes prior to their destruction (Hawking, 1963a). The fact that camels did not develop parasitaemias as high as those observed in rats and mice suggests that, in order to mimic the situation in the field, experimental camels need to be allowed more time for infections to develop parasite counts as high as those that are observed in naturally infected camels. The most important observation that came out of this work is the need of using naive animals which have had no previous exposure to the trypanosomes. This would eliminate the factor of acquired resistance, cryptic

infections, the possibility of parasites in the CNS as well as elucidate the possibility of innate camel resistance. Camels of this nature can only be from a herd of animals born and bred in a confirmed trypanosomiasis-free area. At present, no such animals are available at KETRI. Future attempts to investigate interference in camels would require at least five camels per group in order to allow for individual variations in the response of animals to inoculation of trypanosomes. Thus, larger barn space to accommodate more camels, as well as more funds to maintain them would be necessary in a future study.

The role of interference of suramin-sensitive stocks on the establishment of infection by the resistant ones may be important in controlling the spread of suramin resistance in the field. Assuming that this study illustrates what happens in field conditions, an attempt of a suramin-resistant stock to establish in an animal already infected with a suramin-sensitive may not be successful although the resistant trypanosomes might not be eliminated. The administration of suramin would clear the sensitive trypanosomes thus providing opportunity for the resistant ones to proliferate. Similarly, trypanosomes, either sensitive or resistant to the action of suramin, which may have invaded the CNS might be a source of relapsing infection. Thus, sequestered suramin-resistant trypanosomes may later re-invade the blood and suramin-sensitive trypanosomes would also be able to re-invade the blood when the suramin dose levels decreased to an extent that they might not be destroyed. What this study did not address, however, was the period suramin-resistant trypanosomes would remain in a state of suppression without proliferation in the absence of suramin or the period and factors which would make trypanosomes to remain sequestered in tissues.

If suramin-resistant trypanosomes are selected-out due to their slower growth rates as implied by the results in Chapter Six, then they would eventually be eliminated from the population. On the other hand, the results in Chapter Six demonstrated slower growth rates only in clones from a single stock and not in unrelated stocks. If differences in growth rates did not occur in unrelated trypanosomes in the field, a selective process would still be in operation by interference and transmission would most likely consist of sensitive trypanosomes due to their higher proportion. In the event that suramin-resistant trypanosomes also prevent establishment of sensitive ones, they would be at a selective advantage and be maintained in the population at the advantage of the suramin-sensitive parasites. Thus, there would be competition between suramin-sensitive and resistant trypanosomes and the population that had an overall selective advantage would be transmitted and maintained while the other population would not be transmitted as readily.

CHAPTER EIGHT

DIFFERENCES BETWEEN SURAMIN-SENSITIVE AND RESISTANT *T. EVANSI* AT THE DNA LEVEL

8.1 INTRODUCTION AND OBJECTIVE

The analysis of parasite deoxyribonucleic acid (DNA) sequences would be an ideal method of detecting any intra-specific differences associated with drug sensitivity. Such a method would directly analyse the genome of the organisms instead of gene products or phenotypic differences as in zymodeme analysis or in testing for sensitivity in the rodent model.

The analysis of the genome of many organisms in recent years has been enabled by the development of molecular *in vitro* techniques which provide a means for isolating and manipulation of DNA (Maniatis *et al,* 1982). A technique which compares DNA samples at the chromosome level is pulse field gel electrophoresis (PFGE) which separates DNA of test samples into individual chromosomes. Such a technique may only detect large differences in the DNA since the presence and size of individual chromosomes are used in the comparison. One of the most important techniques currently used in molecular biological work involves the cleaving of large DNA molecules into fragments which can be studied more easily. This is done by the use of restriction endonucleases, enzymes that recognise and cleave DNA at specific sequences. These enzymes, isolated mostly from prokaryotes, cleave DNA leaving either blunt or protruding cohesive ends which can be joined, by the use of specific enzymes called ligases, to other DNA molecules which have complementary nucleotide sequences. After cleavage by enzyme digestion, the fragments are separated by either polyacrylamide or agarose gel electrophoresis. As in other electrophoretic techniques, separation is based on the fact that fragments migrate on gels at different rates depending on, among other factors, their size and the concentration of the gel. In agarose gel electrophoresis, 0.7 to 1.2% gels are optimal for separation of DNA fragments whose size is between 10 and 0.4 kilobases (kb). In order to visualise DNA, a staining method is used which takes advantage of the ability of ethidium bromide intercalating with DNA (Sharp *et al,* 1973). Staining with ethidium bromide provides

a method of visualising DNA which facilitates identifying and purifying fragments as well as determining their size. The DNA strands of these fragments are first separated (denatured), and the fragments can then be transferred by a process of Southern blotting (Southern, 1975) onto membranes which have a high affinity to DNA such as nitrocellulose or nylon as illustrated in Figure 8.1.

In order to compare the nucleotide-base sequences of the separated fragments with sequences of another DNA fragment (a probe), hybridisation techniques are used for it is very unlikely that ethidium bromide stained digests would reveal such details. The probe is first labelled with an enzyme or radioactive material and, by a process of hybridisation, allowed to bind with any complementary sequences on the blotted DNA fragments. Complementary sequences are then located as bands on autoradiographs. When comparing several DNA samples, one of them can be digested, labelled and used as a probe. Hybridisation of the probe with the other DNA samples can then be used to detect similarities and dif. In trypanosomes, such an approach may difficult for the total genome of 3-4 x 10^7 bp (Myler, 1993) may be too large to use. A DNA fragment can also be used as a probe to compare test DNA samples. When this is the purpose of using a probe, any DNA fragment can be labelled and used as a probe and the hybridisation profiles of test DNA samples to the probe, compared.

Plasmids or bacteriophages in which DNA fragments are inserted (vectors) can also be used as a probe especially when the purpose of hybridisation is to compare DNA samples and not to determine complementarity with the probe. In such situation, total DNA which includes both vector DNA as well as insert DNA, is labelled and hybridised with the DNA samples to be compared. Unlike hybridisation with the insert alone, when both vector and insert DNA are used as a probe, it is not possible to compare the complementarity of test DNA with the probe for hybridisation may either be with the insert or the vector. The use of both vector and insert as probe, however, provides more varied DNA sequences to hybridise with test DNA and thus a higher probability of detecting differences between test DNA samples.

Probes used for the detection of complementarity in test DNA can be developed from fragments which are believed to be the source of the phenotypic character being investigated. The P-glycoprotein gene believed to be responsible for resistance to a wide range of drugs in different organisms (Gros *et al*, 1986a, b, c; Bradley *et al*, 1988; Ouellette *et al*, 1990; Ouellette and Papadopoulou, 1993) is an example of DNA that can be used as a probe to study drug resistance. Although an ideal probe would be one that is developed from the same species as the test DNA, the P-glycoprotein probe can still be used for it has been shown to be highly conserved in different species (Bradley *et al*, 1988; Ouellette *et al*, 1990).

A probe can also be developed from a conserved portion of the genome that is unrelated to the phenotypic character being investigated. Ribosomal DNA found as tandem repeats of a single sequence (McCutchan *et al,* 1984) is an example for it has been found to be highly conserved across species (Rollinson and Kane, 1991). Such a probe can be used to detect differences between test DNA samples without regard for complementarity of the probe and test DNA. Ribosomal DNA has been used in the study of intra-specific differences in the snail *Bulinus* (Rollinson and Kane, 1991) as well as in *Schistosoma* (Jasma *et al,* 1977).

The work described in the previous chapters involved comparisons between phenotypic characteristics of suramin-sensitive and suramin-resistant *T. evansi* parasites. The work described in this chapter aimed at investigating genotypic differences between the same stocks of *T. evansi.* So far, there has not been any reported work which has compared the genomes of drug-sensitive and drug-resistant African trypanosomes. This work compared suramin-sensitive *T. evansi* parasites and their induced resistant clones, produced in experiments described in Chapter Five. Ribosomal DNA from *T. brucei* inserted in the plasmid pUC 8 and a P-glycoprotein gene from *Leishmania tarentole* inserted in pGEM 3 were used as probes in hybridisation experiments with the parasite DNA samples.

8.2 MATERIALS AND METHODS

The trypanosomes used in this study were products of the induction experiment (reduced host response method using cloned material, see Table 5.4) described in Chapter Five and are shown in Table 8.1. These trypanosome stocks were prepared for comparison of their DNA as illustrated diagrammatically in Figure 8.6 and described in the following sub-sections.

Table 8.1 Suramin sensitivities of *T. evansi* used for comparing DNA of suramin-sensitive and resistant strains.

Strain identity	Suramin resistance in mg/kg
2454 C1	0.005
2454 C7	0.5
2454 C9	2.0
2476 C1	0.005
2476 C12	5
2476 C18	11
2476 C22	15

8.2.1 DNA ISOLATION

The total DNA from each of the seven stocks was isolated using a method adapted after Bernards *et al* (1981). In this method, each stocks was grown in five rats which were sacrificed and bled at peak parasitaemia. The parasites were separated from blood elements on a column of DE 52 (Lanham and Godfrey, 1970 described in 3.1.1) and pelleted by centrifugation at 1,000g for 10 minutes (MSE Chilspin 2, Fisons England).

For every 1g of trypanosome pellet, 10 ml of NET buffer (Appendix 14) was added to suspend the parasites and 1% sodium dodecyl sulphate (SDS) added to lyse them. To deproteinise the parasites, proteinase K (20 U/mg, Boehringer Mannheim Biochemicals, U.S.A.) was added at a concentration of 50 μg/ml and the suspension incubated at 37^0C for 30 minutes. This was followed by three extractions by phenol, phenol/chloroform (1:1) and chloroform. For each of these extractions, the suspension was mixed with an equal volume of the organic solvent for 5 minutes and the organic and aqueous phases separated by centrifugation at 1,600g for 5 minutes in an Eppendorf centrifuge (Centrifuge 5415 C, Heraeus, Germany). The aqueous phase was gently removed without interrupting the protein interphase, mixed with two volumes of ice-cold ethanol and kept overnight at 20^0C to precipitate DNA . This was followed by centrifugation at 1,600g for 5 minutes in an Eppendorf centrifuge and all the supernatant aspirated to leave the pellet dry. The pellet was dissolved in 5 ml of TE buffer pH 7.5 (Appendix 15) per g of original pellet and incubated at 37^0C for 30 minutes with 20 μg/ml of ribonuclease A (50 U/mg, Boehringer Mannheim Biochemicals, U.S.A.) in the presence of 0.1% SDS. This was followed by a further incubation with 50 μg/ml of proteinase K at 37^0C for 30 minutes, phenol/chloroform extraction and ethanol precipitation as described earlier. The DNA pellet was then dissolved in 300 μl of TE buffer and kept at 4^0C. The amount and purity of the isolated DNA was estimated by determining the wavelength of DNA in TE at 260 and 280 nm using a spectrophotometer (CE 6600 Multimode Computing UV Spectrophotometer, Cecil instruments Ltd, England). An optical density (OD) of 1 at 260 nm is equivalent of 50 μg/ml while a 260/280 nm ratio of 1.8 indicates pure DNA (Maniatis *et al,* 1982).

8.2.2 DIGESTION

Three endonucleases; BamH1, Hind111, and Pst1 (10,000 U/ml, Boehringer Mannheim Biochemicals, U.S.A.) were used to digest each of the DNA fractions. These enzymes were chosen for they have sites in the plasmid vectors in which the

ribosomal and glycoprotein genes were inserted (Figures 8.3 and 8.5). Hind111 and Pst1 also have restriction sites in the two inserts (Figures 8.2 and 8.4). The reaction mixture contained:

16 µl 10 µg DNA in TE buffer
2 µl enzyme buffer
2 µl enzyme

Digestion was carried out in closed microfuge tubes as recommended by the manufactures of the enzymes at 37^{0}C overnight (at least 18 hours).

8.2.3 ELECTROPHORESIS

The reaction mixture with 4 µl of gel-loading dye (Appendix 16) was loaded in each well. DNA fragments were separated in 0.8% agarose (Boehringer Mannheim Biochemicals, U.S.A.) gels for 15 hours in a horizontal electrophoresis tank (Model HE 33 maxi submarine unit, Hoefler Scientific instruments, U.S.A.) at a constant voltage of 30 volts (power pack: EC 600 E-C apparatus corporation, U.S.A.). The tank buffer was TAE (Appendix 17) with 0.5 µg/ml of Ethidium bromide. A molecular weight standard with 23 DNA fragments ranging in size from 75 bp to 12 kbp ("1 kb", Bethesda Research laboratories, U.S.A.) was run alongside the DNA in order to determine the size of the separated fragments. At this stage, the gels were placed on a ultra-violet ray transilluminator (Hoefler Scientific Instruments, U.S.A.) and photographed using a Polaroid camera (MP 4 Land camera, Kodak UVP U.S.A. and 667 Sigma Polaroid film, U.S.A.).

8.2.4 SOUTHERN BLOTTING

Gels were then blotted onto nylon membranes (Duralon UV ^{TM} Stratagene) as described by Southern (1975) and illustrated in Figure 8.1. In this method, the DNA was first denatured by gently shaking the gel (Orbit shaker, Labline, U.S.A.) in denaturing solution (Appendix 19a) twice for 30 minutes each time. In order to wash out the salt from the denaturing buffer, the gel was immersed and shaken in neutralisation buffer (Appendix 19b) for one hour. The gel was then transferred onto the nylon membrane by overturning it on a glass plate platform covered with 3 MM filter paper. The filter paper served as a wick by being immersed in a shallow plastic container with transfer buffer (Appendix 18). Another 3MM filter paper cut to the size of the gel was placed on the membrane and paper towels stacked on it to a height of at least 30 cm. A second flat glass plate was placed on the paper towels to serve as a

platform for a weight of about 2 Kg. To avoid "short-circuiting", whereby the transfer buffer would by-pass the gel and the nylon membrane by the wick touching the paper towels, the upper edges of the gel were lined with parafilm.

The DNA was left to transfer for 48 hours at which time the wick was still immersed in the buffer and all the paper towels were soaked. The wells on the gel were marked on the reverse side of the membrane (the side not touching the gel) and the membrane's bottom-right-hand corner cut-off to indicate the end of the gel where loading of wells started. The nylon membrane was then air-dried for one hour, baked at 80°C for two hours (Joh Achelis and Sohne, Germany) and kept between 3MM papers until needed for hybridisation.

8.2.5 PROBE PREPARATION FOR HYBRIDISATION

Hybridisation was done using two probes. The first probe used was a 1:1 mixture of two ribosomal probes; pTBR1 and pTBR3 inserted in pUC 8. These probes were developed from *T. brucei* by Prof. J. Boothroyd (Stanford University, U.S.A.) and provided to Dr. Rashid A. Aman who kindly availed them to me. As shown in Figure 8.2, pTBR1 and pTBR3 have sizes of 5.1 and 2.8 kb respectively and are cloned into the BamH1 site in the polycloning site of the PUC8 vector (Figure 8.3). The second probe, pM7, is a *Leishmania* P-glycoprotein gene inserted in pGEM 3, was kindly provided by Dr. M. Ouellette (Quebec, Canada). This is a 850 bp fragment (Figure 8.4) cloned into the Pst1 site of the polycloning area of pGEM-3 vector (Figure 8.5).

8.2.5.1 Radio-labelling

The probes consisting of the plasmid vectors with gene inserts were radio-labelled using [^{32}P] dCTP (370 MBq/ml, Amersham, Arlington Heights U.S.A.) by random priming (Feinberg and Vogelstein, 1983). In this method, probe DNA denatured by heating to 95^{0} C, acts as a template and is incubated with a polymerase enzyme (Klenow enzyme) in the presence of a primer and nucleotides which include the radiolabelled one. A new DNA molecule is synthesised starting at different parts of the template resulting in a probe with radioactive nucleotides incorporated.

While handling radioactive material behind a shield (CAWO Rapid Tube, U.S.A.), the probes were labelled using the following reaction mixture:

11.4 μl labelling mix (dATP, dGTP, dTTP each at 0.5 mM/l, R. Aman, IPR)
1.0 μl Bovine serum albumin - BSA-(20 mg/ml, Boehringer Mannheim)
5.0 μl ^{32}P dCTP (370 MBq/ml)
2.0 μl Klenow enzyme (6000 U/ml, Boehringer Mannheim)
3.6 μl sterile re-distilled water
2.0 μl probe DNA solution (1 μg DNA) denatured first by heating at 95° C for 10 minutes.

This reaction mixture was incubated at 37° C for 30 minutes after which the reaction was stopped by addition of 1 μl of 0.5 M EDTA. The volume of the reaction mixture was adjusted to 100 μl using STE (Appendix 20).

8.2.5.2 Purification of the probes

To purify the probes by removal of all un-incorporated nucleotides, gel filtration chromatography using a Sephadex column was done. To do this, Sephadex (Sephadex-50, Boehringer Mannheim, Appendix 21) kept in 0.01% sodium azide was firmly packed in a column (a 1 ml syringe) using STE; a procedure which also washed off the sodium azide. The reaction mixture was then slowly run through the column and two drops per tube collected in 60 tubes using STE as the eluant. The radioactivity of each of solutions in the tubes was determined in counts per second (cps) using a radioactive monitor (Ratemeter Type RM 6 serial no. 1655, Netechnology, U.K.) and plotted on a graph. The first peak on the graph which consisted of the labelled probe was determined, the contents of the relevant tubes pooled and the total radioactivity determined in cps. The radioactive probe was then used for hybridisation.

8.2.6 HYBRIDISATION AND AUTO-RADIOGRAPHY

To hybridise the blotted nylon membranes, a modified method as detailed by Maniatis *et al* (1982) was used. In this method, membranes were first sealed in a polythene bag (Saran wrap) leaving a small hole at one of the corners and 20 ml of 2X SSC (Appendix 17) put in the bag to wet them. Care was taken each time to remove all bubbles from bags before sealing in order to ensure that all parts of the membranes were soaked by the solutions used. Bags were then gently shaken for 5 minutes and the 2X SSC discarded. Twenty five ml of hybridisation solution (Appendix 22) pre-

warmed to 42^{0}C was then sealed into the bags and left overnight (18 hours) at 42^{0}C with gently shaking. This solution was discarded and 25 ml of hybridisation buffer together with the labelled probe mixture (denatured first by heating at 95°C for 10 minutes) sealed in the bags. The hybridisation reaction was then carried out overnight at 42^{0}C with gentle shaking.

The washing of the membranes differed with the probe used. Different stringencies were tried out whereby the salt concentrations, temperature and duration of washing were varied to determine the conditions which would provide optimal visibility of the hybridised bands with little background radioactivity. For the ribosomal probes, the first wash was at room temperature for 30 minutes using buffer 1 (Appendix 23) and at 60°C for 15 minutes each time using buffer 2 (Appendix 24) for the second and third washes. For the P-glycoprotein probe, the washing was less stringent (consisting of lower salt concentration, lower temperature for a shorter time) and consisted of 15 minutes at 42°C using buffer 1 (Appendix 25) and 5 minutes at 42^{0}C using buffer 2 (Appendix 26). All the washings were done with vigorous shaking and the membranes were scanned for radioactivity using the monitor after every wash to ensure that the washings were not too stringent thereby leading to a loss of hybridised label.

The membranes were then rinsed in 2X SSC, placed in cassettes (CAWO U cassette, 35x43 U.S.A.) and exposed to X-ray film (Sigma Kodak X-OMAT, XAR-5 35x43, U.S.A.). The duration of exposure to the X-ray film was varied to determine what was adequate to balance optimal visibility of bands and background staining. Another factor which influenced the duration of membrane exposure to X-ray films was the age of the ^{32}P in respect to its half-life. Membranes were thus exposed for 24-120 hours. The radiographs were processed by immersion into fixative and developer solutions in the dark as recommended by Kodak and air dried. They were viewed on a light box and photographed with a Polaroid camera (Polaroid MP4 Land camera UVP, U.S.A. and 665 Sigma Polaroid film Sigma, U.S.A.).

8.3 RESULTS

8.3.1 DNA ISOLATION

The DNA yields of the seven *T. evansi* strains shown in Table 8.2 indicate that between 0.5 and 0.8 μg/ml of DNA was isolated from the trypanosome pellets. The exception was KETRI 2476 C_{18} whose yield was reduced by losing about a half of the reaction solution at the phenol-extraction stage of isolation due to the cracking of a centrifuge tube.

Table 8.2 The yield and purity of DNA isolated from *T. evansi* strains with different suramin sensitivities.

Stock KETRI no.	DNA yield mg/ml	Volume of buffer with DNA	260/280 nm ratio
2454 C1	0.52	750	1.58
2454 C7	0.56	750	1.62
2454 C9	0.77	750	1.68
2476 C1	0.66	500	1.72
2476 C12	0.72	600	1.75
2476 C18	0.33	400	1.68
2476 C22	0.81	500	1.59

8.3.2 ELECTROPHORESIS

In each of the digests, DNA digested with BamH1, Hind111 and Pst1 stained with ethidium bromide appeared as smears of varying staining intensity ranging in size from about 1 kb to over 12 kb. Digestion of KETRI 2454 C7 and KETRI 2476 C1 DNA with BamH1 and Hind111 resulted in a mixture of large fragments of over 12 kb and smaller fragments appearing as a smear culminating in a band of about 1.5 kb (Figures 8.7 and 8.9). Digestion of KETRI 2454 C1 and C9 as well as KETRI 2476 C12 and C22 resulted in fragments of over 12 kb appearing as two bands of lower intensity than the other two strains previously described and a smear spreading up to about 2 kb. The digested fragments of KETRI 2476 C18 DNA were less intense than those of the other 6 stocks and were seen as two faint bands of over 12 kb and fragments appearing as a smear up to 3 kb. When KETRI 2454 C7 DNA was digested

with Pst1, an intense band of over 12 kb was observed and fragments appeared as a smear up to about 1 kb (Figure 8.8). Digestion of KETRI 2476 C1 produced similar results except the band of over 12 kb did not stain as intensely with ethidium bromide. DNA of KETRI 2454 C1 and C9 as well as KETRI 2476 C12 and C22 were digested with Pst1 to produce a fragment of over 12 kb appearing as a single band and other fragments appearing as a smear up to about 1 kb.

8.3.3 RADIO-LABELLING AND PROBE PURIFICATION

Both of the probes were labelled well and separated from the unlabelled nucleotides as shown in Figures 8.10 and 8.11. The first peak in each of the figures represents the incorporated labelled nucleotides which eluted first in chromatography due to their larger size as compared to the second peak which represents the free nucleotides. Tubes 15 to 25 which had a radioactivity of 4130 cps were pooled for the ribosomal probes and tubes 7 to 14 with a radioactivity of 3460 cps were pooled for the P-glycoprotein probe.

8.3.4 HYBRIDISATION

Figure 8.12 is an autoradiograph of DNA digested with BamH1 and Hind111 (Figure 8.7) and hybridised with pTBR1 and pTBR3 inserted in pUC 8. Lane 1 loaded with KETRI 2454 C1 DNA and digested with BamH1 has intense bands of 9 kb to over 12 kb, single bands of about 8, 6, 4.8, (band 1) 4, and 3.7 kb and another intense band of about 5.5 kb. Other than the intensity of the various bands, the banding patterns of KETRI 2454 C7 and C9 are similar to that of C1 except C9 (lane 3) has an extra band (D) of about 3.4 kb not found in the other two strains. The DNA of KETRI 2476 strains (lanes 4-7) shows similar banding patterns as described for lane 1 major differences being that the bands of over 12 kb are more distinct appearing as 2 or 3 bands and the intense band of 6 kb appearing as two bands. Lanes 2, 5, 6, and 7 loaded with KETRI 2454 C7 and 2476, C12, 18 and 22 have the extra band (D) of about 3.4 kb marked in lane 3 (KETRI 2454 C9) which lanes 1 and 4 do not appear to have.

The autoradiograph (Figure 8.12) shows that KETRI 2454 C1 DNA (lane 1) digested with Hind111 has fragments of DNA of sizes ranging from 9 to over 12 kb seen as bands which hybridised with the pUC 8 vector containing ribosomal gene inserts (Figure 8.12). There was a distinct band of about 8 kb (band B) after which, there were intense but indistinguishable 5-6 kb bands. There were also distinct bands of about 4, 3.2 (band E), 2.8 (band F) and 1.8 (band G) kb. Other than differences in

intensity of bands and the absence of the 8 kb band, the banding patterns of lanes 2 (KETRI 2454 C7), 3 (KETRI 2454 C9) and 4 (KETRI 2476 C1) were similar to that of lane 1. Lanes 5, 6 and 7 loaded with DNA of KETRI 2476 C12, 18 and 22 had a similar banding pattern to those of lanes 2, 3 and 4 expect that they lacked bands of 3.2, 2.8 and 1.8 (E, F and G) and had a band of about 3.6 kb (band C).

Figure 8.13 is an autoradiograph of DNA digested with Pst1 (Figure 8.8) and hybridised with the ribosomal probes inserted in pUC8. Lanes 1,2 ,3, 5, and 7 had bands (3-8) of about 3.5, 3.0, 2.9, 2.5, 2.0, 1.9 and 1.7 kb. Lane 3 (KETRI 2454 C9) was similar to the others except, in addition there were four bands between 4.5-7 kb (A). Lane 6 loaded with less DNA than the other lanes appeared to have similar bands as those in lane 1 except in this lane they were faint.

Hybridisation of test DNA digested with the BamH1, Hind111 and Pst1 (Figure 8.9) with the P-glycoprotein gene inserted in pGEM 3 is shown in Figure 8.14. Digests of BamH1 appear as distinct bands of over 12 kb irrespective of the DNA used. In addition, other fragments appear as a smear between 5 and over 12 kb. Lane 3 loaded with KETRI 2454 C9 had two bands (C and D) of approximately 11 and 7 kb. Band D was found also in lanes 5,6 and 7.

After digestion with Hind111, hybridisation with pM7 inserted in pGEM 3 shows that there are DNA fragments complementary to the probe which appear as a continuous smear of between 4 to over 12 kb. Except for lane 6 which was loaded with less DNA, there is also a distinct band (1) at about 7 kb in all other lanes whose intensity varies with the different DNA samples. In addition, bands (E, G and H) of about 6.5, 2.9 and 2.8 kb are found in lanes 2, 3, 5 and 7 and band (F) of about 4 kb found in lanes 1, 2, 3 and 4.

The results of hybridisation after digestion with Pst1 show that except lane 3, all other lanes are similar, with fragments ranging in size from 1.6 kb to over 12 kb appearing as a smear. There are distinct bands of 6.5, 4.5, 4.2 and 3.4 kb whose intensity varies in the different lanes. In addition to these bands, lane 3 (KETRI 2454 C9) has an intense band (A) of over 12 kb.

These results show that there are some DNA portions that are not found in all the strains used. These DNA fragments, seen as bands, can be classified into three groups:

a. Bands found in all clones of both trypanosome stocks used. An example is bands 1 and 2 of DNA digested with BamH1 (Figure 8.12), and bands 3-8 (Figure 8.13) when the DNA was digested with Pst1 and hybridised with

pUC 8 and ribosomal genes. Band 1 of DNA digested with Hind111 and hybridised with the pGEM 3 and P-glycoprotein probe (Figure 8.14) was also in this group.

b. Bands found in some clones of a particular stock. Examples of these are bands A (Pst1 digests, Figure 8.13) B and C (Hind111 digests ,Figure 8.12) when using pUC 8 and pTBR. Other bands in this group are A, B, and C (Pst1, Hind111 and BamH1 digests respectively, Figure 8.14) when using pGEM 3 and pM7 as a probe.

c. Bands found in strains whose suramin sensitivities are within certain ranges regardless of the stock. These are band D (a BamH1 digest) and bands E to G (Hind111 digests, Figure 8.12). Other bands are D (a BamH1 digest) and E to H (Hind111 digests, Figure 8.14) when using the pUC 8 and ribosomal genes as well as pGEM 3 and P-glycoprotein probes respectively.

Tables 8.3 and 8.4 show bands in these last two categories when using the ribosomal (pTBR1 and 3) or P-glycoprotein (pM7) probes. When *T. evansi* DNA was hybridised with pUC8 and pTBR, bands A and B were found only in clones of KETRI 2476 and 2454 respectively which were resistant to suramin at a dose level of 0.005 mg/kg (Table 8.3). Band C was found in clones of KETRI 2476 which are resistant to dose levels of suramin of 5 mg/kg and above. A 3.4 kb BamH1 digest (band D) was found in clones of both KETRI 2454 and 2476 which are resistant to dose rates of suramin of 0.5 mg/kg and above. Hind111-digests of 3.2, 2.8 and 1.8 kb (bands E, F and G) were found in clones of both trypanosome stocks which are sensitive to the drug at dose rates of 2 mg/kg and below.

Table 8.4 shows that when DNA samples were hybridised with pGEM 3 and pM7, bands A, B and C (Pst1, Hind111 and BamH1 digests respectively) were found only in KETRI 2454 C1. Band D (a BamH1-digest) was observed in clones of both KETRI 2454 and 2476 which are resistant to suramin at dose rates of 2 mg/kg and above while three Hind111-digests (band E, G and H) were found in clones of both trypanosome stocks which were resistant to the drug at 0.5 mg/kg and above. Another Hind111-digest (band F) was observed in clones of both KETRI 2454 and 2476 which were resistant to suramin at dose rates of 2 mg/kg and below.

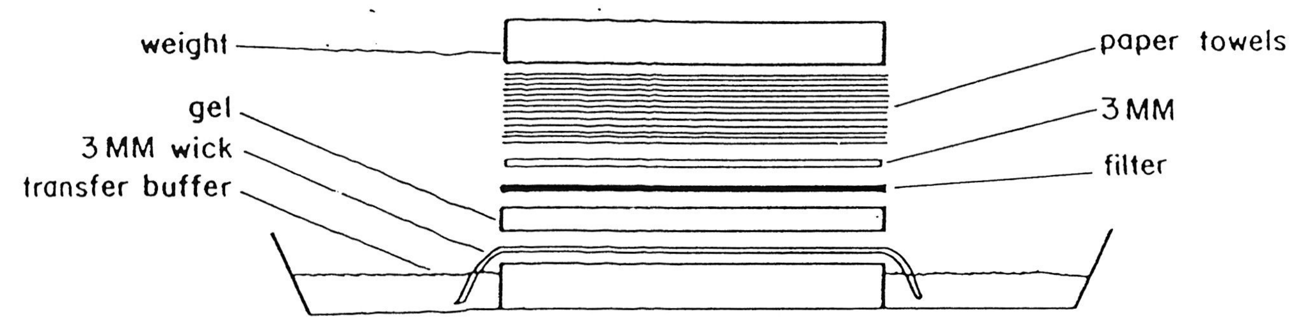
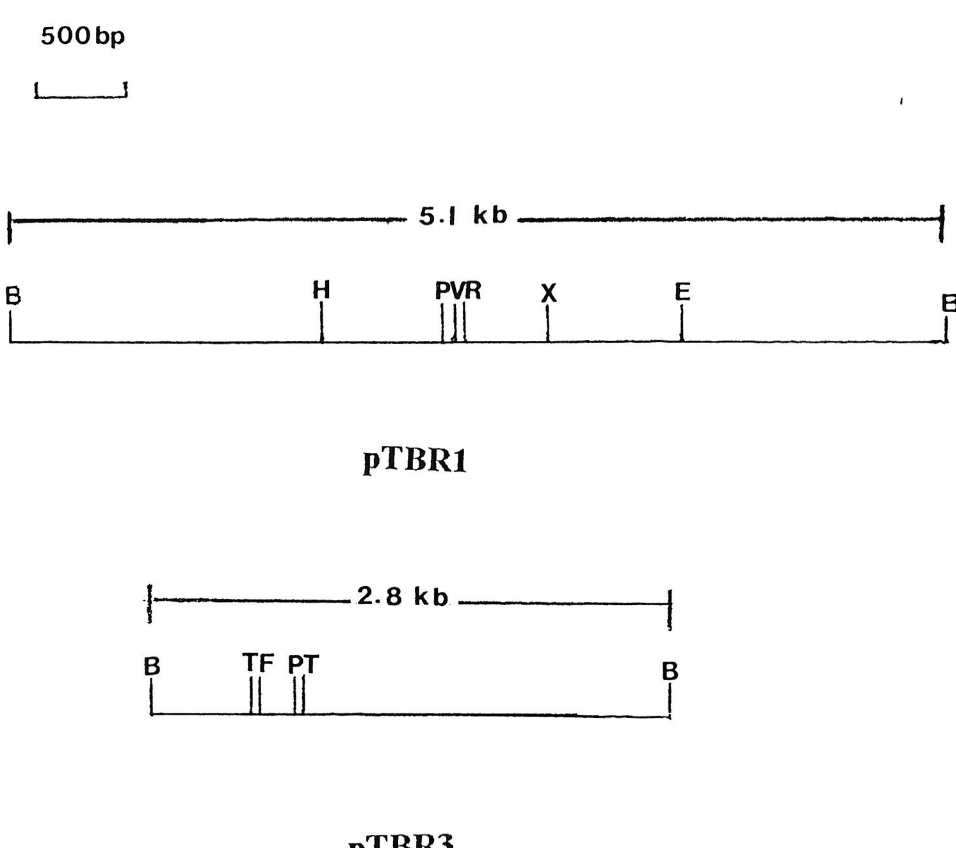
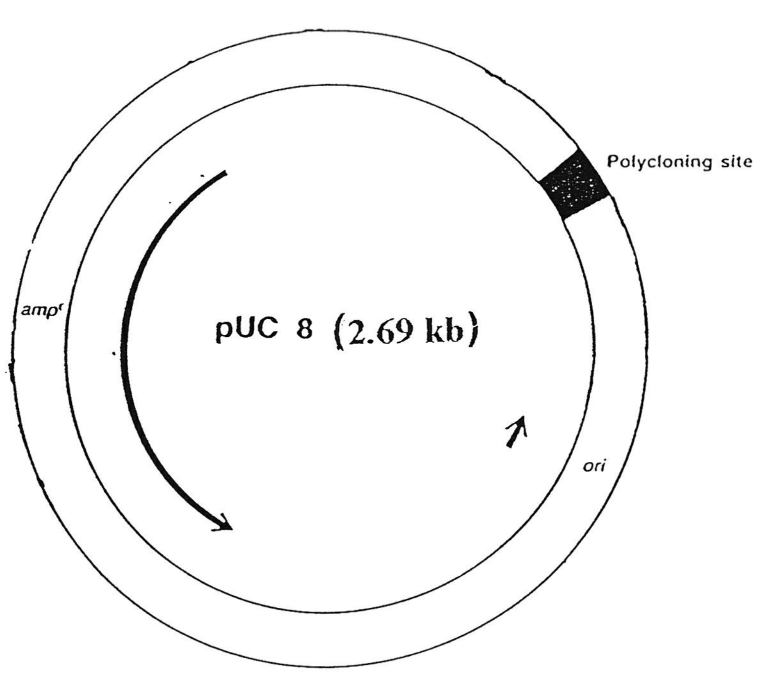


Figure 8.1 Diagrammatic illustration of Southern blotting.



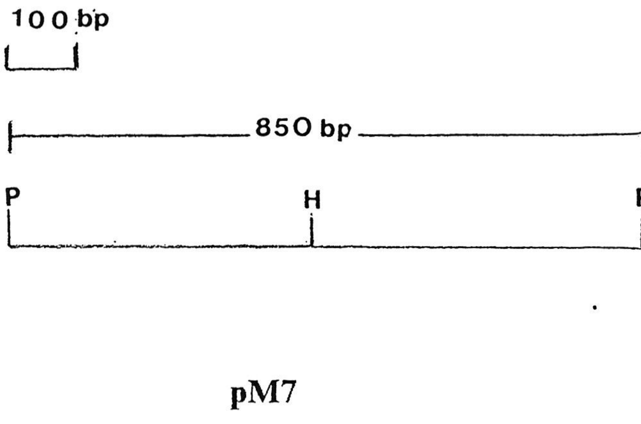
Legend:
B - BglII
E - EcoRI
F - HinfI
H - HindIII
P - PstI
R - EcoRV
T - TaqI
V - Pvu
X - XhoI

Figure 8.2 Physical map of the probes, pTBR1 and pTBR3, from the ribosomal genes of *T. brucei* (Prof. Boothroyd, Stanford University, USA).



Legend: ori - origin of replication.
amp^r - ampicillin resistance gene.

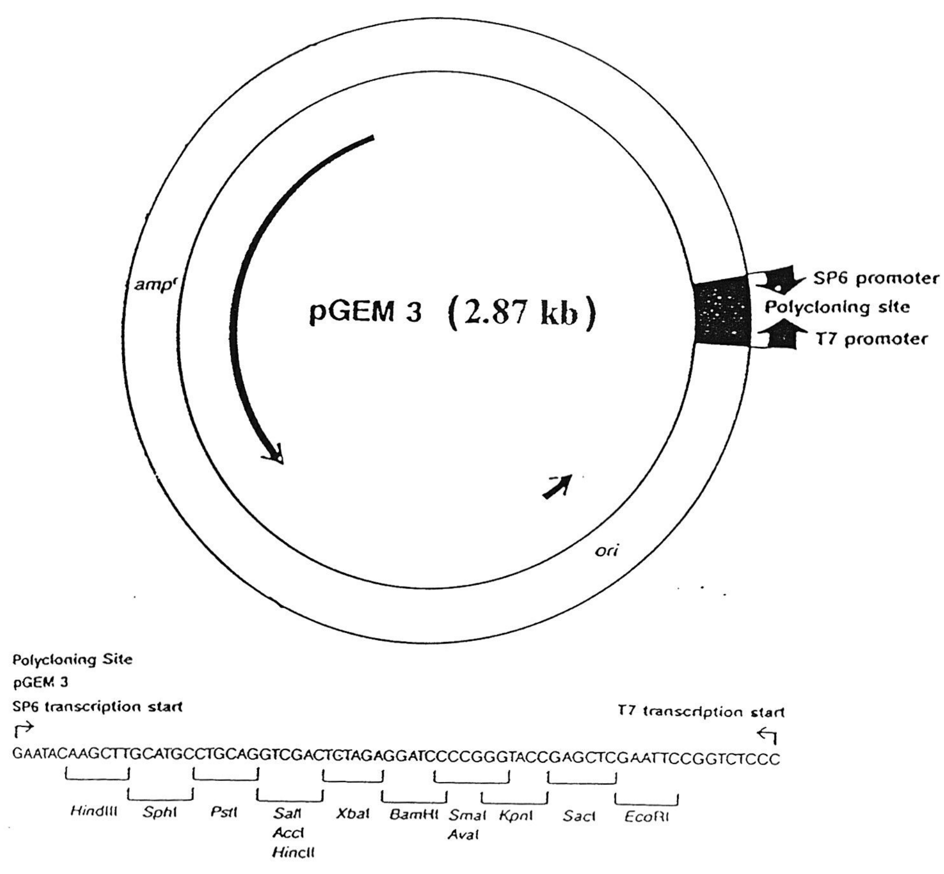
Figure 8.3 Physical map of the pUC 8 plasmid vector in which the ribosomal probes were inserted.



pM7

Legend: **P - PstI**
H - HindII

Figure 8.4 Physical map of the pM7 probe derived from the P-glycoprotein gene of *Leishmania* (Dr. Ouellette, Quebec, Canada).



Legend: ori - origin of replication.
amp^r - ampicillin resistance gene.

Figure 8.5 Physical map of the pGEM 3 plasmid vector in which the P-glycoprotein probe was inserted.

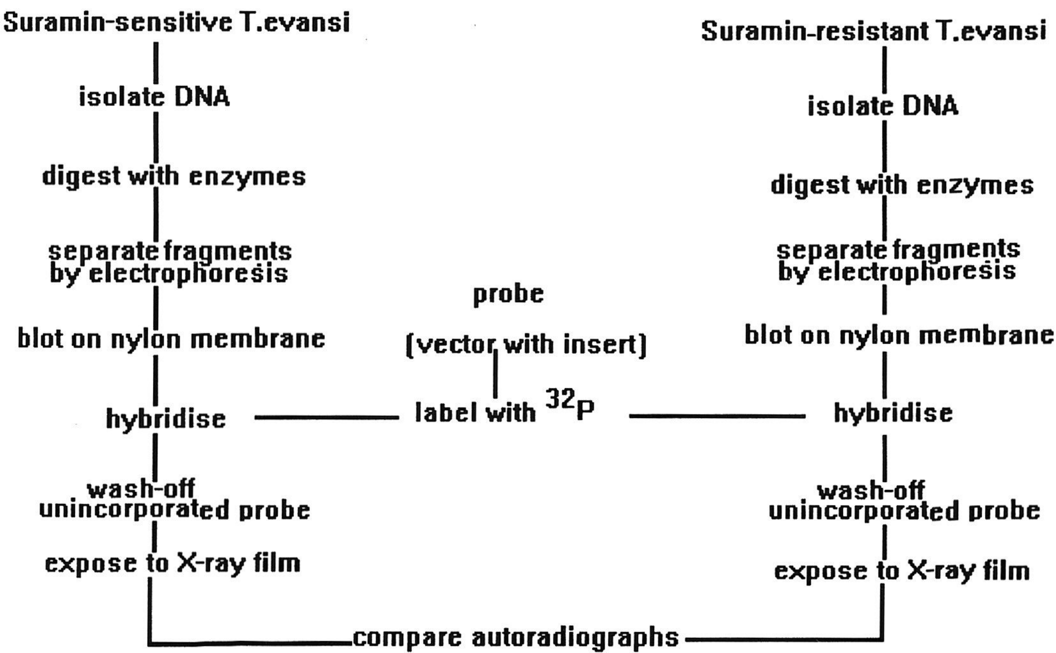


Figure 8.6 Experimental protocol for comparing DNA of suramin-sensitive and resistant *T. evansi.*

Figure 8.7 Photograph showing *T. evansi* DNA digests using Hind111 and BamH1.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).

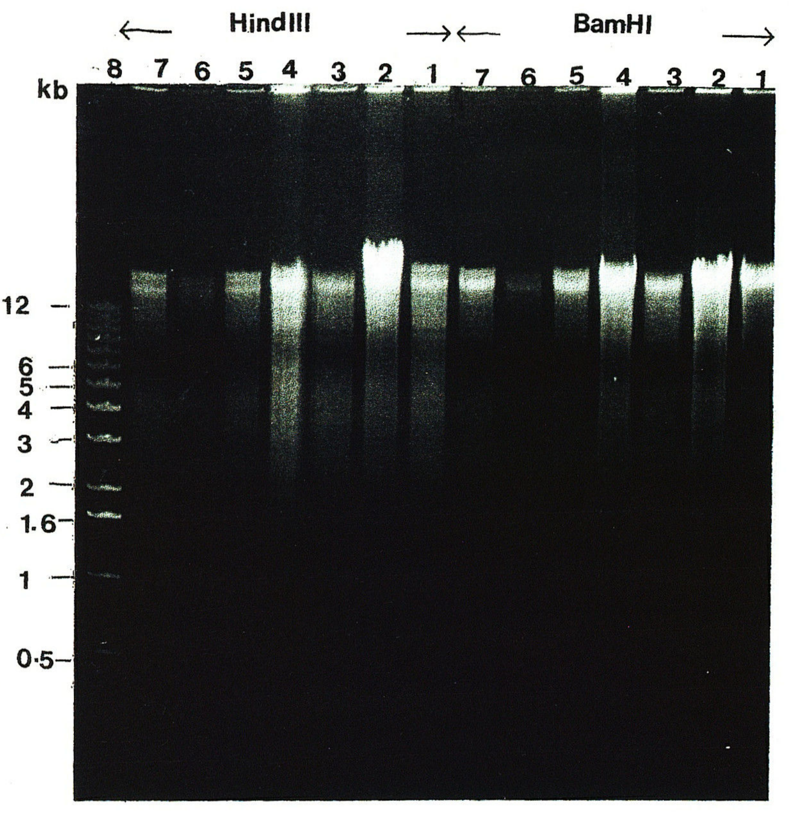

HindIII
BamHI
8 7 6 5 4 3 2 1 7 6 5 4 3 2 1
kb
12
6
5
4
3
2
1.6
1
0.5

Figure 8.8 Photograph showing *T. evansi* DNA digests using Pst1.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).

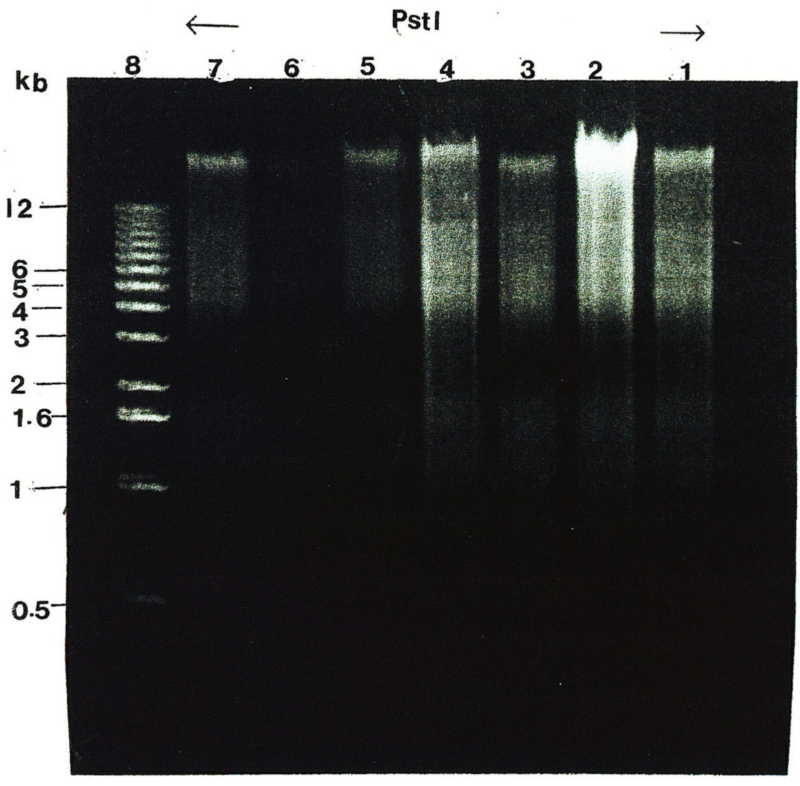

PstI
kb
8
7
6
5
4
3
2
1
12
6
5
4
3
2
1.6
1
0.5

Figure 8.9 Photograph showing *T. evansi* DNA digests using Hind111, BamH1 and Pst1.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

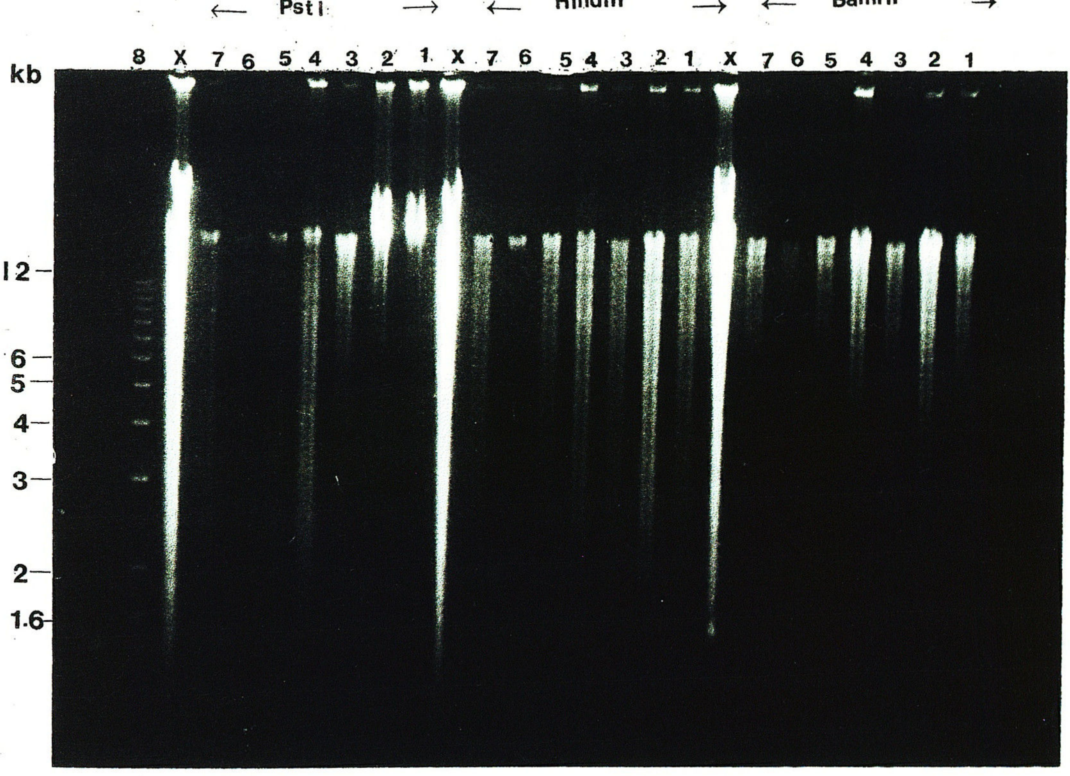
5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).

x unrelated DNA.



← Pst I →
← HindIII →
← BamHI →
8 X 7 6 5 4 3 2 1 X 7 6 5 4 3 2 1 X 7 6 5 4 3 2 1
kb
12
6
5
4
3
2
1.6

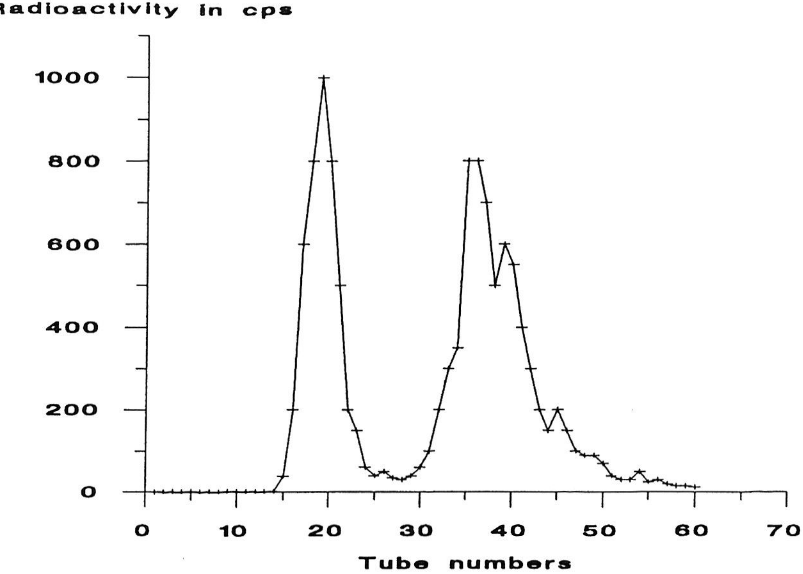


Figure 8.10 Sephadex separation of ^{32}P labelled pTBR1 and 3 inserted in pUC 8.

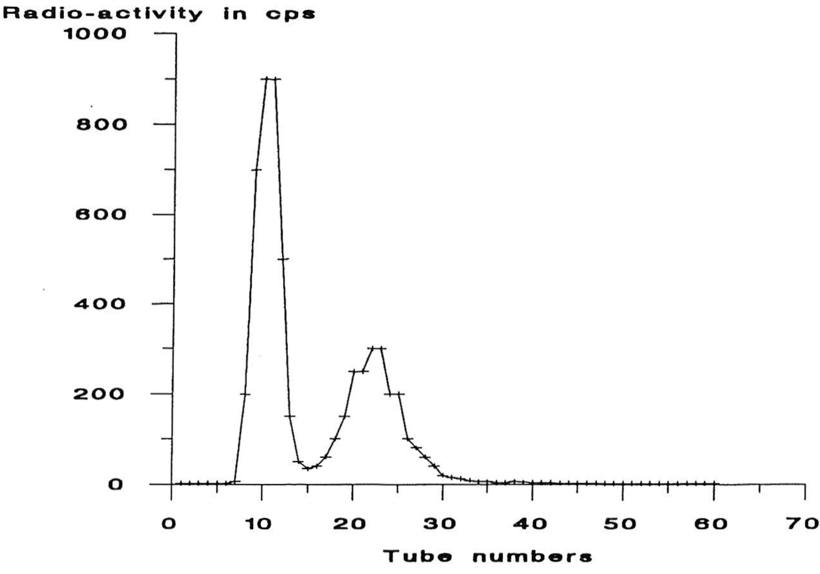


Figure 8.11 Sephadex separation of ^{32}P labelled pM7 inserted in pGEM 3.

Figure 8.12 Autoradiograph showing *T. evansi* DNA digested with Hind111 and BamH1 and hybridised with two ribosomal probes pTBR1 and pTBR3 inserted in pUC 8.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

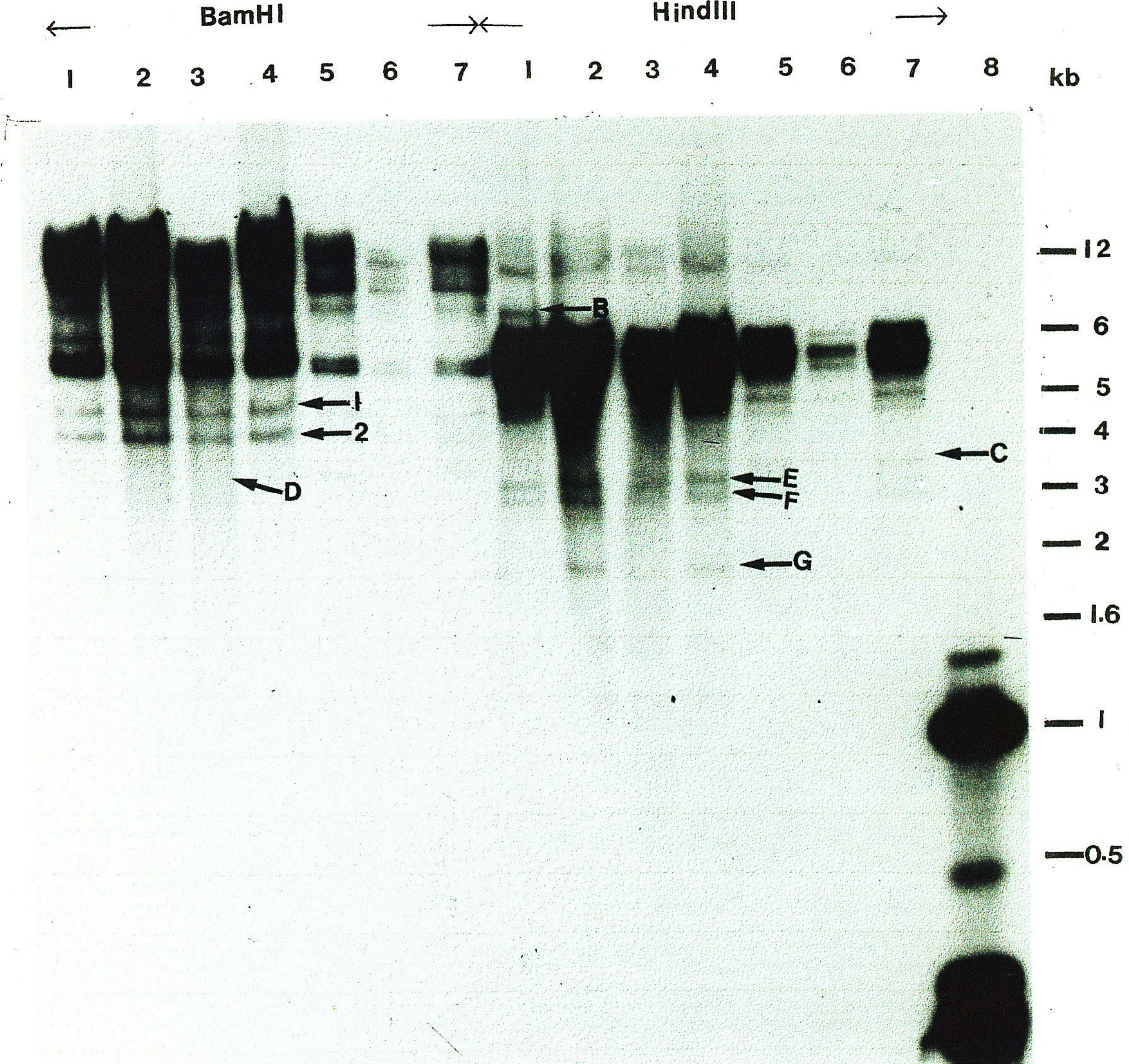
4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).



BamHI
HindIII
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
kb
12
6
5
4
3
2
1.6
1
0.5
B
1
2
C
D
E
F
G

Figure 8.13 Autoradiograph showing *T. evansi* DNA digested with Pst1 and hybridised with two ribosomal probes pTBR1 and pTBR3 inserted in pUC 8.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).

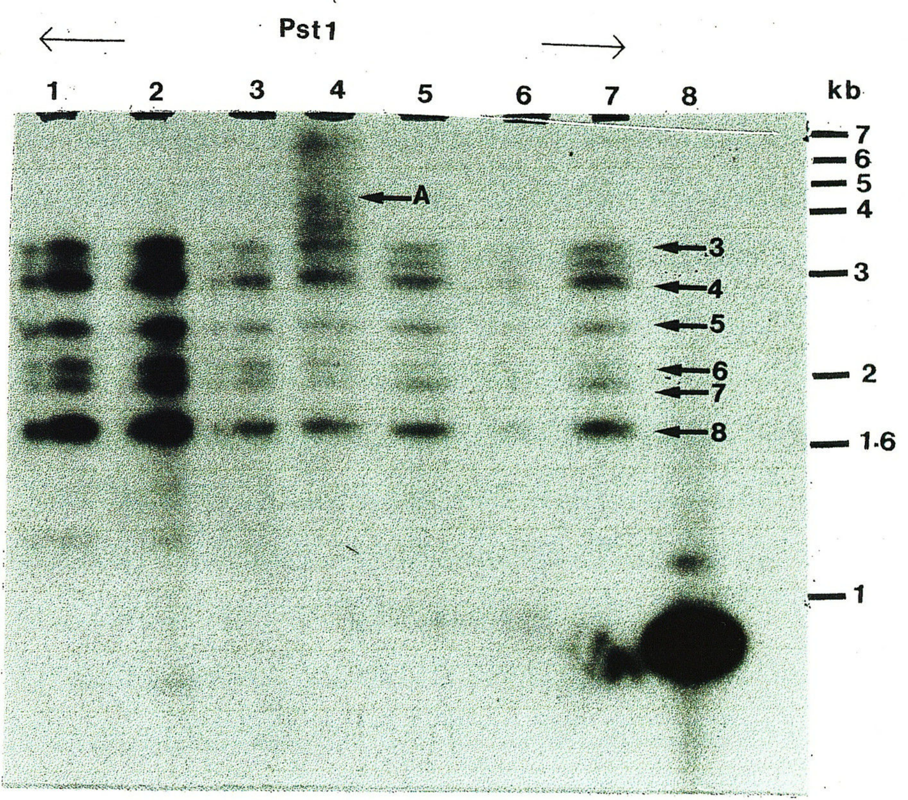

Pst1
1
2
3
4
5
6
7
8
kb
A
3
4
5
6
7
8
7
6
5
4
3
2
1.6
1

Figure 8.14 Autoradiograph showing *T. evansi* DNA digested with Hind111, BamH1 and Pst1 and hybridised with P-glycoprotein gene inserted in pGEM 3.

Lanes: 1 KETRI 2454 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

2 KETRI 2454 C7 resistant to suramin at a dose rate of 0.5 mg/kg.

3 KETRI 2454 C9 resistant to suramin at a dose rate of 2 mg/kg.

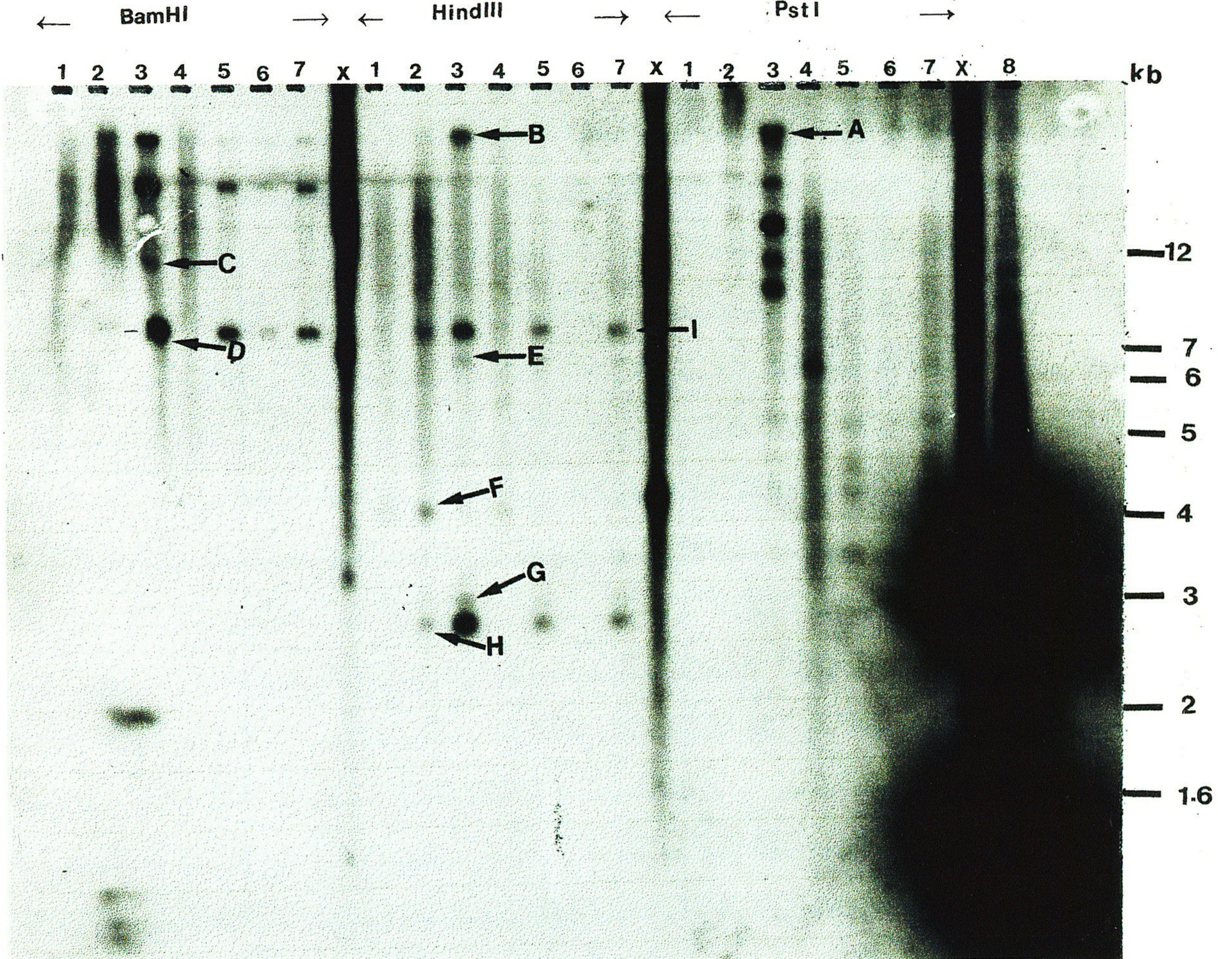
4 KETRI 2476 C1 resistant to suramin at a dose rate of 0.005 mg/kg.

5 KETRI 2476 C12 resistant to suramin at a dose rate of 5 mg/kg.

6 KETRI 2476 C18 resistant to suramin at a dose rate of 11 mg/kg.

7 KETRI 2476 C22 resistant to suramin at a dose rate of 15 mg/kg.

8 1kb molecular weight standard (Bethesda Research Laboratories, U.S.A).



BamHI
HindIII
Pst I
1 2 3 4 5 6 7 X 1 2 3 4 5 6 7 X 1 2 3 4 5 6 7 X 8
kb
A
B
C
D
E
F
G
H
I
12
7
6
5
4
3
2
1.6

Table 8.3 Bands found in the DNA of some *T. evansi* clones of KETRI 2454 and KETRI 2476 when hybridised with pTBRI and pTBR3 probes derived from *T. brucei* inserted in pUC 8

Band identity	Shown in Figure	DNA digested with	Approx. band sizes in kb.	Suramin resistance in mg/kg KETRI 2454 C1 0.005	C7 0.5	C9 2	KETRI 2476 C1 0.005	C11 5	C18 11	C22 15
A	8.13	Pst1	4.5-7	-	-	-	+	-	-	-
B	8.12	Hind111	8	+	-	-	-	-	-	-
C	8.12	Hind111	3.6	-	-	-	-	+	?	+
D	8.12	BamH1	3.4	-	+	+	-	+	+	+
E	8.12	Hind111	3.2	+	+	+	+	-	-	-
F	8.12	Hind111	2.8	+	+	+	+	-	-	-
G	8.12	Hind111	1.2	+	+	+	+	-	-	-

Table 8.4 Bands found in the DNA of some *T. evansi* clones of KETRI 2454 and KETRI 2476 when hybridised with pM7 probe derived from *Leishmania* inserted in pGEM 3.

Band identity	Shown in Figure	DNA digested with	Approx. band sizes in kb.	Suramin resistance in mg/kg KETRI 2454 C 1 0.005	C 7 0.5	C 9 2	KETRI 2476 C 1 0.005	C 11 5	C 18 11	C 22 15
A	8.14	Pst1	> 12	-	-	±	-	-	-	-
B	8.14	Hind111	> 12	-	-	±	-	-	-	-
C	8.14	BamH1	11	-	-	±	-	-	-	-
D	8.14	BamH1	7	-	-	±	-	±	±	±
E	8.14	Hind111	6.5	-	±	±	-	±	?	±
F	8.14	Hind111	4	±	±	-	±	-	-	-
G	8.14	Hind111	2.9	-	±	±	-	±	?	±
H	8.14	Hind111	2.8	-	±	±	-	±	?	±

8.4 DISCUSSION

The results of the work described in this chapter can be explained by the "mutation followed by selection" theory as the basis of the development of resistance. As the cloned starting material in the induction experiment was homogeneous in respect of suramin resistance, any subsequent difference in suramin sensitivity (and indeed in any other phenotypic occurrence) can only be accounted for by a mutational event. Selection of the mutants, either positively or negatively depending on the presence or absence of suramin, would then determine the relative proportions of the mutants within the total population. A single mutational event of this nature is thought to be sufficient for the multi-drug resistant phenotype in neoplastic cells (Ling, 1982).

DNA bands found in all clones irrespective of trypanosome strain could be parts of the genome that are characteristic of the species *Trypanosoma.* The second kind of bands which were observed in some clones of a particular stock (for example bands A, B and C in hybridisation with the ribosomal genes and the P-glycoprotein genes inserted in the respective vectors) may represent unique mutational events at particular points in time during the course of induction of resistance. These mutations were either not inherited or were selected out in subsequent trypanosome generations.

The third kind of bands which are important in the study of suramin resistance were those that were in strains within certain ranges of suramin resistance irrespective of the stock (bands C-G when hybridised with pM7 and bands D-H when hybridised with pTBR). These might represent mutations at particular points in the induction exercise which were inherited and selected for probably due to their importance in conveying a selective advantage in the presence of suramin. The fact that these bands were found in strains irrespective of the stock implies that they were in a position in the genome (locus) common to the two stocks KETRI 2454 and 2476.

The absence of bands with increased resistance to suramin (bands E, F and G in pTBR and band F in pM7) is suggestive of a deletion of DNA as resistance is induced. Such a deletion associated with induction of drug resistance was observed in *Trypanosoma cruzi*; where the amount of kinetoplast DNA, in relation to the total DNA, was reduced in phenotypes which were resistant to ethidium bromide as compared to their sensitive parents (Riou, 1976). The presence of bands associated with increased suramin resistance (band D in pTBR and bands D, E, G and H in pM7) suggest an addition of genetic material as trypanosomes developed resistance to suramin. This kind of genetic change would be as expected in a DNA amplification. Such amplifications of DNA have been linked to drug resistance of the mdr phenotype in human cells (Schimke 1978 *et al*), *Leishmania* (Beverley *et al* 1984) and *Plasmodium* (Foote *et al* 1989). Amplification of genetic material at the P-

glycoprotein locus is at times seen as chromosomal aberrations such as increased length of homogeneously staining regions -HSR- (Bradley *et al,* 1988). Amplification of P-glycoprotein genes has been observed to be the most consistent biochemical alteration in multi-drug resistant phenotypes in many species ranging from bacteria to human cells. This wide range is due the fact that the multi-drug resistance gene is highly conserved across species.

The fact that pM7 probe derived from *Leishmania* hybridised with the DNA in this study means that there is homology in the P-glycoprotein genes of *Leishmania* and *Trypanosoma.* This is expected since the two genera are close phylogenically, both being of the order *kinetoplastida.* It also not surprising that the ribosomal genes of *T. brucei* hybridised with many portions of test DNA for both *T. brucei and T. evansi* are in the same sub-genus and would have a high degree of homology. When test DNA was hybridised with the pM7 and the pGEM 3 vector, trypanosome bands D, E, G and H which were only found in trypanosome samples of a particular level of suramin sensitivity or higher might indicate homology between the suramin-resistant *T. evansi* and the P-glycoprotein gene of *Leishmania*; a linkage which may suggest that this gene is amplified in suramin-resistant trypanosomes. Such a linkage would be especially important in view of the observation that the P-glycoprotein gene, which codes for a membrane protein shown to be important in extrusion of drugs, is over-expressed in drug-resistant cells (Bradley *et al,* 1988; Riordan and Ling, 1979). An over-expression of this gene may be responsible for the decreased trypanocide accumulation observed in *T. brucei, T. rhodesiense* and *T. congolense* (Frommel and Balber, 1987; Sutherland *et al,* 1991, 1992). The study on *T. congolense* (Sutherland *et al,* 1992) indicated that resistance to Samorin was energy-dependent, a description fitting the P-glycoprotein pump observed in mdr phenotypes (Bradley *et al,* 1988) and suggesting further that drug accumulation in resistant trypanosomes is related to the P-glycoprotein gene. The fact that both the P-glycoprotein gene and the vector in which it was inserted were used for hybridisation, however, mean that it is not possible to distinguish if the bands in question hybridised either with the gene or the vector and thus the association between the bands and gene amplification in suramin-resistant *T. evansi* cannot be established.

There have been investigations on genotypic differences of drug-sensitive and drug resistant organisms. A ribosomal gene of *Schistosoma tropica* cloned into a plasmid has been used to compare DNA of strains of *Schistosoma* digested with Pst1, Hind111 and BamH1 (Brindley *et al,* 1989; Viera *et al,* 1991). Intraspecific differences were observed in the various strains investigated; some of which were isolated from patients who were not responding to chemotherapy. In another study of *Plasmodium* malaria, a locus on chromosome 7 was identified as being responsible for

parasites were shown to have amplified extrachromosomal DNA and a Bgl11 fragment observed in the drug-resistant parasites but not in the drug-sensitive ones (Beverley *et al,* 1984). In *Leishmania tarentole,* methotrexate-resistance has been associated with a DNA fragment believed to code for P-glycoprotein (Ouellette and Borst, 1991). In *Trypanosoma,* Riou (1976) compared EcoR1, Hpa11 and Hae111 kDNA fragments of ethidium bromide-sensitive and resistant *T. cruzi.* The results indicated that the proportion of kDNA in relation to total DNA content decreased from 22% in the drug-sensitive parasites to 16% in the resistant ones suggesting DNA deletion as resistance to ethidium bromide was acquired.

There are many phenotypic characteristics associated with drug-resistant trypanosomes; the most important being tolerance to trypanocidal dose levels which destroy drug-sensitive trypanosomes. Other characteristics are slower growth rates (Cantrell, 1956) and decreased drug accumulation in cells (Frommel and Balber, 1987; Sutherland *et al,* 1991, 1992). These phenotypic differences may be as a result of genotypic changes which might be detected as DNA changes such as gene amplifications. The work described in this chapter shows that there are DNA fragments present or absent in *T. evansi* clones which are resistant to suramin when compared to clones sensitive to this drug. Although the DNA fragments seen as bands are not consistent with the degree of suramin resistance induced, these results suggest that suramin resistance may be related to a mutational event whereby there is a change of nucleotide bases such as a point mutation, resulting into different DNA sequences and thus restriction sites for the restriction enzymes used. Since suramin has been shown to act on many different enzymes, it is possible that development of resistance to this drug could be associated with changes at the DNA level which result in a change of enzyme structure.

It is important to note that the association between DNA differences and suramin resistance observed in this study may have been due to other factors during the induction exercise such as trypanosome passage in mice. Furthermore, comparisons of suramin-sensitive and resistant parasites were done using DNA acquired from single isolations. Further investigations are necessary to eliminate the possibility of these factors and to determine whether the differences observed in this study are found also in the DNA of other stocks of known sensitivity to suramin. A study in which the P-glycoprotein gene without the vector would be used for hybridisation is an area of further study which would determine whether the differences observed in this study indicate complementarity with this gene; a linkage which would be important in elucidating further the mechanism of suramin-resistance. It would also be important to investigate the coding role of the DNA fragments associated with suramin resistance to determine whether they are related to phenotypic characteristics of drug resistance parasites such as drug accumulation and growth rates.

CHAPTER NINE

GENERAL DISCUSSION

Although resistance to trypanocidal drugs has been recognised for many years, the mechanisms of its development and maintenance in the field are not properly understood. The aim of the present study was to attempt to elucidate some of the underlying mechanisms which determine the expression of resistance or sensitivity in cameline stocks of *T. evansi*. The work described in this thesis has achieved the following principle observations:

1. Resistance to Berenil, Samorin, suramin and Trypacide is present in stocks of cameline *T. evansi* from Kenya. Resistance to suramin, the drug used for the longest period of time was found in 0% of the stocks isolated from four of the six areas sampled; resistance to Trypacide, the other drug used to control cameline *T. evansi* infections in Kenya, was found in 18% of the stocks isolated from three of the six areas sampled. Surprisingly, resistance to Berenil was widespread, occurring in 47% of the stocks from all of the areas from which trypanosomes were isolated. Resistance to Samorin was found in only 7% of the stocks isolated from two of the six areas sampled.
2. No correlation was found between the malic isoenzyme patterns of the stocks examined and resistance to either suramin, Trypacide, Berenil or Samorin.
3. The timing of suramin administration, and to a lesser extent, the number of trypanosomes inoculated, are important factors which influence the success of treatment of mice infected with *T. evansi*. Thus, early treatment and a small trypanosome inoculum dose result in higher cure rates.
   There is an inverse linear relationship between trypanosome dose and pre-patent periods of stocks of *T. evansi* inoculated in mice; the smaller the number of trypanosomes inoculated, the longer the pre-patent period.
4. It is easier to clone suramin-sensitive trypanosomes than those that are resistant to the drug.

6. Clones of *T. evansi* derived from single stocks have varying sensitivities to suramin. Some clones show a similar level of sensitivity to the parent stock but other clones are either more or less sensitive.
7. Suramin resistance can be induced readily in *T. evansi* strains by exposing them to sub-curative doses of the drug in mice; immunosuppression of the host facilitates this process.
8. The development of suramin resistance in a population of *T. evansi* is due to mutation and selection.
9. Induced suramin-resistance of *T. evansi* is stable in the short term for it does not wane after maintenance of the trypanosome in the absence of suramin for ten passages in mice.
10. Suramin-resistant clones of *T. evansi* grow more slowly than their sensitive parents; a phenomenon observed if the differences in their sensitivity to suramin is fairly large.
11. Infections with suramin-resistant trypanosomes in mice prevent subsequent super-infection with sensitive parasites. To a lesser extent, infections of suramin-sensitive trypanosomes prevent superinfections with resistant trypanosomes in mice and rats.
12. Suramin-resistant and sensitive *T. evansi* differ at the molecular level as seen by the presence or absence of particular DNA fragments. Thus, there are some DNA fragments which are associated with sensitivity to suramin within particular ranges irrespective of the *T. evansi* stock used.

The control by drugs of *T. evansi* infections in camels depends on the outcome of the complex interaction between the parasite, the host, the vector and the drug. *Trypanosoma evansi* infection in the camel occurs following inoculation of parasites by haematophagous biting flies and provokes an immunological response in the host animal. This response leads to the production of trypanosome-specific antibodies which destroy the parasites resulting in a release of trypanosomal antigens. However, due to antigenic variation, some trypanosomes survive to maintain the infection. When an infected animal is treated with a trypanocidal drug such as suramin, trypanosomes which are sensitive to that drug at the dose level used are destroyed. However, trypanosomes which are resistant to the drug survive and continue to thrive. The development of such drug-resistant trypanosomes which arise by mutation and are maintained by selective processes is facilitated by the maintenance of parasites to sub-curative drug dose levels in the infected animal. The time at which treatment is administered might also influence the development. Thus

when treatment is administered late in the infection, trypanosomes may evade the drug by sequestering in inaccessible sites such as the CNS.

The ideal method of determining the extent of *T. evansi* resistance would be the use of the natural host of the trypanosome species, the camel. It is, however, not always possible to carry out such studies due to technical and financial constraints. A better approach at determining drug resistance is by the use of the parasite itself. The DNA bands associated with suramin-resistance in this study could be investigated further and if this association applies to other trypanosome stocks, the DNA fragments could be developed into a probe which would detect suramin-resistance in infected blood samples. In the absence of these ideal methods, the resistance levels observed in this study, which was carried out in mice infected with *T. evansi,* suggest that there is widespread resistance to trypanocidal drugs in Kenya Under field conditions, such resistance levels would increase trypanosome infection rate in camels due to many cases of failed chemotherapy. It is not possible to extrapolate accurately the results of drug-resistance studies obtained from mice and conclude that there is widespread *T. evansi* resistance to trypanocidal drugs in infected camels in Kenya. Reports from the field suggest the situation is not as serious as it appears from experimental studies. The discrepancy observed between results of trypanocide resistance obtained from experimental studies like this one and from the real situation in the field may be due to the lack of a reliable formula which converts drug doses between animals of different species and weights. Moreover, the trypanosome stocks used in this study were isolated from infected camels and maintained by cryopreservation and passage in rodents for more than ten years; procedures whose effect on the sensitivity to drugs is not known. Another possible reason is a sampling bias whereby stocks isolated from the field tend to be those that have not responded to treatment. In the absence of a better system, the results obtained in this study are, however, an indication of the drug-resistance status of *T. evansi* in Kenya.

When there is widespread drug resistance, there is a need to introduce an alternative drug to which trypanosomes respond. In the Kenyan scenario, the introduction of cymelarsen, a newly developed trypanocide, would alleviate the problem of resistance to the two trypanocidal drugs commonly used for the control of cameline trypanosomiasis. If the conditions that promoted the development of resistance to the first trypanocidal drug persist, trypanosomes might also develop resistance to the alternative drugs resulting in a situation whereby trypanosomes are resistant to all available drugs. In the event of such a desperate situation, control of trypanosome infection would be left with the camel and the camel owner; the situation that existed originally before the introduction of drugs. The ability of some

camels to self-cure which was observed in this study, would be a selective advantage amongst animals exposed to natural infection; hence, camels with this trait would withstand infection better than those without it. Such a selective advantage would probably lead to the development of trypanotolerant camels. The camel owner would also place more importance on traditional methods of trypanosomiasis control which avoid animal/vector interaction by movement of animals and burning of shrubs which produce fumes that act as fly repellents in the animal enclosures, "bomas".

In spite of the fact that a vaccine would be the ideal approach for the control of trypanosomiasis, its development in tsetse-transmitted trypanosomes has been difficult due to antigenic variation. In *T. evansi* which has fewer VATs, however, such an a vaccine might be easier to develop from the major antigen types which would be recognised by a majority of trypanosome stocks. Such an approach would provide a solution to drug-resistance.

The ability of suramin to bind to plasma proteins is important for it results in a period of prophylactic activity of the drug. This characteristic, however, can lead to the development of suramin resistance for a gradual reduction of drug levels in the animal results in the exposure of trypanosomes to sub-curative drug doses; one of the ways in which resistance develops. In instances where a drug such as Berenil is eliminated quickly by the animal, there is no prophylactic period and thus little likelihood of development of resistance due to exposure of parasites to gradually decreasing drug levels. So, the high level of Berenil resistance probably did not develop by underdosing in the supervised ranch situations but may be due to reasons such as inefficacy of this drug in treating *T. evansi* as discussed in section 4.4.

The number of trypanosomes a particular vector transmits is important for this study has shown that a low trypanosome inoculum dose increases the chances of cure. This factor may be especially important for different fly species have been shown to have varying transmission efficiencies due to their feeding habits and size of their mouth parts (Foil and Issel, 1992). Thus, an animal may be able to control infection with a small number of trypanosomes compared to infection by a larger number. In experimental infections where transmission is by inoculation of trypanosomes by syringe, the large number of trypanosomes used may work towards the development of resistance by reducing cure rates and exposing parasites to drug doses which may not eliminate them. Such infections may also indicate drug resistance levels which are higher than would be the case in natural infections where the inoculated trypanosomes are probably fewer.

Immunosuppression of experimentally infected mice was shown to facilitate the development of suramin resistance. It is possible that the immunosuppressive nature of trypanosome and other infections such as helminthiasis together with stressful conditions on the camel such as malnutrition would contribute towards the development of drug resistance. Where resistance spreads to such levels that the small number of available drugs is not effective, then camel production will be threatened for the immune systems of animals would be overwhelmed and the animals would be unable to contain trypanosome infection.

Maintenance and spread of suramin resistance under field conditions might be influenced by differences in growth rates and interference between drug-sensitive and resistant trypanosomes. Hence, where both drug-sensitive and resistant parasites are found in a mixed infection, the slower growing drug-resistant trypanosome clones may be selected out thus decreasing their chances of transmission to the next animal host. Similarly, drug-sensitive trypanosomes may prevent drug-resistant ones from establishing infection and thus result in their being selected out. If a drug is used in such a mixed infection, drug-resistant trypanosomes may be at a selective advantage as drug-sensitive trypanosomes are selected out. In such a situation, the drug-resistant trypanosomes would be transmitted more readily and thus resistance would spread. It is possible that there are other factors which influence the development and maintenance of drug-resistant trypanosomes in a population as evidenced by the observation that suramin-resistant *T. evansi* can be found in camel herds which have not been exposed to suramin (R. Boid, personal communication). More investigations into the mechanisms of drug resistance and the characteristics of resistant parasites could elucidate this further.

The aim of chemotherapy is to control pathogens and contain infections to such a level that animal production is economical. However, when the distribution and administration of drugs is not properly controlled by qualified staff, this aim might not be realised for the drugs may be misused leading to the development and spread of resistant pathogens. This study has emphasised the importance of closely monitoring the use of the few trypanocidal drugs available to control camel trypanosomiasis in order to prevent the development and spread of resistant *T. evansi*.

It is my hope that the investigations carried out in the present study and reported in this thesis will increase knowledge resulting to a better use of not only

suramin, but other trypanocides as well. If by this knowledge, the control of cameline *T. evansi* will be better achieved and animal production increased leading to a better-fed Kenyan population, without degrading the environment any more than is necessary, then my labours will not have been in vain.

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APPENDICES

Appendix 1
Phosphate Buffered Saline glucose (PSG) pH 8.0

To make PS stock solution

Anhydrous Disodium hydrogen phosphate	14.38 g
Sodium dihydrogen phosphate	0.78 g
Sodium chloride	4.25 g
Add distilled water up to	1000 ml

To make PS

PS stock	6 parts
Distilled water	4 parts
Glucose to give 1% w/v	

Appendix 2
Diethylaminoethyl Cellulose (DE 52)

Suspend 200 g DE 52 in 1000 ml PSG
Wash by letting it stand and pouring off the PSG
Resuspend in PSG
Adjust pH to 8.0 with Phosphoric acid
Wash two times with PSG
Store in PSG at $4^{O}C$ until needed

Appendix 3
ELISA pre-coating buffer

Glutaraldehyde (50%)	20 ml
Add distilled water up to	1000 ml

Appendix 4
ELISA coating buffer pH 9.6

Sodium carbonate	1.59 g
Sodium bicarbonate	2.93 g
Sodium benzoate	0.1 g
Add distilled water up to	1000 ml

Appendix 5
ELISA washing buffer. PBS/0.005% Tween-20 pH 7.2

Sodium chloride	8.0g
Disodium hydrogen orthophosphate	1.4 g
Potassium dihydrogen orthophosphate	0.2 g
Potassium chloride	0.2 g

Tween 20	0.5 ml
Add distilled water up to	1000 ml

Appendix 6
ELISA blocking buffer pH 7.2.

PBS/0.005% Tween-20 with 1% bovine seurm albumin (BSA).

Appendix 7
ELISA substrate buffer pH 6.0.

Sodium acetate	8.2 g
Citric acid	266 mg
Add distilled water up to	1000 ml

Chromogen solution.

Tetramethylbenzidine (TMB)	50 mg
Dimethylsulphoxide (DMSO)	5 ml

Substrate.

Chromogen solution	100 µl
Substrate buffer	10 ml
3% H_2O_2	40 ml

Appendix 8
Giemsa's stain.

Add 7 g Giemsa's powder to 500 ml flask and shake until dissolved.
Keep at 60°C for two days with shaking twice a day.
Add 500 ml methanol.
Keep at 60°C for seven days with shaking twice a day.

Appendix 9
Giemsa's buffer pH 7.4

NaCl	30 g
Na_2HPO_4	4 g
Add distilled water up to	5000 ml

Appendix 10
Cocktail for lysing trypanosomes

Dithiothreitol (DTT)	8.088 g
Add 200 mM ethylenediamine tetraacetic acide (EDTA) pH 7.0 (7.445 g in 100 ml distilled water)	1.0 ml
Add Amino caproic acid (ACA)	0.0468 g

Appendix 11
Isoenzyme tank phosphate buffer pH 7.4

Na_2HPO_4	23.88 g
NaH_2PO_4	3.99 g

Appendix 12
Isoenzyme gel phosphate buffer

Isoenzyme tank buffer	30 ml
Distilled water	370 ml

Appendix 13
Isoenzyme developer

0.3M Tris-HCl pH 7.4	6.0 ml
1M Malic acid pH 7.0	0.2 ml
1M $MgCl_2$	0.2 ml
NADP	0.4 mg
Methyl thiazolyl tetrazolium (MTT)	10.0 mg
Phenazine methosulphate (PMS)	4.0 mg
Distilled water	3.6 mg
Agar	10.0 mg

Appendix 14
NET buffer

NaCl	2.922 g
EDTA	47.152 g
Tris	0.6055 g

Appendix 15
TE buffer pH 7.5

Tris	0.605 g
EDTA	0.186 g
Distilled water upto	1000 ml

Appendix 16
6x Gel-loading buffer

25% bromophenol blue
25% xylene cyanol
40% (w/v) sucrose

Appendix 17
TAE tank buffer

Tris	4.84 g
Glacial acetic acid	1.14 ml
0.5M EDTA pH 8.0	2.0 ml
Add distilled water upto	1000 ml

Appendix 18
DNA transfer buffer (20 x SSC) pH 7.0

Sodium chloride	175.3 g
Sodium citrate	88.2 g
2 x SSC	
Dilute 20 x SSC 10 times with distilled water	
Add distilled water upto	1000 ml

Appendix 19
a Denaturing solution

NaCl	87.7 g
NaOH	20 g
Add distilled water up to	1000 ml

b Neutralising solution pH 7.0

Tris	60.5 g
NaCl	120.0 g
Add distilled water up to	1000 ml

Appendix 20
STE buffer

0.1M NaCl
10mM Tris-Cl (pH 8.0)
1mM EDTA (pH 8.0)

Appendix 21
Sephadex for purifying probes

Sephadex	10 g
Sterile distilled water	160 ml
Equilibrate with TE buffer to pH 7.6	

Appendix 22
Hybridisation buffer

Deionised formamide	50 ml
20 x SSC Denhardt's solution	25 ml
Salmon sperm DNA 10 mg/ml	1 ml
1M sodium phosphate pH 8.0	2 ml
10 % SDS	2 ml
Distilled water	18 ml

Denhardt's solution

2 % polyvinylphrollidone
2 % Bovine serum albumin (BSA) 20 mg/ml
2 % Ficoll

Appendix 23
Ribosomal probe washing buffer 1

2 x SSC	40 ml
0.5 % sodium dodecyl sulphate (SDS)	10 ml
0.1 % Tetrasodium pyrrophosphate (NaPP)	8 ml
Add distilled water up to	800 ml

Appendix 24
Ribosomal probe washing buffer 2

0.1 SSC	4 ml
0.5 % SDS	20 ml
0.1 % NaPP	8 ml
Add distilled water up to	800 ml

Appendix 25
P-glycoprotein washing buffer 1

5 x SSC	80 ml
0.1 % SDS	20 ml
0.1 % NaPP	8 ml
Add distilled water up to	800 ml

Appendix 26
P-glycoprotein washing buffer 2

2 x SSC	4 ml
0.1 % SDS	20 ml
0.1 % NaPP	4 ml
Add distilled water up to	800 ml

Appendix 27

Determination of generation times of seven *T. evansi* stocks.

Strain KETRI no.	Rat no.	First wave of parasitaemia Trypanosome count Beginning x 10^6 / ml	lev	End x 10^6 / ml	lev	n	G
2454	1	0.25	5.40	37.5	7.57	7.2	3.6
	2	0.25	5.40	2.15	8.33	9.7	5.2
	3	2.0	6.30	190	8.28	6.6	4.9
	4	3.25	6.51	250	8.40	6.3	7.0
	5	2.0	6.30	210	8.32	6.7	7.5
2454 CL1	1	0.25	5.40	6.75	6.83	4.8	6.3
	2	1	6.00	275	8.44	8.1	6.4
	3	1.5	6.20	78	7.89	5.6	10.0
	4	0.50	5.70	20.5	7.31	5.4	5.9
	5	0.25	5.40	45	7.65	7.5	6.4
2454 CL9	1	10.5	7.02	267	8.43	4.7	16.2
	2	0.125	5.10	45.5	7.66	8.5	5.7
	3	2.0	6.30	210	8.32	6.7	7.5
	4	0.25	5.40	45	7.65	7.5	9.1
	5	1.50	6.18	11.25	7.05	2.9	8.3
2476	1	82	7.90	315	8.50	2.0	12.0
	2	1.25	6.10	230	8.36	7.5	6.7
	3	0.25	5.40	5.40	7.76	7.8	7.7
	4	0.50	5.70	20.5	7.30	5.4	5.9
	5	0.25	5.70	67.5	7.83	7.1	7.1
2476 CL1	1	2.25	6.35	33.5	7.52	3.9	5.1
	2	1.25	6.10	230	8.36	7.5	6.7
	3	0.5	5.70	49.5	7.70	6.6	6.1
	4	2.0	6.30	150	8.18	6.3	2.6
	5	1.5	6.18	11.25	7.05	2.9	8.3
2476 CL22	1	3.25	6.51	222	8.35	6.1	11.8
	2	0.25	5.40	190	8.28	8.0	9.0
	3	0.75	5.87	184	8.26	7.9	8.6
	4	1.25	6.10	328	8.52	8.0	8.7
	5	5.25	6.72	198	8.30	5.3	13.7
3136	1	1.25	6.10	2.15	6.30	0.66	2.9
	2	5.0	6.70	12.5	7.10	1.33	6.0
	3	0.75	5.90	40	7.60	5.7	8.4
	4	0.75	5.90	1.25	6.10	0.66	3.0
	5	1.0	6.0	1.5	6.20	0.66	6.1

Legend: lev- log equivalent which is the log^{10} of trypanosome counts per ml.
G - generation times.
n - number of generation

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Sensitivity of Kenyan *Trypanosoma evansi* to the trypanosomicidal drugs suramin and quinapyramine

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Suramin and quinapyramine sensitivities of 44 stocks of *Trypanosoma evansi*, isolated from camels in Kenya, were determined in mice at a dose rate of 5 mg/kg bodyweight. Seven of the stocks showing resistance to either or both drugs were further tested with suramin at dose rates of 4, 8 or 16 mg/kg bodyweight and with quinapyramine at 2, 4 or 7·4 mg/kg bodyweight. The results indicated that 16%, 25% and 4·5% of the 44 stocks were resistant to suramin, quinapyramine, or both drugs, respectively. Thus, of the two trypanosomicides, suramin, in spite of its long and widespread use for chemotherapy in camels, seems to be the more effective against *T. evansi*. Amongst the 7 stocks, little or no cross-resistance was observed between the 2 drugs. This observation, and the fact that only one isolate of the 44 showed multiple resistance, are important since suramin and quinapyramine are the only trypanosomicides which have been available and widely used to control camel trypanosomiasis in Kenya.

International Scientific Council for Trypanosomiasis Research and Control
21st Meeting Yamoussokro, Cote D'Ivore 1991

ABSTRACT

The efficacy of Suramin is dependent on parasite inoculum dose and timing of treatment.

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Three *Trypanosoma evansi* isolates: KETRI 3136, 2455 and 2454 reported to be resistant to Suramin at 80, 10 and 0.005 mg/kg bodyweight, respectively, were used to study the effect of trypanosome inoculum dose and treatment time to the efficacy of Suramin in mice. Three inoculum doses (10^3, 10^4 and 10^6 parasites per mouse) and five treatment times ((day 0, 1, 2 and at the onset of parasitaemia determined by microhaematocrit centrifugation (OMCT) or by wet blood film (OWF)) were used.

The results indicate that treatment was more effective at lower inoculum doses and at early treatment times. This was especially observed the more resistant an isolate was. It was important to note that a highly resistant isolate could be sensitive to Suramin at the lower inoculum doses and when treatment was administered early. The reasons for this may be due to parasite proliferation and invasion to the CNS.

In view of this results, it is suggested that an isolate's sensitivity to Suramin should be described at a particular parasite inoculum dose and drug treatment time. Studies should also be done to determine which of these results best represent the situation in the field.

First International Seminar on Non-Tsetse Transmitted Trypanosomoses
Annecy, France 1992

THE SEARCH FOR A DNA MARKER FOR SURAMIN RESISTANCE IN *TRYPANOSOMA EVANSI*

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Suramin has been the major trypanocide used for the chemotherapy and chemoprophylaxis of surra caused by *T.evansi* in camels. Resistance to Suramin has been reported and is indicated by failure of treatment. The mouse model has also been used to indicate resistance however extrapolation of rodent results to camels is not accurate. The ideal method would be a marker which would directly involve the parasite and therefore avoid the host effect.

Resistance in two Suramin sensitive strains was induced 400 and 3000 fold by using sub-curative doses. The DNA of the naive and induced resistant parasites was then compared. The DNA was first digested with three restriction enzymes; BamHI, Hind III and Pst1; blotted onto nylon membranes and hybridised with two ribosomal probes pTBR1 and pTBR3.

The preliminary results indicate that there are differences in the resistant and sensitive strains which are indicated by the presence or absence of bands. One group of bands observed are unique to either of the two isolates and appear to be strain specific. The other group of bands are found in Suramin sensitive or resistant parasites irrespective of trypanosome strain. In this second group, two bands appear to be specific to parasites resistant to Suramin at 2.0mg/kg bodyweight and above while another two bands appear to be present in parasites resistance to 2.0mg/kg bodyweight and below.

Although this work is preliminary, it is important in the search for a marker for Suramin resistance which might be used for field purposes.

Transactions of the Royal Society of Tropical Medicine and Hygiene (1990) **84**:171

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