DRUG-RESISTANCE AND GENETIC EXCHANGE IN *TRYPANOSOMA BRUCEI*

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SUMMARY

The aims of the work described in this thesis were to generate well characterised stable cymelarsan (melCy)-resistant and suramin-resistant lines of *Trypanosoma brucei*, and to use these lines to investigate the biochemical and genetic basis of drug-resistance. During these studies, different methods were used to assay drug-resistance in the cloned lines and these methods were critically compared to develop a rapid method for determining drug-resistance. MelCy-resistance was investigated by analysing the pattern of cross-resistance to other melaminophenyl arsenicals and two diamidines, and the role of altered drug uptake in the mechanism of melCy-resistance. Genetic exchange between drug-resistant lines provided information on the genetic basis of melCy-resistance and suramin-resistance.

Four drug-resistant lines were generated from two cloned stocks of *Trypanosoma brucei* (STIB 247 and STIB 386) by passaging trypanosomes in immunosuppressed mice in the presence of gradually increasing sub-curative drug doses. Drug-resistant populations were recloned, producing two melCy-resistant lines (247MelCy^R and 386MelCy^R) and two suramin-resistant lines (247Sur^R and 386Sur^R). *In vivo* assays in mice indicated that the levels of melCy-resistance had increased to greater than 40mg/Kg in both 247MelCy^R (130-fold increase) and 386MelCy^R (20-fold increase). Similarly, suramin-resistance was measured at 70mg/Kg for both 247Sur^R (47-fold increase) and 386Sur^R (35-fold increase). No cross-resistance between the two drugs was observed. The drug-resistant phenotypes in all four lines were stable upon passaging in mice for a number of weeks in the absence of drug pressure. Three lines (247MelCy^R, 247Sur^R and 386MelCy^R) were transmitted through tsetse flies, without attenuation of their levels of drug-resistance.

The levels of sensitivity/resistance to each drug were determined in the two unselected stocks (247 and 386) *in vitro* in procyclic culture forms and in short-term axenic bloodstream form cultures. Results were expressed in terms of the effective concentration that inhibited growth by 50% over twenty-four hours (EC_{50}). MelCy-resistance was expressed in procyclic and bloodstream culture forms. Suramin-resistance was not expressed in procyclic culture forms, with EC_{50} values of approximately 0.1mM for both the unselected and suramin-resistant lines, although suramin-resistance was expressed in bloodstream culture forms. There was no convincing evidence for the existence of cross-resistance between melCy and suramin in any of the drug-resistant lines *in vitro*. It was concluded that the short-term bloodstream culture was a suitable method for the rapid assessment of drug-resistance.

Both melCy-resistant lines expressed cross-resistance to melarsoprol (melB) and trimelarsen (melW) *in vivo* at the maximum doses tolerated in mice of 40mg/Kg. Procyclic culture forms of 247MelCy^R expressed resistance to melW and melarsen oxide (melOx), with increases in drug-resistance *in vitro* of 770-fold and 290-fold respectively. However, cross-resistance with melB was considerably lower in procyclic culture forms, with an increase in resistance from 247 to 247MelCy^R of only 2.3-fold. A similar pattern was seen with 386 and 386MelCy^R, with the melCy-resistant line expressing resistance to both melW (79-fold increase) and melOx (310-fold increase) but not to melB (1.3-fold increase) in procyclic culture forms. Furthermore, the melCy-resistant lines did not express melB-resistance in short-term bloodstream culture forms. It was concluded that melCy-resistance and melB-resistance may be brought about through different mechanisms.

The 247MelCy^R line was characterised with respect to its mechanism(s) of arsenical uptake, the relationship between arsenical transport and purine and

diamidine transport, and the alterations in uptake associated with melCy-resistance. Cross-resistance was observed in the 247MelCy^R line to the two diamidines, berenil and pentamidine, *in vivo*. The possibility that melCy, melB and melOx, together with berenil and pentamidine, shared a common adenosine transporter for entry into the trypanosome was investigated *in vitro*. Berenil and pentamidine were able to inhibit lysis by all three melaminophenyl arsenicals in a concentration-dependent manner. MelOx and melCy-induced lysis of 247 were inhibited in a concentration-dependent manner by adenosine and adenine, in agreement with the proposal that these arsenical drugs compete with these purines for transport (Carter and Fairlamb 1993). However, melB-induced lysis could not be blocked by adenosine or adenine, suggesting that melB did not share this adenosine transporter. Furthermore, the melCy-resistant line, 247MelCy^R, showed a marked decrease in susceptibility to melOx and melCy-induced lysis and only a very slight decrease in susceptibility to melB-induced lysis, suggesting that the mechanism(s) of melCy-resistance may differ from those of melB-resistance. Procyclic culture forms of 247 could not be lysed by maximal concentrations of the arsenical drugs over one hour. The adenosine transport system in 247 and 247MelCy^R were further characterised by conducting tritiated adenosine uptake studies. It was proposed that at least four distinct adenosine transporters existed, one of which (termed P2) was responsible for the uptake of melCy and melOx, and that the uptake of lipid soluble melB was either by diffusion or by an as yet unidentified route.

MelCy-resistant and suramin-resistant lines were used in co-transmission experiments to generate hybrid progeny that expressed resistance to both melCy and suramin. Double drug-resistant (putative hybrid) trypanosomes were identified *in vivo* in five metacyclic populations by allowing flies producing mature infections to feed on mice, although no clones were isolated. A single three-way co-transmission experiment was also conducted with 247Sur^R,

247MelCy^R and 386MelCy^R. Twenty-three trypanosome clones were generated from flies producing double drug-resistant infections in mice. Eight of these clones expressed resistance to both melCy and suramin *in vivo*. Four of these clones also expressed melCy-resistance and (partial) suramin-resistance *in vitro*. No evidence of genetic exchange (including self-fertilisation) could be found from isoenzyme and karyotype analysis of these cloned progeny. It was concluded that the double drug-resistant clones were likely to be the products of selfing although the limited number of markers available to detect self-fertilisation were unable to provide supporting evidence for this conjecture.

In conclusion, cloned drug-resistant lines of trypanosomes from well characterised genetic backgrounds can be successfully employed as tools to investigate the inheritance of drug-resistance and genetic exchange. Furthermore, the possibility that melCy does not share a common transport system with melB suggests a potential role for melCy in the treatment of some melB-resistant cases of sleeping sickness.

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LIST OF ABBREVIATIONS

[^{3}H]-hypoxanthine	Tritiated hypoxanthine
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BAL	British Anti-Lewisite
Balb/C	Inbred strain of mice
BSA	Bovine serum albumin
B.S.C.M.	Bloodstream culture medium
$CaCl_2$	Calcium chloride
CBSS	Carter's balanced salt solution
CD1	Outbred strain of mice
CFLP	Outbred strain of mice
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
$dddH_2O$	Double-distilled de-ionised water
DFMO	α-difluoromethylornithine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration inhibiting growth by 50%
EDTA	Ethylenediaminetetraacetic acid
F_1	First filial generation
FCS	Foetal calf serum
g	Acceleration due to gravity
G418	Geneticin (neomycin)
GUP	Glasgow University, Protozoology
H_2O_2	Hydrogen peroxide
HIFCS	Heat-inactivated foetal calf serum
HIV	Human immunodeficiency virus
hph	Hygromycin-resistance gene
ICD	Isocitrate dehydrogenase
i.p.	Intraperitoneal
K_a	Association constants
kb	Kilobase
KCl	Potassium chloride
K_i	Dissociation constant of enzyme-inhibitor complex
K_m	Michaelis constant
LDL	Low density lipoprotein
LMA	Low Melting Point Agarose
Mb	Megabase
ME_B	Malic enzyme B
MelB	Melarsoprol
MelCy	Cymelarsan
MelOx	Melarsen oxide
MelT	Adduct of melarsen oxide and trypanothione
MelW	Trimelarsen
$MgSO_4$	Magnesium sulphate
NaCl	Sodium chloride

Na_2HPO_4	Di-sodium hydrogen phosphate
NaOH	Sodium hydroxide
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
neo	Neomycin-resistnce gene
OD_{750}	Absorbance at 750nm
ODC	Ornithine decarboxylase
PBS	Phosphate-buffered saline
PFGE	Pulsed-field gel electrophoresis
PDT	Mean population doubling time
PSG	Phosphate-buffered saline with glucose
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDM-79	Semi-defined medium (1979)
STIB	Swiss Tropical Institute, Basel
TBE	Tris-borate EDTA buffer
TLB	Trypanosome Lysis Buffer
TO Swiss	Outbred strain of mice
TREU	Trypanosomiasis Research, Edinburgh University
$Try[SH]_2$	Trypanothione
Tyr^3	Tyrosyl-tyrosyl-tyrosine peptidase
VAT	Variable antigen type
VSG	Variable surface glycoprotein
WHO	World Health Organisation

DECLARATION

The results presented in this thesis are my own work except where there is an explicit statement to the contrary.

Some of the isoenzyme analyses of the cloned progeny described in chapter 5 were carried out by Norma Buchanan, W.U.M.P., Glasgow University. Some of the metacyclic populations from infected flies described in chapter 5 were screened in mice by Colin Chapman, W.L.E.P., Glasgow University.

Some of the work in chapter 2 has been published as a meeting abstract: Scott, A., Turner, M. & Tait, A. (1991) The development and characterisation of stable drug-resistant lines of *Trypanosoma brucei* *Annales de la Societe Belge de Médecine Tropicale (supple. 1)*, **72**, 107

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CHAPTER 1
GENERAL INTRODUCTION

1.1 TRYPANOSOMES

1.1.1 Introduction

The genus *Trypanosoma* covers a large range of parasitic protozoa found throughout the world in a variety of both vertebrate and invertebrate hosts. Most species live in the body fluids and tissues of their host without any apparent pathological consequences, but there are a few species that are responsible for a range of diseases collectively known as the trypanosomiases. Of these pathogenic species, it is the tsetse-transmitted trypanosomes of sub-Saharan Africa and the reduviid-transmitted trypanosomes of South America that cause most concern to mankind. In terms of the total number of humans infected, *Trypanosoma cruzi*, the causative agent of Chagas' disease, is by far the most important species with at least 20 million people infected in Central and South America each year. In Africa the annual rate of human infection by trypanosomes is much lower. An estimated 25 000 new cases of sleeping sickness, caused either by *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense*, are reported each year (WHO 1986a), all of which are invariably fatal unless treated. However, the African problem can not be so easily quantified (see review by Kuzoe 1993), since it is not only the direct effect on humans that causes concern, but also the indirect effect trypanosomiasis can have on humans, such as on the domestic livestock, upon which so much depends. Domestic animals become diseased when infected with *T. congolense* and *T. vivax*, which like *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, are transmitted by the tsetse fly. Animal trypanosomiasis, "nagana", is one of the biggest economic problems of African livestock farming, with an immeasurable cost in terms of loss of production, wasting and infertility. The effect upon rural farming is confounded with the fact that the most fertile and well vegetated areas are often

the most ideal habitats for the tsetse, and are therefore avoided by humans and their domestic animals (Jordan 1986a).

The different species of mammalian trypanosomes that have just been mentioned do not all share a common life cycle. Of the species that are pathogenic to mankind and its livestock, most are transmitted by an insect vector, although in some cases, transmission can also occur through other means, such as the venereal transmission between horses of *T. equiperdum*. The insect transmitted trypanosomes can be divided into two groups depending on the method by which transmission from the vector to the new host occurs. These two groups are the *Salivaria* and the *Stercoraria*, with trypanosomes of the former group being transmitted through the insect's saliva as opposed to the latter group in which transmission occurs through the insect's faeces. *T. cruzi* belongs to the *Stercoraria* which is distinguished by having its mammalian-infective forms (metacyclics) in the faeces and urine of the insect vector, the reduviid bug. Transmission is via contamination of a wound, often the insect bite. With the salivarian trypanosomes such as *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. congolense*, and *T. vivax*, transmission occurs through the mouthparts of the insect vector with the metacyclic trypanosomes being present in the saliva. However, there are substantial differences in the development of these trypanosome species in the tsetse fly (see Vickerman *et al.* 1991). For example, whereas both *T. brucei* and *T. congolense* develop in the tsetse midgut before migration to the insect's mouthparts, *T. vivax* development is totally in the mouthparts. *T. evansi* is also a salivarian trypanosome, although transmission differs from that of the tsetse-transmitted trypanosomes in that cyclical development does not occur at all in the insect, and syringe-like, or mechanical transmission occurs from the insect's saliva without the existence of metacyclics.

This study is concerned solely with the tsetse-transmitted trypanosomes of sub-Saharan Africa, and in particular those of the *T. brucei* species. The *brucei* group of trypanosomes consists of three subspecies, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. They are morphologically indistinguishable from each other but can be distinguished in a number of other ways, not least by the nature of the diseases they bring about. *T. b. brucei* is non-infective to humans, and can be distinguished from the other two by its sensitivity to human serum (Rickman and Robson 1970). The two human-infective subspecies, *T. b. rhodesiense* and *T. b. gambiense*, can be distinguished from each other by the course of human infection, with *T. b. rhodesiense* bringing about an acute infection compared to the more chronic infection of *T. b. gambiense*. Furthermore, *T. b. rhodesiense* and *T. b. brucei* are transmitted by *Glossina morsitans morsitans*, *G. m. centralis* and *G. pallidipes* whereas *T. b. gambiense* is transmitted principally by *G. palpalis*, *G. fuscipes* and *G. tachinoides* (Jordan 1986b). Biochemical and molecular analysis of the three different subspecies have shown that *T. b. rhodesiense* and *T. b. brucei* are also closely related with respect to isoenzymes (Tait *et al.* 1985). However this close relationship does not extend to *T. b. gambiense* (Gibson *et al.* 1980; Tait *et al.* 1984). Studies on DNA polymorphisms in ribosomal RNA genes further indicated the close relationship between *T. b. brucei* and *T. b. rhodesiense* and the relative distance of these from *T. b. gambiense* (Hide *et al.* 1990). Despite the fact that on this evidence it is possible to consider *T. b. gambiense* as a separate species, for the purpose of the present study, it will be regarded, together with *T. b. brucei* and *T. b. rhodesiense*, as subspecies of *T. brucei*. References to individual subspecies will therefore be made as *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*, and general comments for the species as a whole will be made as *T. brucei*.

1.1.2 Life Cycle of *T. brucei*

Trypanosomes of the *T. brucei* species share a common life cycle, with development in the mammalian host being followed by transmission in an insect vector to a new host. These salivarian trypanosomes have a life cycle that consists of at least six distinct stages that differ not only in morphology, but also in their biochemistry, particularly their energy metabolism (Opperdoes 1985). The life cycle of *T. brucei* has been well studied (reviewed in Vickerman 1985; Vickerman *et al.* 1991) and is of direct relevance to this study, which involves *T. b. brucei* and *T. b. rhodesiense*. Figure 1 shows the life cycle divided into three different environments (mammalian host, tsetse midgut and tsetse salivary gland). Each environment has two life cycle stages: a dividing form to establish the infection in the current environment, and a non-dividing form that acts as a link from one environment to the next. For simplicity, this life cycle does not include the many intermediate forms previously described (Vickerman 1985), nor does it make any reference to the occurrence of any sexual stage, which will be dealt with later **(section 1.3.2)**.

Briefly, a mammalian host initially becomes infected by non-dividing metacyclic trypanosomes when an infected tsetse fly takes a blood meal. The mature metacyclics in the fly's saliva pass into the mammalian tissues and drain into the blood via the lymphatics. The metacyclics transform to long-slender bloodstream forms which divide rapidly and establish the infection in the host (Figure 1). Long-slender forms also transform to short-stumpy bloodstream forms. These cannot divide and have a finite lifespan, but unlike long-slender forms they are pre-adapted to be able to establish a new infection in a tsetse fly's gut, when a fly takes a blood meal from the infected mammal (Vickerman 1965). The short-stumpy trypanosomes are ingested with the blood meal into the tsetse gut where they transform to procyclics. Procyclics divide and establish a very

dense midgut infection. A salivary gland infection becomes established some weeks after the midgut has been infected. Procyclics transform to the non-dividing proventricular forms that travel up the fly's alimentary canal to the proboscis and then into the salivary gland (Evans and Ellis 1983). The salivary gland infection then becomes established when the proventricular forms transform to dividing epimastigotes that attach themselves to the gland's epithelium. Finally the cycle is completed with the epimastigotes, which can not survive in the mammalian host, transforming to the non-dividing metacyclics (Tetley and Vickerman 1985), which are free in the saliva and can withstand the mammalian host's defences, thereby completing the life cycle upon their entry into a new mammalian host.

1.1.3 Pathology of Human Sleeping Sickness

Humans are susceptible to infection by *T. b. rhodesiense* and *T. b. gambiense*. Infection by either will ultimately bring about death if the disease is left untreated. The pathology associated with early-stage and late-stage sleeping sickness (CNS involvement) is reviewed in Poltera (1985). Briefly, infections of both species begin in the same way, with metacyclic trypanosomes entering the dermis, causing a local inflammatory response at the site of infection and resulting in a noticeable swelling on the skin - the trypanosome-containing chancre (Barry and Emery 1984). From this chancre, the trypanosomes drain into the bloodstream via the lymphatics. Upon establishment of a bloodstream infection, the characteristic fluctuations in parasitaemia can be observed. These parasitaemic waves are brought about by the interaction of the host's immune response with the ability of the long-slender forms to change their surface antigen approximately once every 100 cell divisions (Turner and Barry 1989). Trypanosomes are coated with more than 10^7 copies of a single molecular species of variant surface glycoprotein (VSG) which is present only on

metacyclics and bloodstream forms (Cross 1990). This protective coat not only protects the trypanosome against non-specific immune responses, but also the structure of these VSGs determine the variable antigen type (VAT) of the trypanosome. The long-slender forms have both the ability to divide rapidly and switch their VSG, and consequently their surface antigen, thereby evading the host's specific antibody response, which can only eliminate parasites possessing the VAT against which the antibodies were produced (Vickerman 1969). It is therefore the long-slender forms, able to evade the host's immune response, that are responsible for the gross pathology and ultimate death of infected individuals.

Bloodstream infections have an adverse effect on the blood and vascular system of infected individuals and they are frequently associated with anaemia (Poltera 1985; Jenkins and Facer 1985). Although some damage must undoubtedly be due to the direct invasion of the tissue by the parasite, much of the damage has been attributed to adverse immune reactions. A large increase in B-cell proliferation invariably accompanies infection and is usually accompanied with a reduction in the T-cell response, although the significance of these with respect to their pathological consequences remain unclear (Vickerman *et al.* 1991). It has been proposed that the large-scale destruction of trypanosomes results in the release of a vast number of trypanosome antigens that form immune complexes with circulating antibodies. These immune complexes could play an important role in the pathology of the disease (Galvao-Castro *et al.* 1978; Boreham 1985) and have been found in the blood and CSF of human patients (Lambert *et al.* 1981). Complement activation by the immune complexes, and the subsequent cellular response has been shown to cause damage to host tissues, such as heart and skeletal muscle and the brain (Morrison *et al.* 1983). The migration of the long-slender bloodstream forms from the blood into other body fluids and host tissue (such as the lymph, the

CSF and particularly the brain) not only causes the disease to spread into various host organs but it also causes problems with drug therapy, as some of these areas are partially, if not totally, inaccessible to a number of drugs (Gutteridge 1985). The migration and spread of the dividing forms from the blood is important in both the acute infections of *T. b. rhodesiense* and the chronic infections of *T. b. gambiense*, although the protracted nature of the Gambian form of the disease lends itself to a more extensive spread of the trypanosome infection in the host. There are reports, using experimental models, of trypanosome infiltration into a wide variety of organs and tissues with trypanosomes having been observed in the tissues of the heart, lungs, liver, spleen, kidneys, pancreas, stomach, small intestine and numerous endocrine tissues (reviewed in Poltera 1985).

The effect of trypanosomiasis on extra-vascular sites is arguably most significant in the CNS. Late-stage sleeping sickness is defined when parasites have entered the CNS with the resulting symptoms varying from a slight headache to coma (Giordano *et al.* 1977). As with the infections outside the CNS, trypanosomes have been found in a number of locations in and around the brain (Poltera 1985). It would appear that CNS involvement can occur fairly early in the infection and that it gradually worsens and spreads through the CNS as the infection develops. This may account for some of the relapse infections that occur after melarsoprol treatment, which can pass the blood-brain barrier, but may not be able to reach high enough levels at all the sites of infection in the brain, with a number of drug barriers existing within the brain rather than a single one at the blood-brain barrier (Raseroka and Ormerod 1986). This may be extremely important during arsenical treatment of late-stage infections. The rapid destruction of trypanosomes by melarsoprol will free a huge quantity of trypanosome antigens, and it has been proposed that the adverse effects of the host's immune reactions in response to this release of antigens actually leads to

arsenical encephalopathy (Poltera *et al.* 1984).

1.1.4 Metabolic Differences Between Life Cycle Intermediates and the Mammalian Host

The change in the trypanosome's surface coat to protect it against the host's immune response has already been dealt with. There are a number of equally important adaptations made by *T. brucei* in order to survive in the different environments encountered in the life cycle. Particularly important are the changes in energy metabolism between the insect-stages and the mammalian-stages of the parasite's life cycle (Bowman and Flynn 1976). The mitochondrion of long-slender forms is repressed (Vickerman 1965) and they do not possess a functioning electron-transport chain to generate ATP. They use glucose as their sole source of energy, absorbing glucose from the blood and generating energy through glycolysis carried out in the spherical glycosome, after which pyruvate is then excreted. The glycosome is an organelle unique to Trypanosomatids and it contains all the trypanosome's glycolytic enzymes, the opposite of the situation found in all other eukaryotes in which the glycolytic machinery is present in the cytosol (Opperdoes and Borst 1977). Glycolysis in *T. brucei* also differs from mammalian glycolysis in the way NAD is regenerated. Under anaerobic conditions in mammalian cells, NADH produced during glycolysis is oxidised during the production of lactate from pyruvate. However, in *T. brucei*, NADH from glycolysis is oxidised to NAD indirectly by a unique glycerol-3-phosphate oxidase located in the mitochondrion (Grant and Sargent 1960). These marked differences, together with the fact that a number of the trypanosomal glycolytic enzymes have different kinetic properties to their mammalian equivalents, make glycolysis an ideal target for chemotherapy (Opperdoes 1983).

Due to the relative lack of glucose and abundance of proline in the tsetse

midgut, procyclic trypanosomes utilise this amino acid as their primary energy source. Their ability to oxidise proline coincides with a well developed mitochondrion (Bowman and Flynn 1976) and a complete cytochrome electron-transport chain appears for the production of ATP from oxidative phosphorylation (Njogu *et al.* 1980). Furthermore, the limited amount of oxygen in the tsetse gut, together with the rapid disappearance of glucose from an ingested blood meal in an uninfected fly, is catered for by the reduction of the glycosome from a spherical organelle to an ovoid one, and the decrease in activity of some key glycolytic enzymes (Hart *et al* 1984). Short-stumpy forms, the link between long-slender forms and procyclics, are bloodstream forms that possess some of the biochemical peculiarities of procyclics, in preparation for life in the fly's midgut. Short-stumpy forms have an enlarged mitochondrion when compared to long-slender forms and proline oxidase activity appears in preparation for an amino acid based energy source (Vickerman 1965).

Relatively little is known about the metabolism of proventricular forms and epimastigotes, although it appears that the mitochondrion remains enlarged and the glycosomes are suppressed throughout the life cycle in the fly until a short time before the metacyclics detach from the salivary gland epithelium and become mature metacyclics in the gland's lumen (Vickerman 1985). While still attached to the gland, immature metacyclics acquire a VSG coat, the mitochondria regress and the glycosomes become spherical once more (Tetley and Vickerman 1984). Thus upon detachment, the mature metacyclics possess a protective coat and a metabolism which once again is adapted for life in a glucose rich environment.

1.2 CHEMOTHERAPY

1.2.1 Introduction

A concerted effort has been made to control the problem of African trypanosomiasis, and the problem has been tackled in four ways, each with varying degrees of success. Firstly, avoiding tsetse-infested areas, although an obvious solution, is of little help to the rural African, since the high risk areas are often the best grazing land, with the tsetse fly inhabiting the less arid, more vegetated areas (Jordan 1986a). Secondly, a solution to the problem of animal trypanosomiasis could come from the increased use of stock resistant to trypanosome infection (Murray *et al.* 1979). However, such a solution does nothing to alleviate the human disease of sleeping sickness as such animals would still provide a reservoir host for the human-infective trypanosomes. A third method of control is by controlling the tsetse fly vector. A large amount of effort has been put into this approach with a degree of success, particularly with the use of insecticides and fly traps (Jordan 1986c). Finally, the last and most widely used method of controlling the human disease still lies with the use of trypanocidal drugs, used either to treat infected individuals or to prevent infection by prophylaxis. Although almost a century of research has been carried out into the development of drugs for the treatment of human sleeping sickness, only four (suramin, melarsoprol, pentamidine and DFMO) are routinely used in Africa. The development, mode of action and treatment regimes of each will be dealt with separately, together with the various drawbacks associated with their use.

1.2.2 The History of Trypanocide Evolution

Suramin

The turn of the century saw the first real beginnings of experimental

chemotherapy for the treatment of parasitic diseases, particularly for the protozoal infections of plasmodia and trypanosomes (reviewed in Williamson 1970). Work was pioneered by Paul Ehrlich, following on from his initial research into the selective uptake by cells of certain synthetic cotton-dyes, and the use of these for studies on histology and, in particular, haematology. The cytotoxic effect of some of these dyes, together with the fact they were taken up at different rates by different cells, brought Ehrlich to the logical conclusion that they could be used as the basis for selective chemotherapy, and this soon led to the subsequent discovery, in 1891, of the antimalarial property of Methylene Blue (Hawking 1963a). By the turn of the century, Ehrlich had focused his attention on trypanosomes, as they provided a convenient laboratory model with which he could standardise his findings. In his search for trypanocidal compounds, and with the help of his chemist colleagues, he screened hundreds of synthetic cotton-dyes based on Congo Red, using his standard mouse model infected with *T. equinum*. In 1904, he found the first trypanocidal signs in Nagana Red, from which a more water soluble form called Trypan Red was synthesised (Hawking 1963b).

The subsequent years saw the application of a large amount of effort into the gradual improvement of Trypan Red into a less toxic and more active compound. By 1907, Mesnil and Nicolle from the Institut Pasteur had taken Ehrlich's earlier work a step further, and produced Trypan Blue (Tréfouel 1962). This was then improved further to a colourless analogue by a German firm, Bayer, with whom Mesnil and Nicolle had previously been collaborating. Ultimately, Bayer refined their product and in 1916 produced suramin (Bayer 205, Germanin). Suramin has since proved to be invaluable and is still the first drug of choice used to treat early-stage sleeping sickness, especially in East and Central Africa, and is very occasionally used for prophylaxis (Gutteridge 1985).

Arsenicals

At the same time that suramin was being developed, the potential of arsenical drugs was also being investigated. It had already been shown some decades previously by Livingstone, that "two grains of arsenic in a little barley" had a beneficial effect on a tsetse-bitten mare (Livingstone 1858), although the parasitic nature of nagana was not then known. It was almost another forty years until Bruce, in 1896, showed the susceptibility of trypanosome infections to sodium arsenite and arsenious oxide. In 1902, Lavaren and Mesnil, demonstrated the action of sodium arsenite in their mouse model infected with *T. brucei*, but it was universally accepted that although inorganic arsenic was effective, it was too toxic for the treatment of human sleeping sickness (Hawking 1963a). A revolutionary breakthrough came in 1905, when Wolferston Thomas, working in Liverpool, used an organic rather than an inorganic arsenical to treat trypanosomiasis. This organic arsenical, atoxyl, was forty times less toxic than sodium arsenite, while still retaining the latter's activity against trypanosomes (Hawking 1963a). Atoxyl had been produced a few years earlier as a by-product of parafuchsin synthesis, which was itself a highly active trypanocide although being quite unrelated to atoxyl. The trypanocidal action of atoxyl had at first been overlooked by Ehrlich, who in 1903, had discarded it as having no trypanocidal activity in the "test tube". However, by 1908, Ehrlich and others had independently shown that atoxyl and other pentavalent arsenicals, must first be converted to their trivalent form to become active against trypanosomes, and that this reduction occurred *in vivo* (Himmelweit 1960).

The next major development came in 1919, with the introduction of tryparsamide by Jacobs and Heidelberger from the Rockefeller Institute, New York. Curiously, tryparsamide seemed to have limited success against the early-stages of the disease, but it was the first drug to be able to pass into the CSF and

thus cure late-stage Gambian sleeping sickness (Williamson 1970). It also had the further advantage of being less toxic than atoxyl, which had been shown to have undesirable side effects, especially on the optic nerve. Unfortunately, tryparsamide was of no use against the still incurable late-stage Rhodesian sleeping sickness. Furthermore, its extensive use as the only late-stage cure for the Gambian disease, resulted in widespread tryparsamide-resistance, to the extent that in some areas of West Africa, 100% of all cases were reported tryparsamide-resistant. This occurred at a time when 60% of the 50 000 newly reported cases were already late-stage sleeping sickness (Van Hoof 1947).

The introduction of Friedheim's melaminophenyl arsenicals was therefore a very welcome development in the treatment of trypanosomiasis. Melarsen and its reduced equivalent, melarsen oxide, introduced by Friedheim in 1940, proved to be successful in curing tryparsamide-resistant cases (Friedheim 1948). However, late-stage Rhodesian sleeping sickness was still incurable, and like other arsenicals, melarsen and melarsen oxide were still fairly toxic. This toxicity was reduced in 1949 by Friedheim and Vogel with the addition of British Anti-Lewisite (BAL) to melarsen oxide to produce melarsoprol (melB, arsobal) (Friedheim 1949). BAL, which was originally produced as an antidote to an arsenical warfare agent, allowed a much greater dose of arsenical to be given and consequently, for the first time even late-stage Rhodesian sleeping sickness could be treated. Ten years after the introduction of melarsoprol, Friedheim produced a water soluble alternative in trimelarsen (melW) (De Jongh and Friedheim 1959), and in the last decade, cymelarsan (melCy, RM 110) has also been introduced by Rhône Merieux (Zweygarth and Kaminsky 1990). However, extensive field trials of these last two compounds have not been carried out and they initially appear to have no clear advantage over the established cure of melarsoprol. Consequently, melarsoprol remains the only arsenical in use today for the routine treatment of the late stages of both Rhodesian and Gambian

forms of the disease.

Pentamidine

The only drug that has been proven to be successful as a large scale prophylactic is pentamidine (Demarchi 1958). In the mid 1930s, investigations were carried out into the effect of lowering blood sugar to combat trypanosome infections. Compounds were developed from synthetic insulin analogues, resulting in 1942 with the introduction of pentamidine, which, curiously, does not owe its trypanocidal activity to any hypoglycaemic action (Williamson 1970). In fact, hypoglycaemia resulting from pentamidine treatment is regarded as an unfortunate side-effect. Following its introduction, large scale use of pentamidine as a prophylactic was carried out in West Africa with a large amount of success, although the end of European influence in Africa led to a disintegration of an organised system of routine prophylaxis (Jordan 1986d). The main drawback with pentamidine is that it can not pass the blood-brain barrier and consequently is useless in cases with CNS involvement. However, it still remains highly effective in prevention and cure of early stage sleeping sickness.

DFMO

In recent years many experimental compounds have been screened for their potential as anti-trypanosomal agents. Of these, with the possible exception of nifurtimox (Pepin *et al.* 1989) only the anti-cancer drug, α-difluoromethylornithine (DFMO) has proved to be of any major significance. The last 20 years has seen a large amount of interest in the mechanisms of polyamine metabolism of eukaryotic cells. Polyamines are present in all living cells and are vital for cell differentiation and division. A key enzyme in the biosynthesis of polyamines was found to be ornithine decarboxylase (ODC),

which could be irreversibly inhibited by α-difluoromethylornithine (DFMO), a synthetic analogue of its true substrate, ornithine (Metcalf *et al.* 1978). Since trypanosomes rapidly divide, investigations were begun into the potential of DFMO as a trypanocidal drug, with the initial findings that mice infected with *T. b. brucei* or *T. b. rhodesiense* could be cured by DFMO treatment (Bacchi *et al.* 1980; McCann *et al.* 1981a). Further studies showed that DFMO was able to cure late-stage infections of *T. b. gambiense* in human patients that had relapse infections after melarsoprol treatment (Sjoerdsma *et al.* 1984). However, in clinical trials, DFMO has had limited success against *T. b. rhodesiense* (Van Nieuwenhove 1992). Although the expense of DFMO treatment and the large quantities required for a full course of treatment have put some question marks over its future, it is arguably the most important drug to have emerged since Friedheim's melaminophenyl arsenicals.

1.2.3 Administration, Uptake and Mode of Action of Trypanocides

Suramin

Suramin has been used as a trypanocide for almost eight decades, and is still a first choice drug in the treatment of early cases with no CNS involvement. It is effective against both *T. b. rhodesiense* and *T. b. gambiense* as well as *T. b. brucei*, *T. evansi* and *T. equiperdum*, although relatively ineffective against *T. vivax* and *T. congolense* (Hawking 1963b). Furthermore, it has had some success as an anti-cancer drug (Allolio *et al.* 1989), in the treatment of HIV infections (Mitsuya *et al.* 1984) and also onchocerciasis (Hawking 1978).

The actual treatment regime for human trypanosomiasis with suramin may vary depending on the health of the patient. Generally, a number of doses of up to 20mg/Kg are given intravenously over a 3 week period, with a possible further weekly dose for the next 5 weeks. Suramin binds tightly to serum

proteins (Gutteridge 1985) and it has been recently found that 15% of this bound suramin is in fact attached to the apoprotein of LDL (Vansterkenberg *et al.* 1993). Although about 80% of the suramin administered is cleared by the kidneys, this renal clearance is fairly slow, with suramin having a serum half-life of between 44 and 54 days (Collins *et al.* 1986). As a result of this, a single dose of suramin can give protection against trypanosomes for up to 3 months. Suramin becomes distributed around the body in most tissues at concentrations similar to those in the plasma, with the most important exception being the low absorption into the brain (Voogd *et al.* 1993). It is generally assumed that the high negative charge of the suramin molecule blocks its transport across the blood-brain barrier. However a small amount of the drug can pass into the CSF giving rise to concentrations of less than 1% of the levels in the plasma (Stein *et al.* 1989). Furthermore it has also been reported that these low levels of suramin in the brain may in fact be sufficient to effect a cure if the parasites have only recently entered the CNS (Raseroka and Ormerod 1985).

Although it is not entirely clear how suramin enters the trypanosome, it is assumed that the drug is mainly taken up by receptor-mediated endocytosis bound to protein, possibly on LDLs (Vansterkenberg *et al.* 1993), as it is most certainly too highly charged to diffuse across biological membranes and it accumulates in trypanosomes too rapidly for non-specific fluid endocytosis (Fairlamb and Bowman 1980b). Once inside the parasite, suramin has been shown to interact with a number of enzymes. Particularly significant may be its strong inhibition of almost all the glycolytic enzymes (Misset and Opperdoes 1987). Although the interaction of suramin with these glycolytic enzymes is complex and suggestive of multiple sites at which suramin can bind, it is significant that the inhibition is specific for the trypanosomal and not the mammalian forms of these enzymes. It has already been mentioned (**section 1.1.4**) that bloodstream trypanosomes have no electron transport chain. As a

consequence, the parasite is reliant on a glycerol-3-phosphate dehydrogenase/glycerol-3-phosphate oxidase system to oxidise the NADH formed during glycolysis and it is during this process that the long-slender bloodstream form consumes oxygen (Opperdoes 1985). Suramin was found to inhibit the utilisation of oxygen in a concentration-dependant manner and this inhibition of respiration has been attributed to the inhibition of the glycerol-3-phosphate dehydrogenase/glycerol-3-phosphate oxidase system along with the inhibition of the other glycolytic enzymes (Fairlamb and Bowman 1980a).

Suramin can act synergistically to cure late-stage infections with drugs that are totally ineffective when used singly, such as the permanent cures obtained with a combination of suramin and substituted 5-nitroimidazoles (Jennings *et al.* 1983). Furthermore, it has also been shown that DFMO and suramin act synergistically (Clarkson *et al.* 1984), and this may indicate that the mode of action of suramin may also be involved with polyamine metabolism. A recent development has come with the finding that suramin strongly binds to LDL and thereby inhibits receptor-mediated endocytosis of LDL by 50%. It has been proposed that the trypanosome is reliant on the cholesterol and phospholipids present in LDL for the maintenance of its membranes, and that the action of suramin in inhibiting LDL uptake may be sufficient to cause death (Vansterkenberg *et al.* 1993). In conclusion therefore, the primary target of suramin still remains unclear, and its wide range of uses on a range of cell types may indeed indicate no one target is singly responsible for its trypanocidal nature (Voogd *et al.* 1993).

Arsenicals

The arsenical drugs differ markedly from suramin in pharmacokinetics, mode of action and effectiveness against sleeping sickness. Whereas suramin has

an effective clearance rate of 0.3ml/min (with a measured volume of distribution of 40 litres) and a half-life of approximately 50 days (Collins *et al.* 1986), melarsoprol has a total clearance of 50ml/min (volume of distribution was 100 litres) and a serum half-life of 35 hours (Burri *et al.* 1993). A typical full course of treatment for melarsoprol consists of two or three sets of intravenous injections, with each set of injections being separated by 2 weeks, and consisting of 4 daily injections at gradually increasing doses between 1.2 and 3.6mg/Kg (WHO 1986b). This treatment regime results in serum concentrations of approximately 5μg/ml (10μM) 30 minutes after drug injection which gradually drops to approximately 0.2μg/ml (0.4μM) 120 hours post-treatment (Burri *et al.* 1993). In the same study, the levels of melarsoprol in the CSF averaged approximately 50-fold lower than those in the serum.

It is generally thought that the trypanocidal activity of melarsoprol is largely due to its interaction with trypanothione (Fairlamb *et al.* 1989) and possibly also with its inhibition of pyruvate kinase, due to the trypanosome's sole dependence on continuous glucose catabolism to generate ATP in the long-slender bloodstream forms (Flynn and Bowman 1974). At melarsoprol concentrations equivalent to those found in the serum, it was found that trypanosomes were lysed rapidly *in vitro*, and that this lysis was not a result of the inhibition of pyruvate kinase (Van Schaftingen *et al.* 1987). The active form of melarsoprol is thought to be the trivalent moiety of melarsen oxide, which forms a stable adduct with trypanothione (melT) *in vivo* and it is for this reason that trypanothione has been proposed as the primary target of arsenical compounds (Fairlamb *et al.* 1989). Trypanothione in conjunction with trypanothione reductase carries out a vital role in trypanosomes in maintaining the intracellular redox balance and detoxifying H_2O_2, in a similar way to glutathione and glutathione reductase in mammalian cells. Therefore, it is possible to account for the extremely toxic nature of melarsoprol (and

presumably the other melaminophenyl arsenicals such as melCy) through the interaction of melarsen oxide with trypanothione. This mechanism also provides an explanation for the specificity of melarsoprol for trypanosomes, as mammalian cells do not possess trypanothione. Furthermore, the synergistic effect of DFMO with melarsoprol (Jennings 1988) further lends itself to the proposal of trypanothione being the primary target, since the proposed pathway for trypanothione biosynthesis is blocked by DFMO at ornithine decarboxylase (reviewed in Henderson and Fairlamb 1987). During the early development of melaminophenyl arsenicals by Friedheim and others, it was known that they had a high affinity for sulphydryl groups. Consequently, melarsoprol may in fact have many more important targets, such as lipoic acid. This has been found to have an affinity for melarsen oxide 500 times greater than the affinity of trypanothione for melarsen oxide and may be involved in arsenical uptake (Fairlamb *et al.* 1992a). There is however, stronger evidence to suggest that arsenicals are taken up via an adenosine transporter at the cell surface (Carter and Fairlamb 1993). The rapid lysis of trypanosomes in the presence of arsenicals was found to be blocked by adenosine and adenine, and radiolabelled adenosine uptake was found to be inhibited by melarsoprol (K_i = 0.28μM). Furthermore, this study also found that a particular type of adenosine transporter (termed P2) may be involved in melarsen oxide uptake (**chapter 6**).

In light of the recent data on the pharmacokinetic properties of melarsoprol (Burri *et al.* 1993), it would appear that concentrations in the serum are sufficiently high to bring about the rapid lysis *in vivo* that is seen *in vitro.* However, the levels in the CSF were estimated at being around 30ng/ml (0.06μM) and these may be insufficient to cause this rapid lysis in some strains (**chapters 3 and 6**). Furthermore, the investigations carried out on the interaction of arsenicals and trypanothione (Fairlamb *et al.* 1989) were carried out at much higher concentrations of arsenical (10μM melarsen oxide and 50μM

melB) than that found in the CSF. This difference may indicate that the trypanosomes in the CSF are killed by arsenicals at a lower rate than in the bloodstream, and it is also possible that these lower concentrations of arsenicals in the CSF are insufficient to interact with trypanothione to the same extent as in the bloodstream.

Pentamidine

Pentamidine is the only drug in current use that has been used for large-scale prophylaxis (see Dukes 1984). A typical treatment regime consists of a series of intramuscular injections once every 3-6 months. In this respect it is much easier to administer than the other trypanocides in current use. Each series of injections comprises of daily doses of 3-4mg/Kg for 7-10 days. Similar regimes are adopted for the treatment of individuals already infected that do not have trypanosomes present in the CNS, since pentamidine cannot pass the blood-brain barrier. Not only is pentamidine easier to administer, but the adverse side-effects associated with treatment are also relatively mild compared to other drugs, with hypertension due to histamine release being one of the most common (Gutteridge 1985).

There has been very little work carried out on the mode of action of pentamidine in recent years. Earlier studies showed that pentamidine inhibits biosynthetic processes, particularly nucleic acid synthesis (Gutteridge 1969) although the effect of this binding of pentamidine to DNA has not been evaluated and may in fact have little or no physiological significance. Pentamidine was also found to inhibit S-adenosyl-L-methionine decarboxylase in *T. brucei* (Bitonti *et al.* 1986a), although more recent work suggested that this interference with polyamine metabolism was not the primary mode of action of the drug (Berger *et al.* 1993). Most work carried out on the mode of action of

pentamidine has been carried out on drug uptake. A number of studies showed pentamidine to be accumulated within the trypanosome at levels higher than those found in the serum. Furthermore, a temperature- and pH-dependant transporter has been identified with a K_m for pentamidine of 158μM for bloodstream forms of *T. b. brucei* (Damper and Patton 1976). It was interesting to note that this same study, using a pleomorphic line, showed pentamidine uptake to be much reduced in short-stumpy forms and even more so in cultured procyclic forms. More recently it has been proposed that the P2 type adenosine transporter, responsible for the uptake of the melaminophenyl arsenicals, is also responsible for the uptake of pentamidine thereby accounting for the patterns of cross-resistance observed between diamidines, like pentamidine, and melaminophenyl arsenicals (Fromell *et al.* 1987; Fairlamb *et al.* 1992b).

DFMO

In comparison to the other trypanocides in current use, the mode of action of DFMO is extremely well characterised, and indeed, it is the only drug that has been developed with a specific target in mind. DFMO is activated by its target (ornithine decarboxylase) which it irreversibly inhibits, thereby blocking polyamine biosynthesis (Metcalf *et al.* 1978). Studies *in vivo* on the morphology of *T. b. brucei* after treatment with DFMO indicated that the drug caused a predominantly monomorphic line to become mainly short-stumpy in morphology, and that this alteration in morphology was preceded by a depletion in polyamine levels (Bacchi *et al.* 1983). It has since been shown that a T-cell independent antibody response was necessary to fully cure infections after DFMO treatment (Bitonti *et al.* 1986b). The overall mode of action of DFMO would therefore appear to be a block in replication, by inhibiting polyamine metabolism, with a resultant block in the cell's ability to switch its antigenic coat (Turner and Barry 1989). Trypanosome specific antibodies then enable the

immune system to clear the infection.

It is now firmly established that DFMO is the most important drug to be introduced for treating human sleeping sickness, particularly arsenical-resistant cases, since Friedheim's arsenicals in the 1940s. Some of the more recent studies have investigated the use of combination therapy with other trypanocides such as suramin (Zweygarth and Kaminsky 1991), melarsoprol (Jennings 1988) and berenil (Onyeyili and Anika 1989) each with some degree of success. Therapy with DFMO alone is expensive and requires up to 1Kg per patient for a full course, with the drug being administered either orally or intravenously at doses of up to 400mg/Kg/day for about 6 weeks (Van Nieuwenhove *et al.* 1985). These large amounts of DFMO can be difficult to administer due simply to the solubility of the drug and the physical limitations of the patient. Despite these problems, the side effects are remarkably mild, with the most common being diarrhoea, which can be avoided with either lower oral doses or intravenous administration.

1.2.4 Drug-Resistance in Parasites

Definition of Drug-Resistance

Drug-resistance has been studied in a wide range of organisms with most work having been concerned with drug-resistance in bacteria, tumour cells and insects. With respect to drug-resistance in parasites, a large amount of attention has been focused on chloroquine-resistant (and multiple drug-resistant) *Plasmodium falciparum* and on the drug-resistant nematodes of ruminants.

Stable drug-resistance that can be inherited from one trypanosome generation to the next is brought about through one or more genetic mutations. The results of these mutations are expressed through an increased ability for the

parasite to survive in the presence of drug pressure. In 1963, the World Health Organisation defined resistance as the "ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication. Such resistance may be relative (yielding to increased doses of the drug tolerated by the host) or complete (withstanding maximum doses tolerated by the host)." However, an important distinction was made between this and "drug failure", which is defined as the "absence or insufficiency of drug action after administration of a normally effective dose." That there is a difference between the two is of fundamental importance in that although it is true that drug-resistance will bring about drug failure, it may not necessarily be true that drug failure is a result of drug-resistance since resistance is only one of many factors affecting drug failure. Throughout this study, the meanings of both drug-resistance and drug failure will be consistent with the above definitions.

Resistance to Trypanocides

Resistance to the various trypanocidal compounds can be rapidly achieved in the laboratory either *in vitro* or *in vivo*, and much of the work carried out investigating mechanisms of drug action has centred on the comparison of drug-sensitive and laboratory-derived drug-resistant lines. In the field there have been many reports of natural and developed resistance to the drugs used (Bacchi 1993).

Definite reports of resistance to suramin in *T. brucei* are relatively scarce, and there appears to be more evidence of suramin-resistance in *T. evansi* than in *T. brucei*. The lack of definite reports of suramin-resistance in *T. brucei* is probably due to the fact that relapses after suramin treatment are often designated as having resulted from trypanosomes being present in the central

nervous system. These relapses are thus ascribed to drug failure and not the presence of suramin-resistant trypanosomes. Of the reported cases using mouse models, levels of suramin-resistance of greater than 40mg/Kg have been recently shown for field isolates of *T. brucei* and greater than 100mg/kg in *T. evansi* (Zweygarth and Kaminsky 1990). Of particular interest was the finding that out of 29 stocks of *T.evansi* isolated from Egypt, Sudan and Indonesia, all of the stocks that expressed levels of suramin-resistance greater than 64mg/Kg also had a type VII isoenzyme banding pattern for malic enzyme. The other more sensitive stocks had a type II banding pattern (Boid *et al.* 1989). Whether this can be used as a genuine indication of suramin-resistance and/or has some significance for the mechanism of suramin-resistance remains to be seen.

Relapses after melarsoprol treatment are arguably the most serious of all the problems associated with trypanosomiasis chemotherapy. Arsenical chemotherapy fails in an estimated 10% of field cases (Kazyumba *et al.* 1988), with this failure being attributed to melarsoprol-resistance. The position is worsened with an equal number of people suffering from arsenical encephalopathy after treatment, which is frequently fatal (Pepin *et al.* 1989). Experimental studies have been carried out both on field isolates and laboratory generated resistant lines using a variety of *in vivo* and *in vitro* methods to measure the levels of arsenical-resistance (discussed in more detail in **chapter 3**). Field isolates from patients with relapse infections after one or more courses of melarsoprol treatment have levels of resistance of greater than 25mg/Kg in experimental mouse models (Yarlett *et al.* 1991). However, resistant field isolates do not always express resistance *in vitro*, with 24 hour growth inhibition assays showing no difference between some resistant and sensitive clones (Brun *et al.* 1989), whereas melarsoprol levels required to induce rapid lysis do show differences between resistant and sensitive clones and therefore appears to be a more reliable method for assessing drug-resistance (Yarlett *et al.* 1991). A

number of studies have been carried out to identify the mechanisms of arsenical-resistance. Although the role of trypanothione and the formation of melT upon exposure to melarsoprol and melarsen oxide has been established (Fairlamb *et al.* 1989), alterations in trypanothione and polyamine metabolism have not been shown in trypanosomes which express arsenical-resistance. In one well characterised line and its resistant derivative, the levels of trypanothione and glutathione were not significantly different, and although significant differences were found in the levels of trypanothione reductase (Fairlamb *et al.* 1992b), the mechanism of arsenical resistance was attributed to an altered purine transporter (Carter and Fairlamb 1993). Similar findings were made independently in a study of a number of *T. b. rhodesiense* isolates expressing different levels of arsenical-resistance. It was found that different levels of trypanothione did not correlate with levels of arsenical-resistance, and that uptake of melarsen oxide was found to be reduced in resistant isolates (Yarlett *et al.* 1991). It has been proposed that the cross-resistance patterns seen between the different melaminophenyl arsenicals (melOx, melB, melCy and melW) and the diamidines (berenil and pentamidine) can be accounted for by alterations in the P2-type adenosine transporter which is thought to be responsible for arsenical uptake as well as berenil and pentamidine uptake (Frommel and Balber 1987; Zweygarth and Kaminsky 1990; Fairlamb *et al.* 1992b; Carter and Fairlamb 1993).

Current evidence not only suggests that pentamidine uptake is via a transporter but also that pentamidine-resistance, like arsenical-resistance is brought about through an alteration in drug uptake. A recent study investigating pentamidine-resistance failed to find alterations in polyamine levels (thought to be a drug target) or differences in the rate of metabolic conversion of pentamidine between sensitive and resistant trypanosomes (Berger *et al.* 1993). Furthermore, a comparison of a number of isolates with different levels of resistance to pentamidine showed a correlation between drug accumulation and

pentamidine-sensitivity (Damper and Patton 1976).

Altered uptake also appears to be involved in some cases of laboratory generated DFMO-resistance. A study with a DFMO-resistant line of *T. brucei* selected in procyclic culture showed decreased uptake of DFMO in the resistant line after the removal of drug pressure (Phillips and Wang 1987). A separate study also using an *in vitro* generated DFMO-resistant line of procyclic *T. brucei* showed a 2.5-fold decrease in DFMO accumulation in the resistant line after DFMO had been removed for one week. However, in this study, DFMO induced an increase in the levels of ornithine decarboxylase (4-fold) and a marked increase in ornithine (24-fold) in the resistant line. It was proposed that these changes in ornithine metabolism negated the inhibition of ornithine decarboxylase by DFMO and accounted for DFMO-resistance in this line (Bellofatto *et al.* 1987). A similar study carried out on *Leishmania donovani* promastigotes also found DFMO-resistance to be brought about through increased ornithine decarboxylase activity (Coons *et al.* 1990).

1.3 GENETIC EXCHANGE

1.3.1 The Role of Non-Obligatory Mating in Natural Populations

Trypanosomes reproduce by binary fission. Evidence for a sexual process also occurring during the life cycle was first observed in a study of cloned trypanosome stocks isolated from a single focus in Uganda over a 2 year period. Extensive isoenzyme analysis of these stocks showed the population to be in Hardy-Weinberg equilibrium, which, by analogy to similar studies on other organisms, indicated that trypanosomes are diploid and undergo random mating (Tait 1980). Following this finding, mating has been demonstrated in the laboratory, and it has been shown by a number of independent studies that mating is a non-obligatory event in the trypanosome life cycle (**section 1.3.2**).

However, in the last few years, opinions are divided as to the relevance of a sexual stage in natural populations of trypanosomes (Tibayrenc *et al.* 1990; Maynard Smith *et al.* 1993). It has been proposed that although genetic exchange can take place, it has little or no consequence to natural populations (Tibayrenc and Ayala 1991).

The role of mating in trypanosome populations has mainly been studied by analysing isoenzyme electrophoretic variability within trypanosome populations. Seventeen *T. b. brucei* isolates from Lugala, Uganda, were found to have variations in their isoenzymes that indicated that the population had undergone random mating and was in Hardy-Weinberg equilibrium (Tait 1980). A more extensive study was conducted on a population of *T. brucei* using 220 isolates, taken over a period of years up to and including a time of marked increase in cases of sleeping sickness. These stocks were isolated in the Lambwe Valley, Western Kenya, from tsetse flies and a variety of mammalian hosts, including humans. Isoenzyme analysis of trypanosomes taken from humans during the epidemic, revealed new zymodemes (trypanosome strains) that had not been observed previously. Since these were also different from those observed in neighbouring sleeping sickness foci, it was concluded that genetic exchange provided the most plausible explanation for the emergence of these new zymodemes (Gibson and Wellde 1985). The possibility that mutations alone could account for the observed isoenzyme variability was discounted since, in both the Lugala study and the Lambwe Valley study, a relatively high number of recurrent mutations would be needed to explain the patterns observed (Cibulskis 1988). However, a further analysis on the isoenzyme data from the Lambwe Valley suggested that, although genetic exchange played an important long-term role in the evolution of the parasite, this was unlikely to be the cause for the emergence of the previously unobserved zymodemes during the epidemic (Mihok *et al.* 1990). Rather, it was proposed that these new zymodemes had in

fact been present in animal hosts, and the increase in tsetse fly numbers prior to the epidemic allowed the transmission of these strains from the animal reservoirs to new human hosts.

Although the population from Lugala, Uganda was found to be in Hardy-Weinberg equilibrium (i.e. undergoing random mating) (Tait 1980), a number of later studies on different trypanosome populations showed marked deviations from Hardy-Weinberg expectations (Tait *et al.* 1985; Gibson and Wellde 1985; Stevens and Welburn 1993; Hide *et al.* 1994). The fact that a number of populations do not show signs of genetic exchange occurring at random led to the proposal that populations of *T. brucei*, together with a number of other parasitic protozoa including *Plasmodia*, were in fact clonal and that mating did not have any significant impact on natural populations (Tibayrenc *et al.* 1990). A more rational explanation for the deviations from Hardy-Weinberg equilibria was given by Cibulskis (1992), who interpreted the data from the Lambwe Valley, Kenya, by taking into account selection pressures imposed by hosts and geographical location. Thus, it was proposed that certain zymodemes were better adapted to survive in certain hosts, and that this brought about selection pressures for different zymodemes, thereby accounting for the deviations from Hardy-Weinberg equilibria. A given set of ecological conditions would therefore favour certain zymodemes, which would propagate by asexual, or clonal, reproduction. However, a change in these conditions would bring about different selection pressures. Genetic exchange would therefore be favoured to allow the generation of new zymodemes that were more capable of thriving under this new set of conditions (Cibulskis 1992). Although this may appear to contradict the initial finding that a natural trypanosome population can undergo random mating (Tait 1980), it is more likely that, due to the non-obligatory nature of mating, both asexual reproduction and sexual reproduction play significant roles in the epidemiology of natural populations, with some geographical locations

consisting of largely clonal populations and others consisting of randomly mating trypanosomes depending on the ecological conditions. The need for further investigations into the exact nature and frequency of genetic exchange is therefore extremely important in order to elucidate the factors affecting "fertility" and how this may affect the spread and inheritance of human infectivity and drug-resistance.

1.3.2 Experimental Genetic Exchange

Direct evidence of genetic exchange occurring in trypanosomes was first found in a cross between two cloned lines of STIB 247 and STIB 386 (Jenni *et al.* 1986). These stocks are of the subspecies *T. b. brucei* and *T. b. rhodesiense* respectively (Hide *et al.* 1990). Cloned populations were made from trypanosomes that had been cyclically transmitted through a tsetse fly infected with a mixture of 247 and 386. These clones showed phenotypes and genotypes (detected by isoenzyme electrophoresis and restriction fragment length polymorphism (RFLP) analysis respectively) that differed from the isoenzyme patterns of either parent and were consistent with these clones being F_1 hybrid progeny having resulted from the two parental lines having undergone genetic exchange during cyclical transmission in the tsetse fly (Jenni *et al.* 1986). The patterns of inheritance of the isoenzyme phenotypes and the RFLPs suggested a simple model of genetic exchange involving meiosis of the diploid parents, followed by recombination of the haploid "gametes", although it was accepted that other models could also explain these results. A number of other genetic crosses have since been carried out independently in attempts to elucidate the exact processes involved during mating. In order to gain further evidence that genetic exchange involved meiosis and syngamy, it was necessary to analyse a greater number of cloned progeny for a wider variety of phenotypic and genotypic markers. Evidence showing that segregation of alleles occurred at

several loci, and with recombination occurring between loci, was demonstrated independently in a further cross between 247 and 386 (Sternberg *et al.* 1989) and between *T. b. rhodesiense* (058) and *T. b. brucei* (196) (Gibson 1989). Following this, three more crosses were carried out with all possible combinations, of 247, 386 and another *T. b. brucei* clone, TREU 927/4. These crosses analysed the inheritance of a number of markers providing further evidence of allelic segregation and recombination (Turner *et al.* 1990). However, whether a classical Mendelian system exists with meiosis creating haploid gametes which subsequently fuse, or whether meiosis occurs after the fusion of diploid nuclei is still not clear (Tait and Turner 1990).

It is still unclear what factor(s) determine a trypanosomes ability to mate. Several studies have now demonstrated that genetic exchange is non-obligatory. Flies producing hybrid clones also produced clones with the same phenotype and genotype as one or the other parent (Sternberg *et al.* 1989; Gibson 1989; Turner *et al.* 1990). In an attempt to investigate the presence of a mating system, all three pairwise combinations of 247 (*T. b. brucei*), 386 (*T. b. rhodesiense*) and 927/4 (*T. b. brucei*) have been transmitted through tsetse flies with the result that all three combinations of parents produced hybrid progeny (Turner *et al.* 1990). Thus, a simple mating system involving two sexes or fixed mating types (i.e. + and -) could not explain these findings, and it has been proposed that there may be no barriers to mating (Turner *et al.* 1990). However, if this were the case, self-fertilisation, or genetic exchange between trypanosomes of the same stock, would be expected during transmission of a single stock through tsetse flies. Self-fertilisation would bring about segregation of heterozygous alleles at some loci, bringing about progeny that were homozygous at these loci. However, no evidence of segregation at a number of heterozygous loci could be found during the single transmission of five stocks through flies (Tait *et al.* 1989). Evidence for self-fertilisation has, however, been obtained by examining

cloned progeny from a mixed transmission of 247 and 386. Three metacyclic clones that were initially believed to have the 247 parental genotype, were re-examined, and found to be homozygous at the peptidase Tyr^3 locus, which is heterozygous in parental 247. Further evidence for self-fertilisation has been shown by the alteration of karyotype in four non-hybrid clones from this same cross (Tait *et al.* 1993).

The timing and location of mating is also a matter of some debate. It has been proposed that mating actually occurred during transmission of the trypanosomes from the tsetse fly to the mammal. This was based on measurements of the DNA content of the metacyclic trypanosomes, which appeared to have approximately half that of procyclics and bloodstream forms, and therefore were thought to be haploid (Zampetti-Bosseler *et al.* 1986). However, since bloodstream forms are diploid, infections derived from single haploid metacyclics should be homozygous at all loci and this is clearly not the case (Tait *et al.* 1989; Sternberg *et al.* 1989; Turner *et al.* 1990). Furthermore, independent measurements using a different method failed to identify metacyclics that had a DNA content significantly less than other life-cycle stages (Shapiro *et al.* 1984; Kooy *et al.* 1989). There is, therefore, overwhelming evidence to show that mating occurs within the tsetse fly stages and that this genetic exchange involves a reduction division either before or after nuclear fusion. The only positive evidence identifying a location for this process has been provided with the identification of hybrid trypanosomes isolated from the posterior midgut of a tsetse fly infected with 247 and 386 (Schweizer and Jenni 1991). This has been further substantiated in the same laboratory, with the formation of hybrid procyclic trypanosomes *in vitro* (Schweizer *et al.* 1991). However, although it would therefore appear on this evidence that genetic exchange occurs in the midgut between procyclic trypanosomes and that this hybrid formation can occur within the first seven days of the establishment of a mixed midgut infection

(Schweizer *et al.* 1991), a number of attempts by different groups to repeat the *in vitro* mating experiments have proved unsuccessful (Gibson and Whittington 1993; Buchanan pers. comm.). Furthermore, it is unfortunate that the isoenzyme analysis used for the positive identification of procyclic hybrids both *in vivo* and *in vitro* was carried out on uncloned material, and until a hybrid procyclic clone has been identified, it is possible to explain the observed hybrid-type isoenzyme bandings as experimental artefacts (Schweizer and Jenni 1991; Schweizer *et al.* 1991). In a separate study, procyclic trypanosomes and metacyclic trypanosomes were cloned from a 10-week old fly that was producing hybrid trypanosomes. None of the sixteen procyclic clones were hybrid, whereas nine out of ten metacyclics were hybrids (Turner *et al.* 1990). Furthermore, a recent study has used antibiotic-resistance as selectable markers for genetic exchange. (Gibson and Whittington 1993). In this study, metacyclic infections were found to contain double drug-resistant hybrids whereas, although mixed infections were found in the midgut, no double drug-resistant procyclic trypanosomes could be identified. Both these studies indicate that mating occurs in the salivary gland rather than the midgut of the tsetse fly, contrary to the findings of Schweizer and colleagues. The exact location and timing of genetic exchange therefore still requires clarification.

In conclusion, therefore, many of the details concerning mating in *T. brucei* remain unresolved. Analysis of the vast majority of the progeny clones from mixed transmission experiments, are consistent with a model of non-obligatory mating, in which hybrid progeny are the products of a diploid Mendelian system of genetic exchange. Future work exploiting drug-resistance as selectable markers should allow classical genetics to be used in investigating the inheritance of particular phenotypic traits such as drug-resistance.

1.4 OVERALL AIMS

The ultimate aim of this study was to elucidate the genetic and biochemical mechanisms that determine drug-resistance in *T. brucei*. The work presented here can be considered as four separate stages:

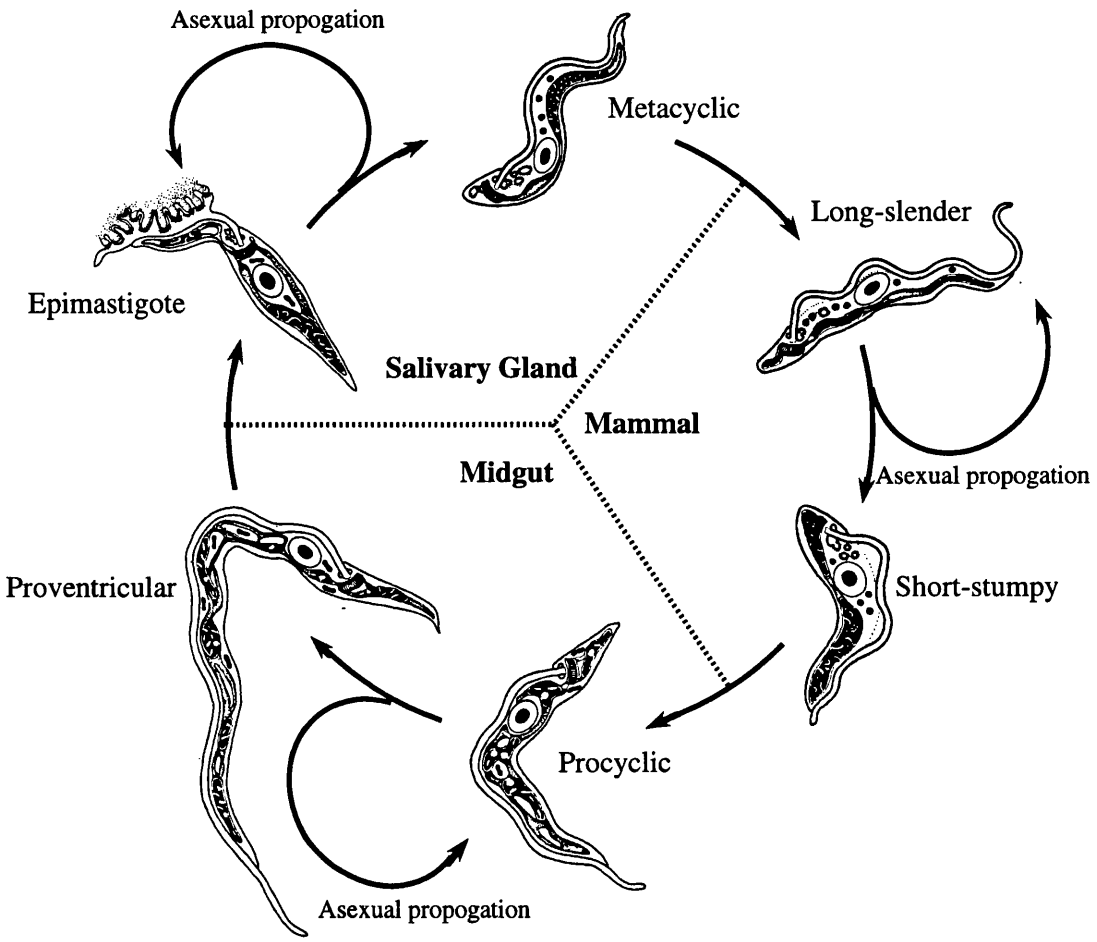
1. Generation of cloned lines of trypanosomes *in vivo* expressing resistance to suramin and cymelarsan and the subsequent characterisation of these lines to ensure there is no cross-resistance between these two drugs, and the drug-resistance phenotypes are stable in the absence of drug pressure during transmission through tsetse flies (**chapter 2**).

2. Development of suitable *in vitro* assays that could be applied to the rapid and reliable measurement of the level of drug-resistance in a number of clones generated from mixed transmission experiments in order to determine the inheritance of drug-resistance during genetic exchange (**chapter 3**).

3. Demonstration that the cloned drug-resistant lines are 'fertile' and cloned progeny, expressing resistance to both cymelarsan and suramin, can be generated by co-transmitting a cymelarsan-resistant and a suramin-resistant line through tsetse flies (**chapters 4 and 5**).

4. Investigation of the role of adenosine transporters in the uptake of the melaminophenyl arsenicals, with particular emphasis on any potential differences between melB and melCy (**chapter 6**).

Figure 1.1
Life Cycle of *T. brucei*



The life cycle of T. brucei consists of at least 6 distinct stages. It can be considered in terms of 3 different environments (tsetse salivary gland, mammalian host, and tsetse midgut) each of which contains two life cycle stages. In each environment, one life cycle stage is able to undergo asexual reproduction, thereby establishing a dense infection in the current environment. The other stage is unable to divide but is capable of migrating or being transmitted to a new environment, whereupon it changes morphology to a dividing form, capable of establishing a new infection (adapted from Vickerman 1985).

CHAPTER 2
GENERATION AND CHARACTERISATION OF CLONED DRUG-RESISTANT LINES *IN VIVO*

2.1 INTRODUCTION

The primary aim of this study was to determine the genetic and biochemical basis of drug-resistance in *T. brucei*, with particular emphasis on the mechanisms of resistance to the melaminophenyl arsenicals. It has long been recognised that the prolonged use of trypanocides for the treatment of infected individuals promotes the development of drug-resistant trypanosomes. In 1929, the occurrence of atoxyl-resistant and tryparsamide-resistant strains in clinical cases led Yorke to investigate drug-resistance. By generating drug-resistant lines of *T. b. rhodesiense* in laboratory mice using gradually increasing sub-curative doses he examined the stability and patterns of cross-resistance of a number of trypanocides (Fulton and Yorke 1941). Since these early studies, drug-resistance has been investigated using either naturally occurring drug-resistant strains or, more frequently, laboratory generated lines.

Melarsoprol-resistance has been shown to develop rapidly in clinical cases that suffer relapse infections after the first course, and subsequently receive further courses, of melarsoprol treatment (Robertson 1963). It is widely believed that such resistance will invariably be associated with resistance to the other melaminophenyl arsenicals. There are a number of recent examples, with both naturally occurring and laboratory derived drug-resistant lines, of cross-resistance between the different melaminophenyl arsenicals *in vivo* (Yarlett *et al.* 1991; Fairlamb *et al.* 1992b). This cross-resistance often extends to the diamidines, berenil and pentamidine (Williamson and Rollo 1959; Frommel and Balber 1987), and although a degree of cross-resistance between suramin and melarsoprol has been observed (Fairlamb *et al.* 1992b), arsenical-resistance does

not necessarily confer resistance to suramin (Fulton and Yorke 1941; Gill 1971).

In order to carry out studies on the molecular and biochemical basis of drug-resistance, it was necessary to generate and characterise cloned drug-resistant lines of trypanosomes. Two cloned trypanosome stocks were chosen for use in these studies (STIB 247 and STIB 386) that have been well characterised for a range of genetic (karyotype and RFLP) and phenotypic (isoenzyme) markers. Previous studies have successfully mated these two stocks by co-transmitting them through tsetse flies, and from these co-transmission experiments, the inheritance of markers during genetic exchange has been determined (Jenni *et al.* 1986; Sternberg *et al.* 1989; Tait *et al.* 1989; Turner *et al.* 1990). Therefore, the generation of drug-resistant lines of STIB 247 and STIB 386, followed by genetic crosses between these two drug-resistant lines would give us the opportunity to examine the pattern of inheritance of the drug-resistance phenotypes on a well characterised genetic background.

This chapter describes the generation and preliminary characterisation of cloned lines of these two stocks that have each been selected for resistance to each of the two drugs, cymelarsan and suramin. Figure 2.1 summarises the procedure through which each unselected, drug-sensitive cloned stock was selected for drug-resistance. Each cloned drug-resistant line was subsequently characterised in terms of the levels of drug-resistance and the stability of this resistance in the absence of drug pressure in mice and after cyclical transmission through tsetse flies. The characterisation of each resistant line included a check for cross-resistance between the different melaminophenyl arsenicals and suramin.

2.2 MATERIALS AND METHODS

2.2.1 Drugs

The two trypanocidal drugs, cymelarsan (melCy) and suramin, were used to generate drug-resistant lines. Two further drugs, melarsoprol (melB) and trimelarsan (melW) were used in the *in vivo* drug-resistance assays. Suramin, melCy and melW were dissolved in $dddH_2O$ (double-distilled de-ionised water) and melB was suspended (by vigorous vortexing to create a milky suspension) in peanut oil (Sigma) immediately prior to use. All drugs were administered by intra-peritoneal injection. Any unused drug was discarded. Suramin (Bayer AG, Batch 738233/1181) was a kind gift from Bayer, Germany. MelCy (Melarsomine Dihydrochloride, Batch CP4189) was a kind gift from Rhone Merieux, France. MelW (Melarsonyl Potassique) and melB (Arsobal) were kind gifts from Dr. Frank Jennings, Glasgow University.

2.2.2 Trypanosome stocks and mice

Two cloned trypanosome stocks were used. These were STIB (Swiss Tropical Institute Basel) 247 and STIB 386, hereafter called 247 and 386 respectively. The 247 line (*T. b. brucei*) was originally cloned from the primary isolate taken from a hartebeest, in 1971, in the Serengeti National Park, Tanzania (Jenni *et al.* 1986). The 386 line was cloned from a stock isolated from a man in Daloa in the Ivory Coast and was originally grouped with other West African stocks as *T. b. gambiense*. It has since been found to be indistinguishable from East African *T. b. rhodesiense* stocks and will be considered as such (Hide *et al.* 1990).

Selection of drug-resistance and routine growth of trypanosomes were carried out in adult female CFLP, CD1 or TO Swiss mice, or adult male CFLP

mice. *In vivo* assays to determine levels of drug-resistance and cloning were conducted using immunosuppressed (cyclophosphamide at a dose of 150mg/Kg by i.p. injection 24 hours prior to trypanosome inoculation) adult female Balb/C mice.

2.2.3 *In Vivo* Generation of Drug Resistant Lines of Trypanosomes

All selections were carried out in female adult CFLP or CD1 mice immunosuppressed with 250mg/Kg cyclophosphamide 24 hours prior to inoculation with trypanosomes. Starting with the cloned unselected trypanosome stocks (either 247 or 386), trypanosomes were grown to a parasitaemia of greater than $3.2x10^7$ trypanosomes/ml and then treated with a sub-curative drug dose by i.p. injection causing recrudescence of the parasitaemia. Trypanosomes from subsequent relapse infections were transferred to one or more immunosuppressed mice and when the parasitaemia rose to $3.2x10^7$ trypanosomes/ml they were again drug treated at the same or a slightly increased dose. Repetition of this sequence eventually generated a population of trypanosomes with a high level of drug-resistance. The homogeneity of this population could not be guaranteed after the drug-resistance selection, therefore each population was recloned by isolating single trypanosomes taken from a drop of infected mouse tail blood suspended in approximately 100-200µl guinea pig serum. Optical cloning of bloodstream trypanosomes was carried out in a cool (10^0C) room as described previously (McLintock 1990), with the exception that guinea pig serum instead of PSG was added to drops containing single trypanosomes.

2.2.4 Cyclical Transmission of Trypanosomes through Tsetse Flies

Three drug-resistant trypanosome lines were cyclically transmitted

through a strain of *Glossina morsitans* that was susceptible to trypanosome infection (Maudlin and Dukes 1985). Tsetse flies were infected with bloodstream trypanosomes taken either from a cryopreserved stabilate or direct from an infected mouse. The infective feeds were prepared as follows:

247MelCy^R and 247Sur^R bloodstream stabilate

1ml aliquots of bloodstream trypanosomes taken from infected mice were cryopreserved with 7.5% DMSO. Infective feeds were prepared by rapidly thawing one aliquot which was then washed once, to remove DMSO, by mixing with 10ml Hank's balanced salt solution containing heparin (50units/ml) and centrifuging at 1000g for 10 minutes at 4^0C. The trypanosome containing pellet was resuspended in approximately 10ml citrated sheep's blood (Advanced Protein Products).

386MelCy^R bloodstream forms

Infected blood was taken from infected mice with parasitaemias greater than $3.2x10^7$ trypanosomes/ml (preferably with a high proportion of short-stumpy forms) with a syringe in which was approximately 0.4ml Hank's balanced salts containing heparin (50units/ml) for every 2ml of blood. Infected blood was either fed directly to flies or mixed with up to 10ml citrated sheep's blood depending on the volume of infective feed required.

Infective feeds were warmed on a tray to 37^0C and given to teneral flies at trypanosome densities of greater than $2.5x10^5$ trypanosomes/ml by allowing the flies to feed through a silicone rubber membrane. Flies were maintained as described previously (Hajduk *et al.* 1981, Turner *et al.* 1990) in a temperature controlled room (26-27^0C) in cupboards with a tray of water to create a humid

environment. Citrated sheep's blood was used for maintenance feeds three times each week.

Flies harbouring mature infections were identified by allowing flies over 18 days old to probe onto microscope slides heated to 37^0C. The dried saliva was then examined under phase-contrast microscopy for the presence of phase-bright metacyclics. Bloodstream infections were re-initiated by allowing flies with metacyclic infections to feed directly on Balb/C mice (anaesthetised with 1 part Hypnovel : 1 part Hypnorm : 2 parts $dddH_20$ - 0.1ml/20g mouse by i.p. injection).

2.2.5 *In Vivo* Assay to Determine Drug-Resistance

An *in vivo* drug assay was used to assess the levels of drug-resistance of the cloned trypanosome lines. Adult female (approximately 20g) Balb/c mice were immunosuppressed with 150mg/Kg cyclophosphamide by intraperitoneal (i.p.) injection. Twenty four hours later each mouse was inoculated i.p. with 10^5 trypanosomes. After a further 24 hours the mice were drug-treated by i.p injection. Identical treatment was given to all mice within a group of six, with a number of groups allowing a range of drug doses to be given. Starting 3 days post-infection and continuing for the next 11 days, tail blood was monitored for patent infections using the rapid matching technique (Herbert & Lumsden 1976). Drug failure due to drug-resistance was judged to have occurred upon the appearance of a patent infection (i.e. greater than 2.5×10^5 trypanosomes/ml).

2.3 RESULTS

2.3.1 Selection History

Figure 2.2(a-d) shows the selection histories for the four drug-resistant

lines (247MelCy^R, 247Sur^R, 386MelCy^R and 386Sur^R). Drug-resistance was selected by incremental increases in the drug pressure. Since the sub-curative dose at any given stage of selection could only be estimated, relapse trypanosomes were often transferred to more than one mouse so that a range of sub-curative doses could be given at each step. So for example, in Figure 2.2(a), three mice were initially inoculated with 247 and subsequently treated with melCy at 0.05, 0.08 and 0.1mg/Kg. Trypanosomes multiplying in the highest drug dose, in this case those from the mouse treated with melCy at 0.1mg/Kg, were transferred to three more mice and the procedure was repeated at higher doses. The selections with each drug were stopped when the sub-curative dose had reached the maximum dose tolerated by the mice. These maximum tolerated doses were 40mg/Kg for melCy (Jennings pers. comm.) and 70mg/Kg for suramin (doses above which the physical appearance of the mice deteriorated).

2.3.2 *In Vivo* Drug-Resistance Assay

The *in vivo* drug-resistance assay was carried out at four points during the process of generating and characterising the drug-resistant clones: before selection for drug-resistance; after selection for drug-resistance and recloning; after mouse passaging in the absence of drug pressure; and after cyclical transmission through the tsetse fly. Figure 2.3(a-d) shows the results of the drug-resistance assays carried out on the cloned lines. The bars in the figure represent the proportion of mice in each drug-dose group that produced a patent infection (i.e. those mice in which the trypanosomes "resisted" drug treatment). The results show the large increases in the level of drug-resistance in the cloned drug-resistant lines when compared to the unselected stocks. To determine whether the drug-resistance phenotypes were stable in the absence of drug pressure, drug pressure was removed and the levels of drug-resistance were subsequently rechecked. The removal of drug pressure had no measurable effect on the levels

of drug-resistance, with all four drug-resistant lines expressing stable resistance after passaging in mice. 247MelCy^R was passaged through 10 mice for 52 days; 247Sur^R, 7 mice for 34 days; 386MelCy^R, 17 mice for 51 days; and 386Sur^R, 11 mice for 41 days. Furthermore, three of the four lines (247MelCy^R, 247Sur^R and 386MelCy^R) were successfully transmitted through tsetse flies, with no loss in the levels of their resistance.

The results of the *in vivo* assays can be expressed in terms of the minimum curative dose i.e. the lowest dose required to completely clear the infection from all six mice in a group. Table 2.1 expresses the results from Figure 2.3(a-d) shown as minimum curative doses. MelCy-resistance has increased from 0.3mg/Kg in 247 and 2.0mg/Kg in 386 to over 40mg/Kg in both melCy-resistant lines. Similarly, suramin-resistance has increased from 1.5mg/Kg and 2.0mg/Kg in 247 and 386 respectively to over 70mg/Kg in both suramin-resistant lines Both melCy-resistant lines and both suramin-resistant lines show stable levels of drug-resistance of 40mg/Kg and 70mg/Kg for melCy and suramin respectively.

The existence of cross-resistance between the melaminophenyl arsenicals is shown in Table 2.2(a and b). MelCy-resistance has increased by over 130-fold for 247 and this gave rise to increases in melB-resistance and melW-resistance of over 125-fold and over 50-fold respectively. The 247MelCy^R line expressed resistance to melB and melW at the maximum tolerated dose of 40mg/Kg for both drugs. Table 2.2(b) shows similar patterns of cross-resistance for 386MelCy^R. Although the levels of melB-resistance and melW-resistance for 386MelCy^R were at the maximum tolerated dose (40mg/Kg), the values for the relative increases in drug-resistance were lower than 247MelCy^R. This is due to unselected 386 expressing higher levels of resistance to all three melaminophenyl arsenicals compared to unselected 247.

It is clear from Table 2.3(a-d) that there was no cross-resistance between suramin and melCy in any of the four cloned resistant lines. Although 386MelCy^R has shown a very slight increase in resistance to suramin from 2.0mg/Kg to 2.5mg/Kg (Table 2.3(b)) and 386Sur^R has shown a similarly small increase in melCy-resistance from 2.0mg/Kg to 3.0mg/Kg (Table 2.3(d)), these represent increases of only 1.3-fold and 1.5-fold respectively.

2.4 DISCUSSION

Four cloned drug-resistant lines have been generated through the selection of drug-resistant sub-populations by incremental increases in drug pressure. An *in vivo* selection was chosen in preference to an *in vitro* selection to more closely parallel drug pressures that would occur in natural populations in the field. The *in vivo* selection fulfils this criterion in a number of ways. Firstly, it was carried out in a mammalian murine host in which each drug was more likely to retain the same pharmacological activity as in the human host. An *in vitro* selection would not have this advantage. Secondly, the two lines chosen (247 and 386) were not available in adapted long-term bloodstream culture (although they have since been adapted (Sutherland, personal communication)). Therefore, an *in vitro* selection would have had to have been carried out on procyclic culture forms, which, on account of the known differences between bloodstream and procyclic metabolism, could have resulted in a different mechanism of drug-resistance.

The selection procedures were carried out in immunosuppressed mice (cyclophosphamide at a dose of 250mg/Kg by i.p. injection 24 hours prior to trypanosome inoculation)). In attempting to mimic drug-resistance selection in the field, it was difficult to ascertain whether or not the mice should be immunosuppressed. Trypanosomiasis occurs in areas of Africa in which there are

often also a number of other infections, such as malaria and toxoplasmosis (Strickland *et al.* 1972) that compromise the host immune system, as well as the immunosuppression caused by trypanosomiasis itself (Goodwin 1970). Therefore, it could be argued that some degree of immunosuppression would inevitably be present in infected patients receiving drug therapy in the field, and therefore the laboratory selection should be carried out in immunosuppressed mice. On a practical level, it has been shown previously that the efficacy of melCy treatment in *T. evansi* is markedly reduced (and the subsequent development of melCy-resistance increased) in irradiated mice compared to immunocompetent mice (Osman *et al.* 1992). This loss of efficacy in immunosuppressed hosts was also shown with the melaminophenyl arsenical, melB (Frommel 1988). The role of suramin and the host immune system has been established during the early years of suramin use, with studies using mice that were immunosuppressed by splenectomy and blocking their reticuloendothelial system with colloidal copper. Similar to the situation with the melaminophenyl arsenicals, immunosuppression led to a decrease in suramin efficacy and an increase in the rate of development of suramin-resistance (Von Jancso and Von Jancso 1934; Von Jancso and Von Jancso 1935). It was suggested that immunosuppressed hosts in the field play a significant role in the development of drug-resistance, and it was for this reason and for the speed at which drug-resistance could be generated that immunosuppressed mice were used.

A number of further points deserve to be mentioned with reference to the selection procedures. Firstly, the general selection procedure that was employed selected for two phenotypes: the desired drug-resistance phenotype; but also a monomorphic phenotype. The selection procedure was, in effect, rapid passaging of a trypanosome line from one mouse to the next, and this selected for trypanosomes with a higher overall growth rate in mice (i.e. either a shorter cell

cycle or a reduction in the ability to transform to short-stumpy forms, or both (Turner 1990). Although no accurate measurements of doubling time were made, the resistant clones undoubtedly grew more rapidly in mice, and this was especially marked for 386Sur^R. Furthermore, the drug-resistant lines proved more difficult to transform from bloodstream to procyclic culture forms compared to the unselected stocks (**section 3.3.1**).

More specifically, two problems were associated with the generation of the suramin-resistant lines. In the immunosuppressed mice, suramin required approximately 2 days to clear the trypanosomes from the blood. The first attempt to generate a suramin-resistant line was with 386. Trypanosomes were taken from mice one day after being treated with suramin, on the assumption that they were resistant to the level of drug administered, when in fact the drug had not had time to take full effect. Thus, when the trypanosomes were transferred to another mouse they had time to recover and multiply. Repetition of this procedure resulted in mice being treated with suramin at 70mg/Kg and the trypanosomes in the blood one day later were cloned. However these clones proved to only have a low level of resistance to suramin (10mg/Kg) when they were rechecked. The procedure for selection described in section 2.2.4 where trypanosomes were only taken from subsequent relapse infections after drug treatment solved this problem. The second problem associated with the selection for suramin-resistance was due to the pharmacology of the drug. Suramin binds to plasma proteins and has a half-life of approximately 50 days (Collins *et al.* 1986). Consequently, the process of passaging trypanosomes from a suramin treated mouse, which will transfer approximately 0.5ml of blood, to another mouse, will also transfer suramin bound to blood proteins. This accumulation of suramin with the addition of a fresh dose one day after infection could account for the finding that 247Sur^R has a minimum curative dose of greater than 70mg/Kg although it was apparently only selected to a level of 50mg/Kg. A

further explanation for this observation could simply be that the mutation that was selected for at 50mg/kg also confers resistance at a higher dose than the one at which it was selected. This is almost certainly the case with the arsenical-resistance in 247MelCy^R **(section 3.4)**

The *in vivo* assay used was designed so that drug failure would only be seen if the trypanosomes had multiplied in the presence of the drug and were therefore drug-resistant. The rapid matching technique (Herbert and Lumsden 1976), used to assess the mouse parasitaemia, was only sensitive to parasitaemias greater than $2.5x10^5$ trypanosomes/ml, and since each mouse received only 10^5 trypanosomes, a patent parasitaemia would only be seen if the trypanosomes had multiplied. The parasitaemias were monitored for a full 14 days following the initial trypanosome inoculation as an immunosuppressed mouse inoculated with a single trypanosome of these lines will always produce a patent infection within 6-12 days. It may have been possible for some of the faster growing lines to multiply at such a rate that there would have been a patent infection one day after inoculation. For this reason, tail blood was monitored 48 hours after drug administration to allow the trypanocidal action of the drugs to take full effect. Finally, to ensure the drug was acting in the circulation and not locally in the peritoneal cavity, drug treatment was given 24 hours after trypanosome inoculation as it takes up to 24 hours for the trypanosomes to pass from the peritoneal cavity into the blood circulation.

Table 2.2(a and b) show the minimum curative doses of the melaminophenyl arsenicals melCy, melB and melW for the unselected stocks. Treatment with melB in clinical cases is carried out at drug doses of between 1.2g and 3.6mg/Kg (WHO 1986b) and this would be sufficient to cure infections of 247 and 386 with minimum curative doses of 0.32 and 0.64mg/Kg respectively. In a patient from Uganda infected with *T. b. rhodesiense*,

trypanosomes were isolated that required a dose of melB of more than 10mg/Kg to cure the infection in mice (Robertson 1963). This patient suffered relapse infections after melB and melW treatment, and the persistent use of drug-treatment led to the development of melB-resistant trypanosomes that were resistant to a dose of melB of 30mg/Kg in mice. Therefore, the minimum curative doses presented here for 247MelCy^R and 386MelCy^R compare favourably with the levels of melB-resistance in clinical cases. Minimum curative doses for melCy in mice infected with isolates of *T. b. brucei* vary from 1.6mg/Kg to 6.3mg/Kg (Zweygarth and Kaminsky 1990). Doses above 0.5mg/Kg have been found to be sufficient to cure buffaloes naturally infected with *T. evansi* (Lun *et al.* 1991) and above 0.3mg/Kg for *T. evansi* infected camels (Zelleke *et al.* 1989). Suramin, on the other hand, is administered to infected individuals at doses of approximately 20mg/Kg (Voogd *et al.* 1993). This is far in excess of the minimum curative doses of 1.5 and 2.0mg/Kg for 247 and 386 respectively (Table 2.1). However, infections with the suramin-resistant lines would not be cured by this treatment. Suramin-resistant isolates of *T. b. brucei* have been identified with minimum curative doses greater than 40mg/Kg (Zweygarth and Kaminsky 1990) and isolates of *T. evansi* have been found with minimum curative doses of 20mg/Kg compared to sensitive stocks that were susceptible to doses between 0.5 and 4mg/Kg (Zhang *et al.* 1992).

The levels of resistance to the melaminophenyl arsenicals were consistently higher in 386 than 247 (Table 2.2(a and b)) The most marked difference occurred with melCy, with 247 expressing a level of melCy-resistance of 0.3mg/Kg compared to 2.0mg/Kg for 386. One possible explanation for this could be that the human-infective nature of 386 (*T. b. rhodesiense*) would make it more likely to have been exposed to sub-curative doses of melB than 247 (*T. b. brucei*) since the melaminophenyl arsenicals are not used to treat *T. b. brucei* infections in animals. Consequently, 386 may have developed a slight degree of

melB-resistance during its evolution in Africa. Alternatively, this difference may reflect only a naturally occurring variation in metabolism between trypanosome field populations from which these two isolates were taken.

The results of the *in vivo* assays show that the four cloned drug-resistant lines (247MelCy^R, 247Sur^R, 386MelCy^R and 386Sur^R) express stable resistance after they have been passaged through mice for a number of weeks in the absence of drug pressure. More significantly, three lines (247MelCy^R, 247Sur^R and 386MelCy^R) were cyclically transmitted through tsetse flies. This process took over 3 weeks and involved at least five distinct differentiation steps (Vickerman 1985) all undertaken in the absence of drug pressure. All three lines maintained their drug-resistance, thus demonstrating that the drug-resistance phenotypes were stable (Table 2.1).

Cross-resistance between the melaminophenyl arsenicals was observed in both of the melCy-resistant lines (Table 2.2(a and b)). The *in vivo* assay was unable to measure minimum curative doses above 40mg/Kg, and consequently it was impossible to determine possible higher levels of resistance to the melaminophenyl arsenicals. Important differences between the levels of drug-resistance between the melaminophenyl arsenicals may therefore exist, evidence for which can be seen *in vitro* (**chapters 3 and 6**). There was no conclusive evidence for the existence of cross-resistance between suramin and melCy, in agreement with previous findings for suramin and the melaminophenyl arsenicals (Gill 1971; Pospichal *et al.* 1994). One previous study showed a 5.8-fold increase in suramin-resistance in a melarsen-resistant line, although no explanation was given for this (Fairlamb *et al.* 1992b). Two lines in the present study showed very slight degrees of cross-resistance (a 1.3-fold increase in suramin-resistance in 386MelCy^R and a 1.5-fold increase in melCy-resistance in 386Sur^R). However, the nature of the *in vivo* assay and the extremely small

extent to which these two lines expressed cross-resistance does not allow any biological significance to be placed on these data with any confidence. It was therefore concluded that whatever biochemical mechanisms were being employed by the trypanosomes to resist drug treatment, the mechanism(s) conferring suramin-resistance are different to those for melCy-resistance. This lack of cross-resistance between the two drugs was vital for future studies using the drug-resistance phenotypes as selectable markers for genetic exchange.

Figure 2.1
***In Vivo* Generation and Characterisation of a Stable Drug-Resistant Clone**

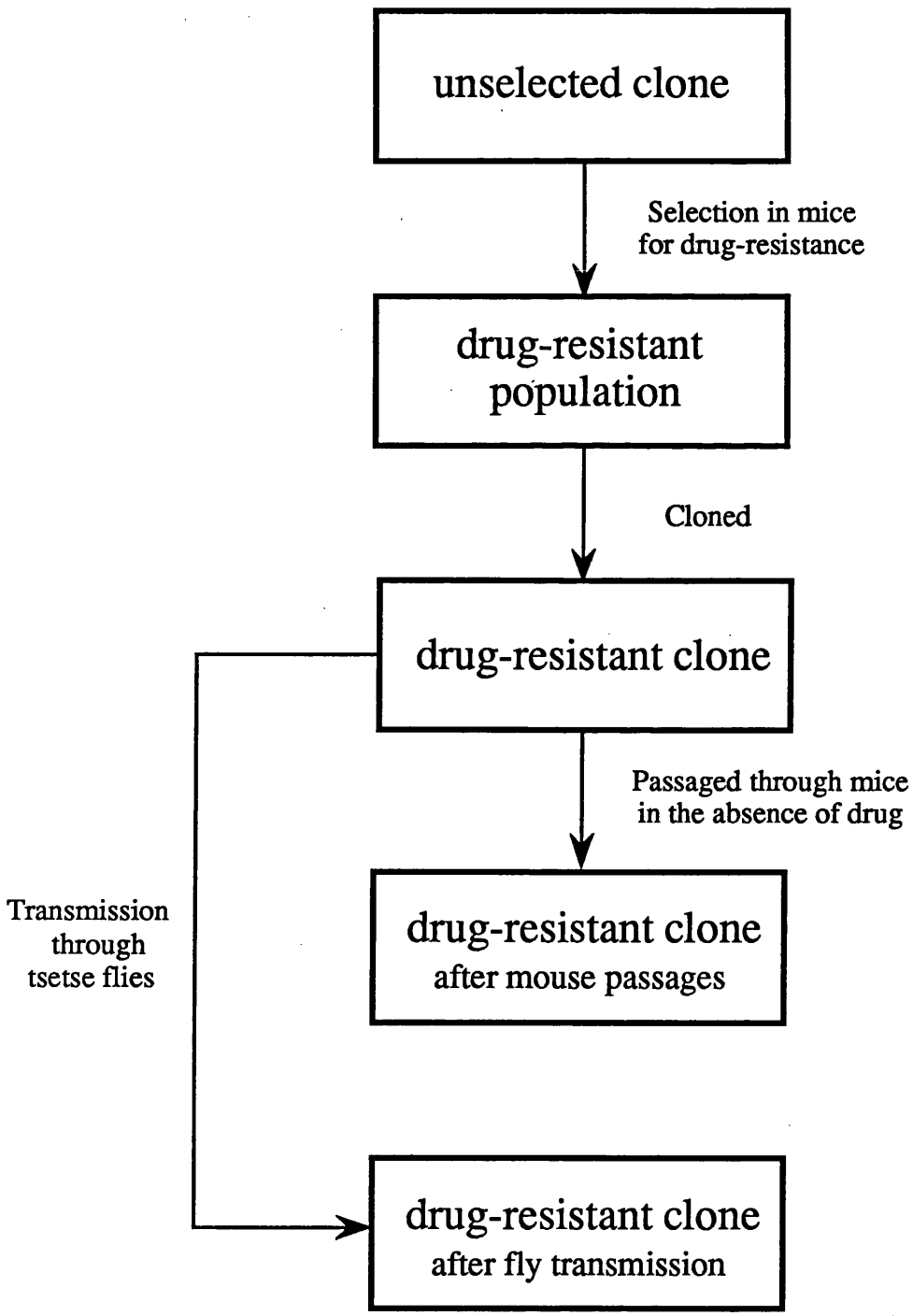


Figure 2.1
***In Vivo* Generation and Characterisation of a Stable Drug-Resistant Clone**

The flow diagram shows the stages through which a cloned, stable drug-resistant line was generated from an unselected drug-sensitive stock. The drug-resistant population was generated in mice through gradual increases in sub-curative drug treatment and subsequently cloned to ensure the homogeneity of the drug-resistant line. This cloned line was then passaged through mice in the absence of drug pressure and cyclically transmitted through tsetse flies to demonstrate the stability of the drug-resistance phenotype.

Figure 2.2(a)
Selection History for 247MelCy^{R}

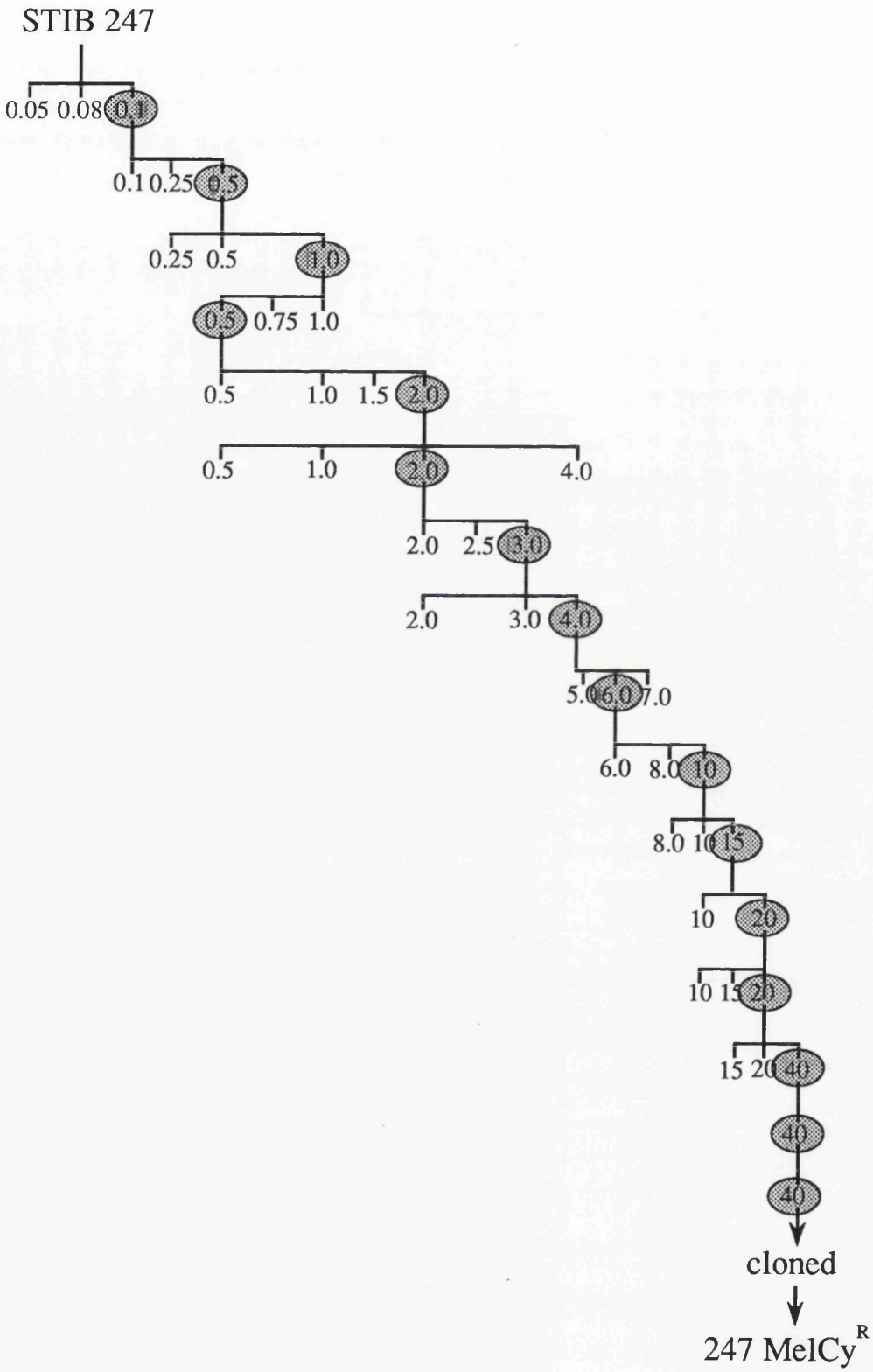


Figure 2.2(a)
Selection History for 247MelCy^R

This diagram plots the course through which the 247 unselected stock was passaged in mice and exposed to gradually increasing sub-curative drug doses to generate the 247MelCy^R clone. The numbers in the figure represent these sub-curative doses of melCy in mg/Kg body weight. The longer vertical lines indicate the point at which trypanosomes from a relapse infection were transferred to a further group of mice, with the range of drug doses they subsequently received being indicated by the horizontal lines.

Figure 2.2(b)
Selection History for 247Sur^{R}

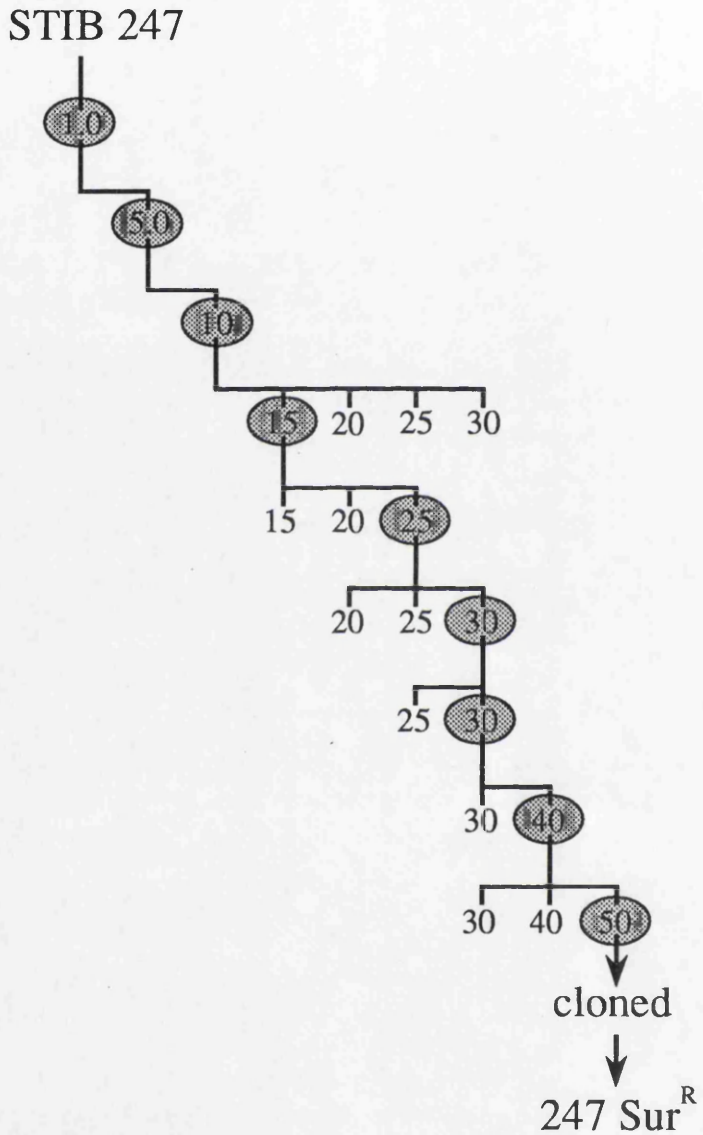


Figure 2.2(b)
Selection History for 247Sur^R

This diagram plots the course through which the 247 unselected stock was passaged in mice and exposed to gradually increasing sub-curative drug doses to generate the 247Sur^R clone. See legend for Figure 2.2(a) for an explanation.

Figure 2.2(c)
Selection History for 386MelCy^{R}

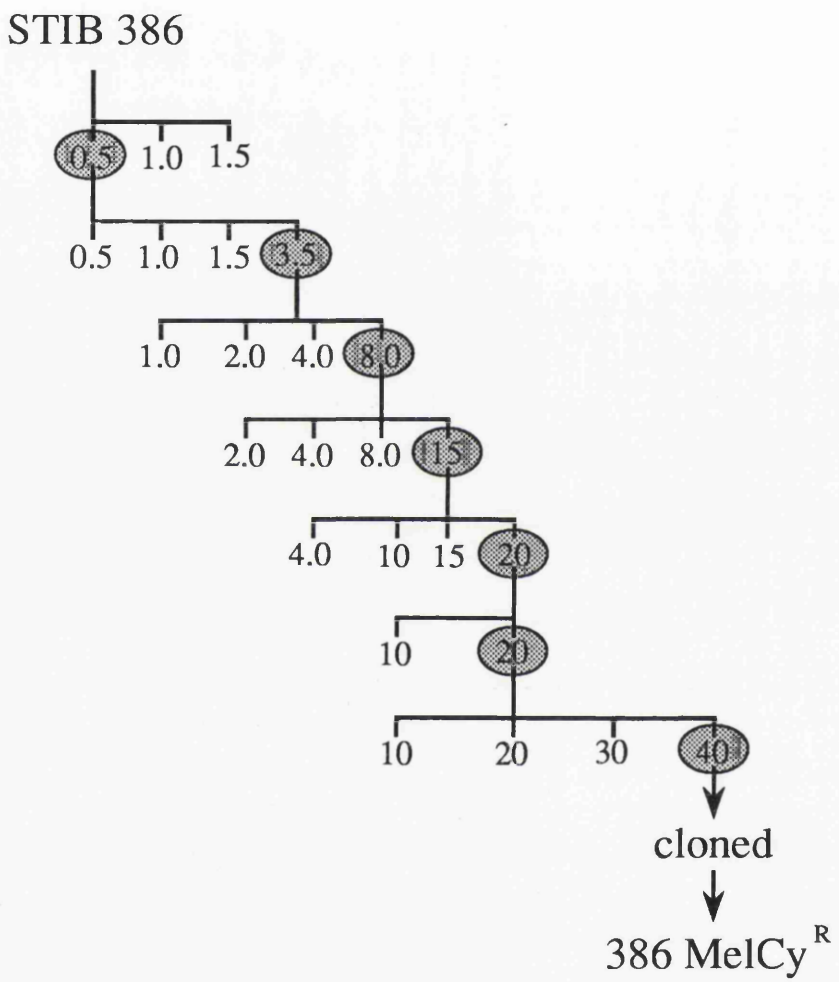


Figure 2.2(c)
Selection History for 386MelCy^R

This diagram plots the course through which the 386 unselected stock was passaged in mice and exposed to gradually increasing sub-curative drug doses to generate the 386MelCy^R clone. See legend for Figure 2.2(a) for an explanation.

Figure 2.2(d)
Selection History for 386Sur^{R}

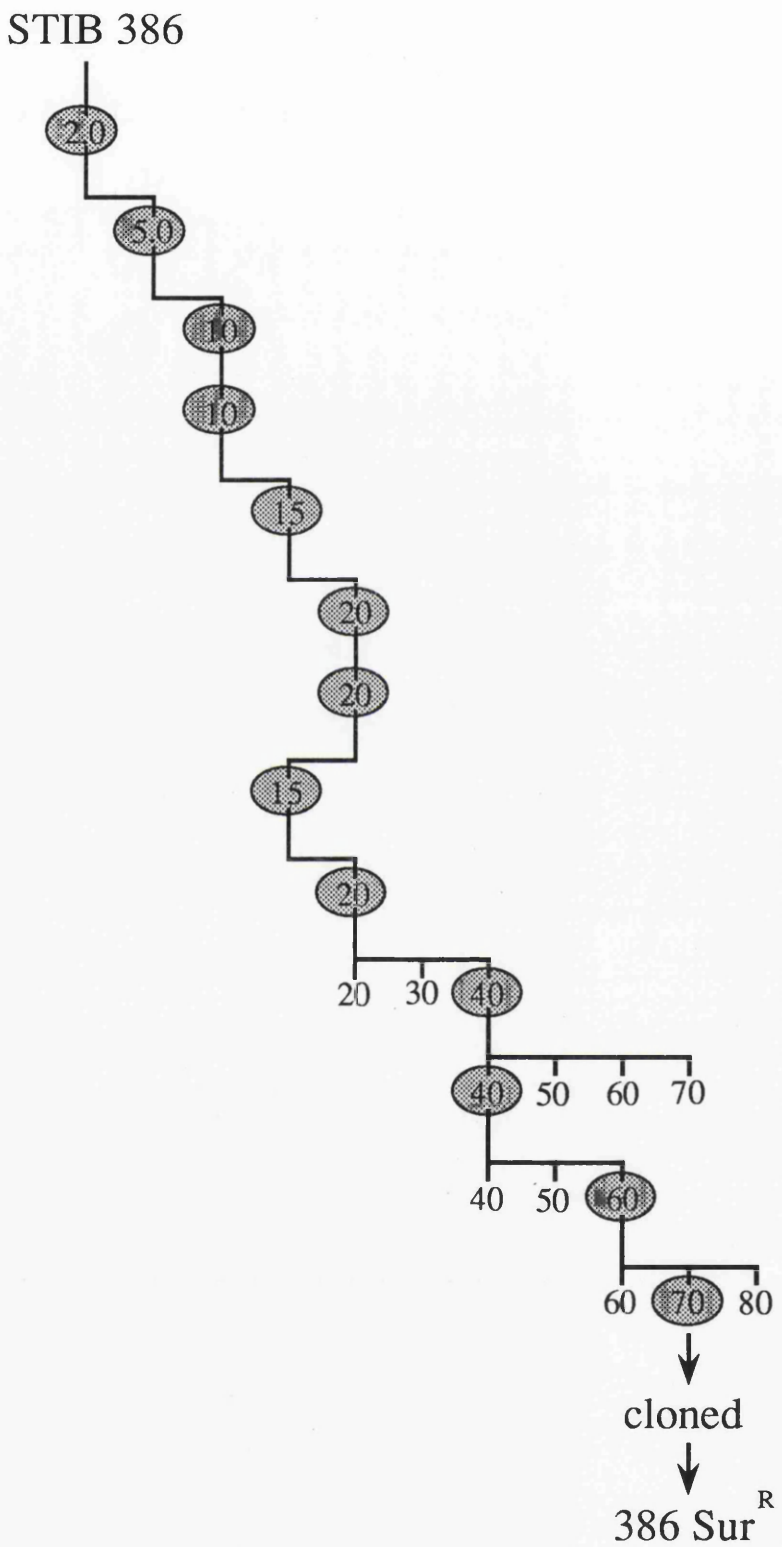


Figure 2.2(d)
Selection History for 386Sur^R

This diagram plots the course through which the 386 unselected stock was passaged in mice and exposed to gradually increasing sub-curative drug doses to generate the 386Sur^R clone. See legend for Figure 2.2(a) for an explanation.

Figure 2.3(a)
***In Vivo* Assay for MelCy-Resistance in 247 and 247MelCy^{R}**

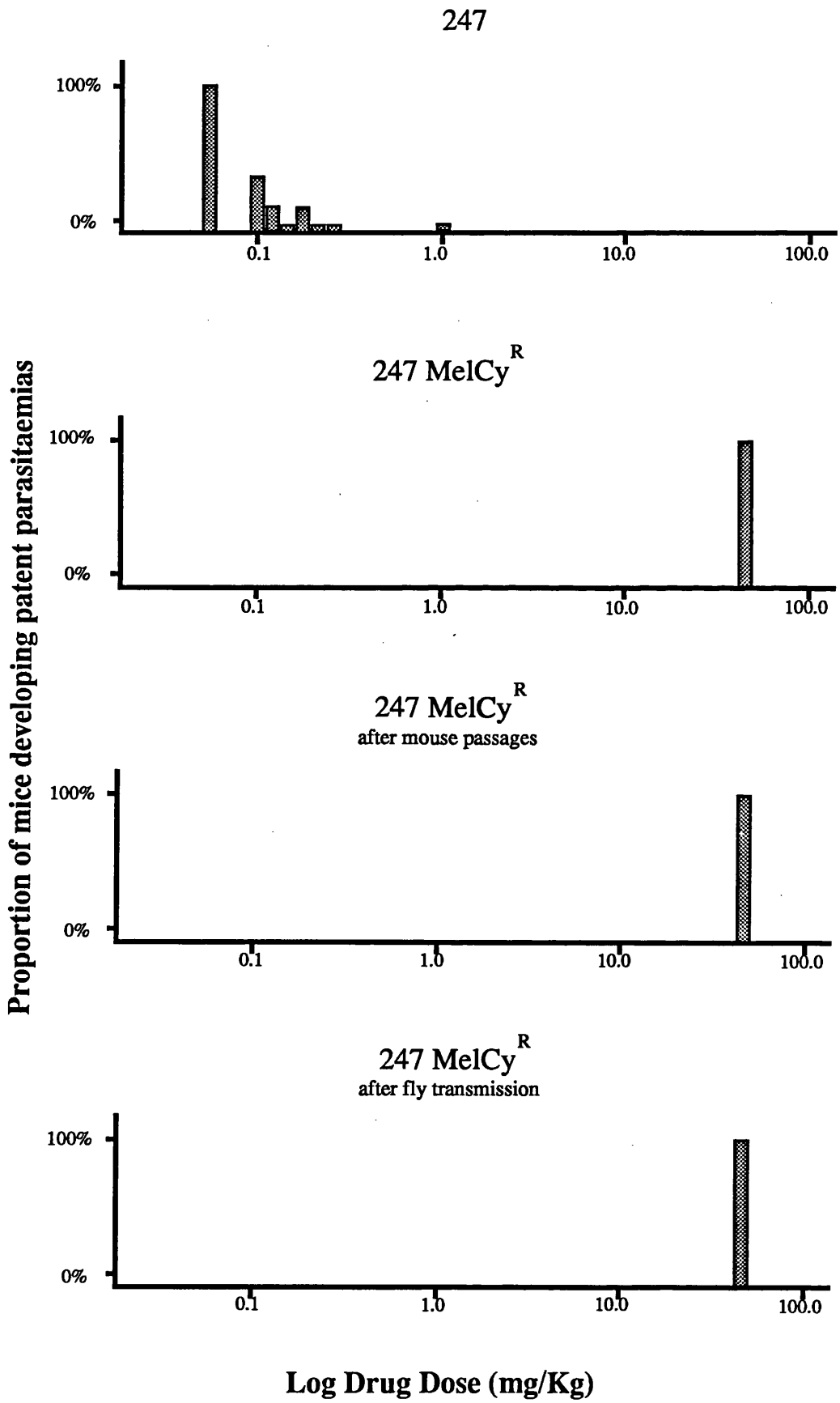


Figure 2.3(a)
***In Vivo* Assay for MelCy-Resistance in 247 and 247MelCy^R**

The results of the *in vivo* drug-resistance assays for 247 and 247MelCy^R are shown graphically as the proportion of mice in each group of six that developed patent infections (indicated by the vertical bars). The horizontal axis is a logarithmic scale and indicates the dose of melCy (mg/Kg body weight) that was administered to each group of mice. The assays were carried out at four points during the process of generating and characterising 247MelCy^R: before selection for drug-resistance; after selection for drug-resistance and recloning; after mouse passaging in the absence of drug pressure; and after cyclical transmission through the tsetse fly.

Figure 2.3(b)
***In Vivo* Assay for Suramin-Resistance in 247 and 247Sur^R**

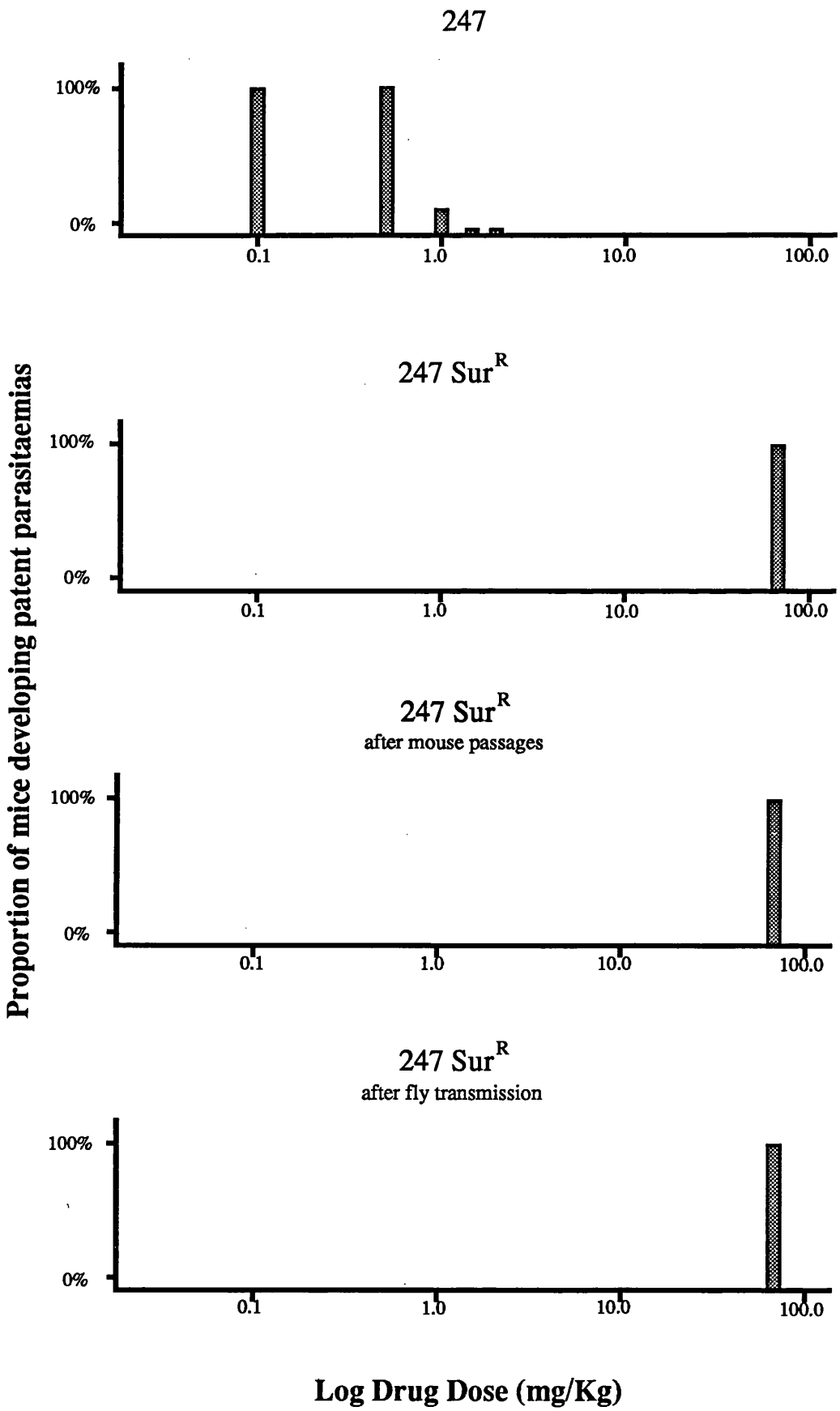


Figure 2.3(b)
***In Vivo* Assay for Suramin-Resistance in 247 and 247Sur^R**

The results of the *in vivo* drug-resistance assays for 247 and 247Sur^R are shown graphically. See legend for Figure 2.3(a) for an explanation.

Figure 2.3(c)
In Vivo Assay for MelCy-Resistance in 386 and 386MelCy^{R}

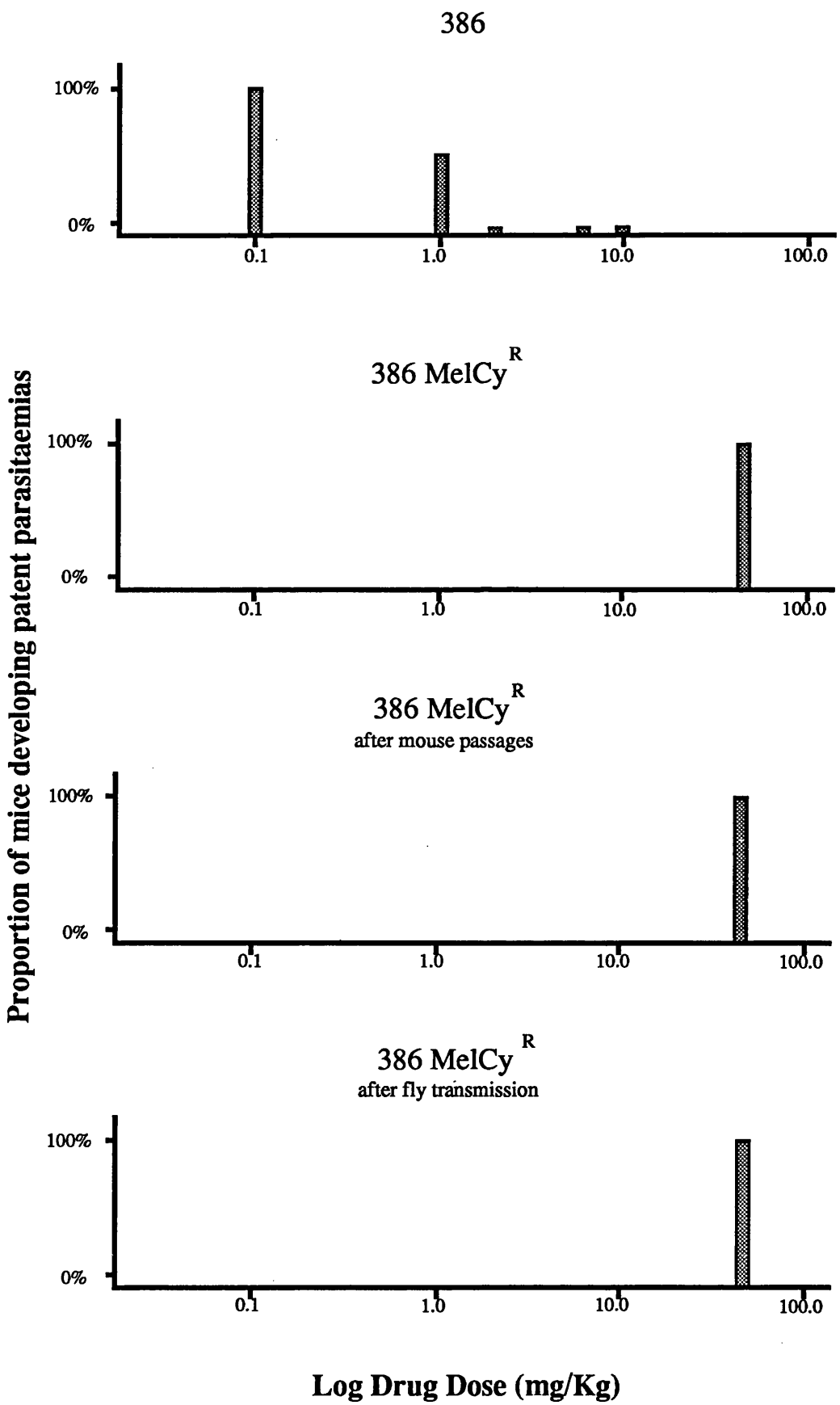


Figure 2.3(c)
***In Vivo* Assay for MelCy-Resistance in 386 and 386MelCy^R**

The results of the *in vivo* drug-resistance assays for 386 and 386MelCy^R are shown graphically. See legend for Figure 2.3(a) for an explanation.

Figure 2.3(d)
***In Vivo* Assay for Suramin-Resistance in 386 and 386Sur^R**

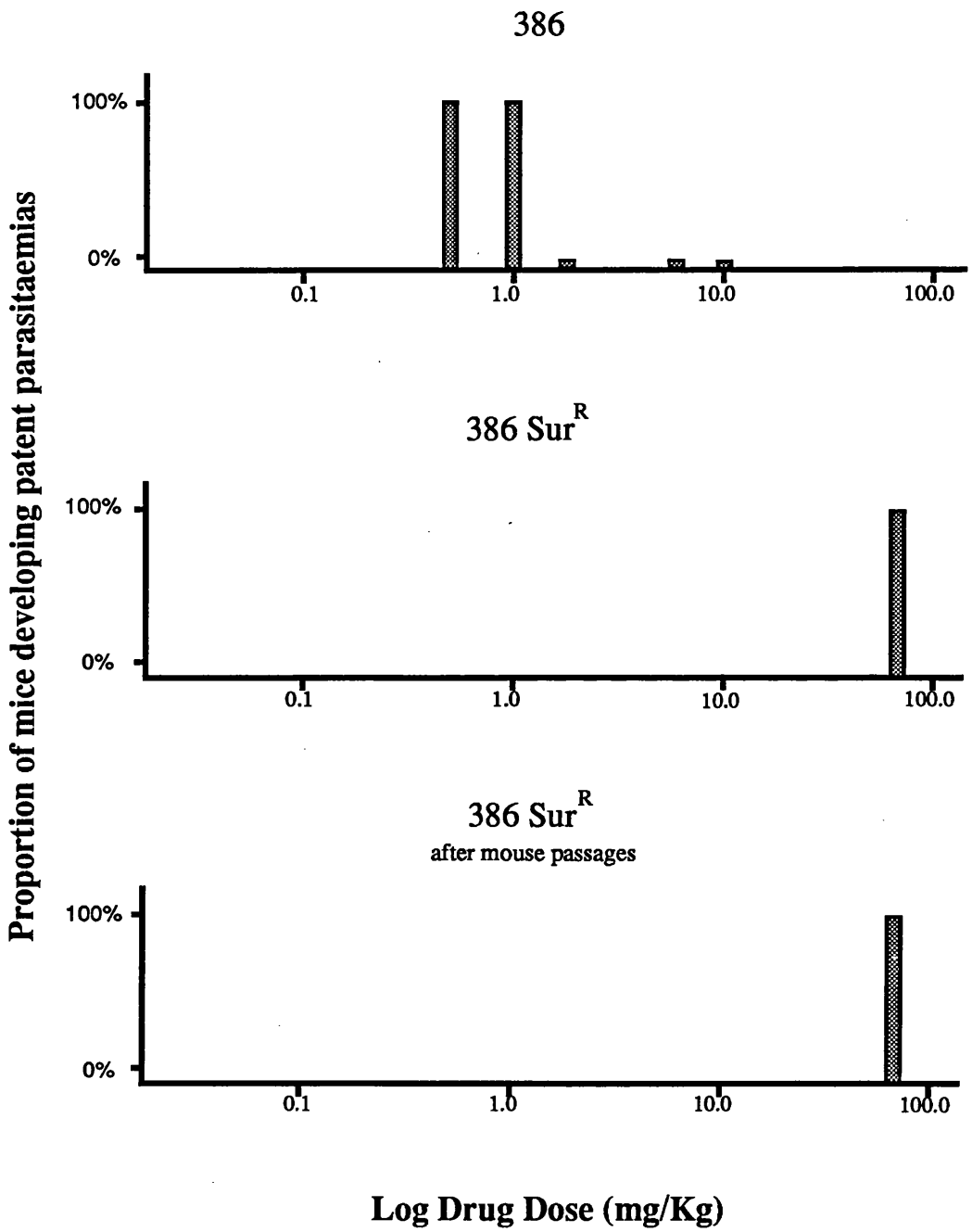


Figure 2.3(d)
***In Vivo* Assay for Suramin-Resistance in 386 and 386Sur^R**

The results of the *in vivo* drug-resistance assays for 386 and 386Sur^R are shown graphically. See legend for Figure 2.3(a) for an explanation with the exception that 386Sur^R was not cyclically transmitted through tsetse flies.

Table 2.1
Minimum Doses of Drug Required to Cure Infections before and after Selection for Drug-Resistance and after Passaging of Resistant Lines through Mice and Fly-Transmission

	Minimum Curative Dose			
	MelCy (mg/Kg)		Suramin (mg/Kg)	
	247MelCy^R	386MelCy^R	247Sur^R	386Sur^R
Unselected Line	0.3[a]	2.0[b]	1.5[c]	2.0[d]
Resistant Line	>40[e]	>40[e]	>70[f]	>70[g]
Resistant Line (passaged)	>40[e]	>40[e]	>70[f]	>70[f]
Resistant Line (fly-transmitted)	>40[e]	>40[e]	>70[f]	ND

The results of the *in vivo* assays for melCy and suramin are expressed in terms of the minimum curative doses required to cure mice infected with the unselected stocks and the four drug-resistant lines. For each drug-resistant line, the level of resistance is given for the drug against which it was generated, and also the level of resistance in the unselected line from which it was generated.

[a] Doses tested were 0.05, 0.1, 0.2, 0.25, 0.3, 0.4, 0.5 and 1.0mg/Kg
[b] Doses tested were 0.1, 1.0, 2.0, 5.0 and 10mg/Kg
[c] Doses tested were 0.1, 0.5, 1.0, 1.5, 2.0mg/Kg
[d] Doses tested were 0.5, 1.0, 1.5, 2.0 and 2.5mg/Kg
[e] Dose tested was 40mg/Kg
[f] Dose tested was 70mg/Kg
[g] Doses tested were 20, 30, 40, 50, 60 and 70mg/Kg

Table 2.2
Cross-Resistance between Melaminophenyl Arsenicals
(a) in 247MelCy^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	247	247MelCy^R	
MelCy	0.3	>40	>130
MelB	0.32[a]	>40[b]	>125
MelW	0.8[c]	>40[b]	>50

(b) in 386MelCy^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	386	386MelCy^R	
MelCy	2.0	>40	>20
MelB	0.64[d]	>40[b]	>63
MelW	3.2[e]	>40[b]	>13

Minimum curative doses are shown for (a) 247 and 247MelCy^R and (b) 386 and 386MelCy^R for melCy, melB and melW. The resistance factor shows the degree to which the resistant lines have increased their level of drug-resistance and is a ratio of the minimum curative doses for the resistant compared to the unselected lines.

[a] Doses tested were 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and 1.3mg/Kg
[b] Dose tested was 40mg/Kg
[c] Doses tested were 0.1, 0.2, 0.4, 0.8 and 1.6mg/Kg
[d] Doses tested were 0.08, 0.16, 0.32, 0.64 and 1.3mg/Kg
[e] Doses tested were 0.2, 0.4, 0.8, 1.6 and 3.2mg/Kg

Table 2.3
Lack of Cross-Resistance between Melaminophenyl Arsenicals and Suramin
(a) in 247MelCy^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	247	247MelCy^R	
MelCy	0.3	>40	>130
Suramin	1.5	1.5[a]	1.0

(b) in 386MelCy^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	386	386MelCy^R	
MelCy	2.0	>40	>20
Suramin	2.0	2.5[b]	1.3

(c) in 247Sur^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	247	247Sur^R	
Suramin	1.5	>70	>47
MelCy	0.3	0.3[c]	1.0

(d) in 386Sur^R

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	386	386Sur^R	
Suramin	2.0	>70	>35
MelCy	2.0	3.0[d]	1.5

Table 2.3
Lack of Cross-Resistance between Melaminophenyl Arsenicals and Suramin

Minimum curative doses together with the resistance factors are shown for (a) 247 and 247MelCy^R, (b) 386 and 386MelCy^R, (c) 247 and 247Sur^R and (d) 386 and 386Sur^R for melCy and suramin.

[a] Doses tested were 0.5, 1.5 and 2.0mg/Kg
[b] Doses tested were 1.5, 2.0, 2.5 and 3.0mg/Kg
[c] Doses tested were 0.3 and 0.4mg/Kg
[d] Doses tested were 2.0, 2.5 and 3.0mg/Kg

CHAPTER 3
IN VITRO CHARACTERISATION OF CLONED LINES

3.1 INTRODUCTION

Since the earliest beginnings of trypanocide development, starting with Ehrlich's mouse model infected with *T. equinum*, there has been a large number of different methods used to assess and compare the trypanocidal activity of different compounds. More importantly for the present study, a similarly extensive array of methods have been used to assess and compare the levels of trypanocide-resistance/sensitivity in a wide range of field isolates and laboratory-derived trypanosome lines (Kaminsky and Brun 1993). A huge variety of screening systems have been employed using both *in vivo* and *in vitro* methods, with the use of different trypanosome lines or life cycle stages, with different hosts or culture systems, and with different criteria, including long-term viability (Kaminsky *et al.* 1989), [^{3}H]-hypoxanthine incorporation (Brun *et al.* 1989; Pospichal *et al.* 1994) and growth inhibition (Zhang *et al.* 1992), having been used to measure the level of drug-resistance, and this has ultimately made the task of comparing data from different sources very difficult if not, in some cases, impossible.

The last 20 years has seen the gradual improvement of semi-defined media capable of sustaining trypanosome growth *in vitro* for indefinite periods. Particularly important for the present study has been the work initiated by Hirumi for bloodstream forms (Hirumi *et al.* 1977) and improved by Baltz (Baltz *et al.* 1985) and the work by Brun and colleagues for procyclics (Brun and Schonenberger 1979). This has led to the design of many *in vitro* drug-resistance assays for *T. brucei* and other trypanosome species (reviewed in Kaminsky and Brun 1993). Ideally, the perfect assay should mimic the environment in which

the drug interacts with the trypanosome, in other words the body fluids of an infected human, and the results should correlate closely with clinical findings. Since clinical data is not available for laboratory generated lines (and most of the field isolates), the validity of each *in vitro* assay system should therefore, where possible, be judged with reference to parallel *in vivo* assays, usually carried out in mice. Bloodstream cultures offer a distinct advantage over procyclic cultures, not only because this *in vivo* comparison can be made more directly, but more importantly, because of the biochemical differences that are known to exist between the mammalian and insect stages of trypanosomes, particularly with respect to energy metabolism (reviewed in Opperdoes 1985). This point is particularly valid in the case of assays involving suramin, which inhibits at least 9 glycolytic enzymes (Willson *et al.* 1993) at physiological concentrations (Stein *et al.* 1989) with the activity of many of these glycosomal enzymes being altered in the procyclic glycosome compared to the bloodstream glycosome (Hart *et al.* 1984). Furthermore, it is believed that suramin is taken up by receptor-mediated endocytosis (Fairlamb and Bowman 1980a; Fairlamb and Bowman 1980b), a process which does not occur in procyclics (Vickerman 1985). Despite these differences, insect-stage trypanosomes are still useful tools for *in vitro* assays, as these differences between bloodstream and procyclics can be exploited in investigations into mode of drug action. On a practical level, *in vitro* growth of procyclics is also more readily achieved compared to bloodstream forms. The majority of *in vitro* assays conducted in recent years, however, have involved the use of bloodstream cultures (Kaminsky and Brun 1993). One important problem associated with many of the bloodstream culture assay systems that have been employed has been the necessity of adapting the trypanosomes to culture before conducting the assay. This has biological implications with regards to the effect this process of adaptation may have upon the biochemistry of the trypanosome. It has been shown previously that a line of *T. evansi* kept in bloodstream culture for a prolonged period lost both its kinetoplast and its ability to infect mice

(Zweygarth *et al.* 1991). Not only does *in vitro* adaptation have biological implications, but there are practical implications too, due to the time it takes for the trypanosomes to adapt to culture, which may take a month or longer. This is unsatisfactory in clinical cases, where the rapid diagnosis of drug-resistant trypanosomes could have extremely important consequences on the health, and perhaps life, of the patient.

The various methods used for *in vitro* drug-resistance assays have been applied to a number of different trypanosome species including *T. brucei*, *T. evansi*, *T. congolense*, *T. vivax*, *T. simiae* and *T. equiperdum*. The validity of each assay system and their applicability to different drug/strain combinations have been reviewed recently (Kaminsky and Brun 1993). Briefly, the conclusion of this review was that the "assay to be applied depends largely on the specific aim of the study, on the trypanosome stocks available and on the equipment of the laboratory". It was the principal aim of the present study to further characterise the six lines described in the last chapter (247, 386, 247Sur^R, 247MelCy^R, 386Sur^R and 386MelCy^R) in terms of their levels of resistance to the melaminophenyl arsenicals and suramin and the absence or presence of cross-resistance between these compounds. A longer term objective was to study the inheritance of drug-resistance in a number of cloned progeny from a cross between two lines each expressing a different drug-resistance phenotype. Consequently, it was necessary to develop a reliable *in vitro* assay system that closely reflected the results of the *in vivo* assay for melCy and suramin, and one that did not involve the extensive adaptation of cloned lines to axenic culture. Furthermore, the *in vitro* characterisation provided further information about the possible mode of action of the drugs.

3.2 MATERIALS AND METHODS

3.2.1 Drugs

MelCy, melB, melW and suramin were supplied and stored as described previously (**section 2.2.1**). Melarsen oxide (melOx) was a kind gift from Dr. Frank Jennings, Glasgow University. MelOx was stored as a solution in propylene glycol at 4^0C.

3.2.2 Trypanosome Stocks and Mice

Six cloned lines were used: the two unselected sensitive lines (247 and 386) and the four drug-resistant clones described in the previous chapter (247Sur^R, 247MelCy^R, 386Sur^R and 386MelCy^R). Growth of trypanosomes was carried out in immunosuppressed (cyclophosphamide at a dose of 250mg/Kg by i.p. injection) adult female CFLP, CD1 or TO Swiss mice, or adult male CFLP mice.

3.2.3 *In Vitro* Cultivation of Trypanosomes

The six cloned trypanosome lines described in chapter 2 were further characterised *in vitro* using two culture systems: an established procyclic culture; and a short-term non-adapted bloodstream culture that sustained growth for 48 hours.

Growth of Procyclic Trypanosomes

Bloodstream trypanosomes were grown in mice, harvested under sterile conditions and transformed to procyclic forms in SDM-79 supplemented with 10% heat-inactivated foetal calf serum (Gibco) (Brun & Schonenberger 1979)

plus 3mM cis-aconitate (10µl of cis-aconitate from a 52mg/ml stock solution per 1ml complete medium) at 26^0C. Procyclic cultures were then maintained in complete SDM-79 (SDM-79 supplemented with 10% heat-inactivated foetal calf serum) at 26^0C at a trypanosome density between 10^6/ml and 10^7/ml. Trypanosome growth for the six cloned lines (247, 386, 247MelCy^R, 386MelCy^R, 247Sur^R, and 386Sur^R) was measured with established procyclic cultures that had been transformed and maintained for less than 2 months. Trypanosome counts were carried out using an Improved Neubauer haemocytometer in octuplicate, every 24 hours, over 5 days from a starting density of 10^6 trypanosomes/ml. The rate of growth, b, over the first 3 days was calculated by least squares regression analysis (Minitab Release 8.2 Extended). The mean population doubling times (PDTs) were calculated from the growth rates using the following equation:

$$PDT = \frac{\ln 2}{b}$$

In order to determine if the growth rates of the unselected lines were significantly different from those of the drug-resistant lines, statistical comparisons were made using analysis of covariance

Procyclic trypanosome stabilates were cryopreserved using procyclics from a dense culture (>10^7 trypanosomes/ml) which were centrifuged at 900g for 10 minutes at 4^0C. Pellets were resuspended in heat-inactivated foetal calf serum containing 10% DMSO to give a final trypanosome density of 10^7-10^8 trypanosomes/ml. Aliquots of these were then frozen and stored in liquid nitrogen.

Growth of Bloodstream Trypanosomes

A short-term bloodstream culture system was used that supported growth of trypanosomes for at least 48 hours. The medium used was based upon that described by Baltz *et al.* (1985) modified by Mhlanga & Shall (unpublished data). The bloodstream culture medium used (hereafter called B.S.C.M) was made up in two parts as follows:

INCOMPLETE MEDIUM

Minimum Essential Medium with Earle's salts (Gibco)	1 litre
Non-Essential Amino Acid Concentrate (x100) (Gibco)	10ml
Hepes (Sigma)	6.0g
Glucose (BDH)	1.0g
Sodium Bicarbonate (BDH)	2.2g
L-Glutamine (Sigma)	0.3g

This was made to a final volume of 1100ml with $dddH_2O$ and the pH was adjusted to 7.3 with 4M NaOH. It was then filter sterilised through a 0.22μm filter and could be stored for up to a maximum of 3 months at 4^0C. Immediately before use, the incomplete medium was further supplemented to give the following final concentrations:

Thymidine (Gibco)	0.1mM
Hypoxanthine (Gibco)	0.016mM
Dithiothreitol (BDH)	0.4mM
Sodium Pyruvate (Gibco)	2.0mM
Catalase (Sigma)	1.7μg/ml
Adenosine (Sigma)	0.04mM
Guanosine (Sigma)	0.02mM
Foetal Calf Serum (Gibco)[a]	20%(v/v)

[a] Techgen foetal calf serum was used for a small number of growth inhibition assays.

To obtain these final concentrations for the supplements, approximately 5ml of the following solutions were initially made up in incomplete medium. These solutions were made up immediately before use. Any unused solutions were discarded:

Dithiothreitol (BDH)	6.17mg/ml
Sodium Pyruvate (Gibco)	22.0mg/ml
Catalase (Sigma)	0.17mg/ml
Adenosine (Sigma)	1.07mg/ml
Guanosine (Sigma)	0.566mg/ml

Equal volumes of these five solutions were mixed together and filter sterilised through a 0.22μm nylon filter. 100ml of complete medium was then made up from 5ml of this sterile solution, 73ml incomplete medium, 1ml Thymidine (x100) (Gibco), 1ml Hypoxanthine (x100) (Gibco) and 20ml FCS (Gibco).

Bloodstream trypanosomes were then harvested from a mouse, with a parasitaemia (in logarithmic growth) greater than 3.2×10^7 trypanosomes/ml, by cardiac puncture under sterile conditions into a syringe containing 0.1ml heparin (500units/ml) in Earle's balanced salt solution. The infected blood was then mixed with two volumes of complete B.S.C.M. and spun at room temperature for 10 minutes at 150g. The resulting supernatant containing trypanosomes was then removed and the trypanosomes were resuspended in complete B.S.C.M. at a density of 10^5 trypanosomes/ml. Cultures were incubated in 5%CO_2/95%air at 37^0C. Growth of the two sensitive lines, 247 and 386, was measured using an Improved Neubauer haemocytometer in octuplicate, approximately every 12 hours, for 3½ days.

3.2.4 *In Vitro* Growth Inhibition Assay

The cloned trypanosome lines were assessed for their levels of resistance to a number of trypanocides using both established procyclic cultures (suramin, melCy, melW, melB and melOx), and short-term, unadapted bloodstream cultures (suramin, melCy and melB). For both assay systems, drugs were dissolved in the appropriate complete medium and filter sterilised through a 0.22μm nylon filter immediately before use. Microtitre plates were used to give a range of drug concentrations by doubling dilutions, each well receiving a final volume of 50μl drug solution, and wells being grouped in fours for quadruplicate counts.

Growth Inhibition of Procyclic Trypanosomes

All dilutions of both trypanosomes and drugs were carried out in complete SDM-79. The trypanosomes were resuspended at a density of $2x10^6$ trypanosomes/ml. 50μl of this was added to each well containing 50μl of drug giving a starting density in drug at 0 hours of 10^6 trypanosomes/ml. The plates were covered and incubated at 26^0C for 24 hours after which the trypanosomes in each well were counted using an Improved Neubauer haemocytometer.

Growth Inhibition of Bloodstream Trypanosomes

Bloodstream trypanosomes were harvested from a mouse and purified as described above. All dilutions of both trypanosomes and drugs were carried out in complete B.S.C.M. The trypanosomes were resuspended in complete B.S.C.M. to a density of $2x10^5$ trypanosomes/ml. 50μl of this was added to each well containing 50μl of drug giving a starting density in drug at 0 hours of 10^5 trypanosomes/ml. The plates were covered and incubated in 5%CO_2/95%air at

37^0C for 24 hours, after which the trypanosomes were counted using an Improved Neubauer haemocytometer.

3.3 RESULTS

3.3.1 *In Vitro* Growth of Trypanosomes

Procyclic Growth Curves

Figure 3.1(a-f) shows the growth curves for the six cloned lines described in the previous chapter. Complete SDM-79 supported growth for at least 5 days in all six lines. From observing and maintaining the cultures (i.e. anecdotal evidence), there appeared to be a tendency for the drug-resistant lines (particularly 386Sur^R) to be more difficult to transform than the sensitive lines (particularly 247). The drug-resistant lines also appeared to require fresh medium less often than the unselected lines. This slower growth of the drug-resistant lines is indicated from the mean population doubling times (PDTs) for the six lines shown in Table 3.1, with 247 and 386 having PDTs of 15.7 and 24.7 hours respectively, compared to PDTs in the drug-resistant lines between 28.2 and 43.8 hours. Statistical analysis of the data by comparison of regression coefficients from the growth curves, showed that these differences in growth rate were significant between 247 and 247MelCy^R ($p<0.001$) and 247 and 247Sur^R ($p<0.01$) although the differences in the growth rates between 386 and both 386MelCy^R and 386Sur^R were not significant ($p>0.1$).

Bloodstream Growth Curves

The growth curves over 3½ days for the two sensitive lines (247 and 386) from which all the drug-resistant lines were generated are shown in Figure 3.2(a and b) respectively. In both lines, the complete B.S.C.M. supported growth over the first 24 hours and maintained cell viability for the following 24

hours. Death of the trypanosomes occurred approximately 48 hours after the initiation of the culture.

3.3.2 *In Vitro* Growth Inhibition Assay

The effective concentrations that inhibited growth by 50% (EC_{50} values) were calculated from the raw data using a method based on that previously described (Kaminsky and Zweygarth 1989; Zweygarth and Kaminsky 1990). The number of generations (i.e. the number of times the trypanosomes had undergone cell division) at different drug concentrations was calculated and expressed as a percentage of the number of generations present in the absence of drug after 24 hours, using the following equation (Kaminsky and Zweygarth 1989):

$$\% \text{ Growth Inhibition} = \frac{[\log n_{24} \text{ (with drug)} - \log n_0]}{[\log n_{24} \text{ (control)} - \log n_0]} \times 100$$

n_0 = number of trypanosomes at time = 0

n_{24} = number of trypanosomes after 24 hours

The percentage growth inhibition values at different drug concentrations were then used to generate a growth inhibition profile as shown schematically in Figure 3.3. A drug concentration range was therefore arrived at with limits defined by the growth inhibition curve. Below a certain concentration of drug, there was no measurable effect upon growth (100% growth). Conversely, above a certain drug concentration, no measurable growth could be seen (0% growth). EC_{50} values were calculated using only the data points lying between 10% and 90% growth inhibition and approximating these to a straight line calculated by linear regression (Minitab Release 8.2 Extended).

Growth Inhibition due to Arsenicals

Four lines (247, 386, 247MelCy^R and 386MelCy^R) were assessed for the levels of their resistance to four different melaminophenyl arsenicals (melCy, melW, melB and melOx) using procyclic culture forms. Each line/drug combination was carried out in triplicate. Figure 3.4(a-d) shows examples of growth inhibition profiles in procyclic culture of the four melaminophenyl arsenicals with 247 and 247MelCy^R. It can be seen that in the drug-resistant line, resistance to melB was expressed to a much lesser extent than the expression of resistance to melCy, melW and melOx. This low level of expression of melB-resistance seen in procyclic culture was also seen in bloodstream culture (Figure 3.5(a-b)). Furthermore, a similar pattern of growth inhibition profiles was seen with 386 and 386MelCy^R. The effective concentrations required to inhibit growth by 50% were calculated from the growth inhibition assays, and these EC_{50} values are shown in Table 3.2. The r^2 values shown indicate how closely the data points between 10% and 90% growth inhibition in each assay could be approximated to a straight line. Most of the r^2 values from the regression analyses lie in the region of 0.8 or greater. In most instances, comparison within triplicate experiments shows the range of EC_{50} values from the highest to lowest to be equivalent to one doubling dilution or less. The one exception to this can be seen with the combination of 386MelCy^R and melB. Here, the range of 0.25μM to 1.8μM represents almost three doubling dilutions. Comparison of values between different lines and drugs can be more easily seen in Table 3.3, with only the average EC_{50} values from triplicate experiments being shown. For both 247 and 386, the data suggests that melCy was the most potent inhibitor of growth, with values of 0.060μM and 0.025μM for 247 and 386 respectively, compared to 7.0μM and 0.98μM for the same lines with melB. This relatively high concentration required for melB to inhibit growth in the sensitive lines is particularly noticeable with 247. However, the most noticeable findings from the

growth inhibition studies shown in Table 3.3 was the differences seen in the degree of cross-resistance between the different melaminophenyl arsenicals. MelCy-resistance increased by 520-fold from 0.060μM for 247 to 31μM in its resistant derivative, 247MelCy^R (Table 3.4). Similarly, a 560-fold increase in melCy-resistance was seen between 386 and 386MelCy^R. This accounts for increases in resistance in both cases that is equivalent to more than 9 doubling dilutions (a 512-fold resistance factor being equivalent to 9 doubling dilutions in the growth inhibition assays). A large amount of cross-resistance was also seen to melW and melOx in both melCy-resistant lines. However, this was not the case with melB, the melaminophenyl arsenical used to treat sleeping sickness in clinical cases. For both resistant lines, comparison with the sensitive lines, from which they were derived, showed only slight increases in melB-resistance (2.3-fold and 1.3-fold for the 247MelCy^{R}and 386MelCy^R lines respectively).

The growth inhibition profiles in bloodstream culture of 247 and 247MelCy^R for melCy and melB are shown in Figure 3.5(a and b), illustrating the point that although the sensitive line has similar low levels of resistance to both drugs, the resistant line expresses resistance to melCy to a much greater extent than it does to melB. Table 3.5 shows the EC_{50} and r^2 values for the bloodstream growth inhibition assays for all six lines with melCy, melB and suramin (which will be dealt with separately). The experiments were carried out in triplicate except in three cases where only duplicates were carried out. Most of the data between repeat experiments for the two melaminophenyl arsenicals were within a 2-fold range, although in some cases the variability was greater than this. The EC_{50} values for 386 with melCy ranged from 0.012μM to 0.11μM, which was a 9-fold range and equivalent to more than three doubling dilutions. Examination of the average EC_{50} values (Table 3.6) show that the two sensitive lines required similar concentrations of both melCy and melB (approximately 0.05μM) to inhibit growth by 50%. MelCy-resistance was

expressed in bloodstream culture forms with differences in resistance between the resistant and sensitive lines being 87-fold and 24-fold for 247:247MelCy^R and 386:386MelCy^R respectively (Table 3.4). However these differences in melCy-resistance were not as marked as they were in procyclics, being an order of magnitude lower (EC_{50} values of 2.7μM for 247MelCy^R bloodstream forms compared to 31μM for 247MelCy^R procyclics). The lack of cross-resistance between melB and melCy that was found in procyclics was also found in bloodstream culture forms, with concentrations of melB of only 0.13μM for 247MelCy^R and 0.12μM for 386MelCy^R required to inhibit growth by 50%. These accounted for increases in resistance of 2.1-fold and 1.4-fold respectively (Table 3.4).

Growth Inhibition due to Suramin

Figure 3.6(a and b) illustrates the distinct lack of expression of suramin-resistance in procyclic culture for both 247Sur^R and 386Sur^R. An extremely high concentration of suramin was required to inhibit growth in procyclic cultures (Table 3.7). Taking the data as a whole, the EC_{50} values range from 64μM to 320μM for all four lines assayed (247, 386, 247Sur^R and 386Sur^R). The EC_{50} values from within a set of triplicate experiments are again generally within a 2-fold range although 386 shows a range greater than 4-fold, equivalent to two doubling dilutions. Six out of twelve r^2 values are greater than 0.9, with the lowest being 0.557. The average EC_{50} values are shown in table 3.8. It is clear that not only were procyclics extremely insensitive to suramin with EC_{50} values of the order of 100μM, but also that the levels of resistance did not increase in the suramin-resistant lines (Table 3.8).

A very different picture was seen with the effect of suramin on bloodstream culture forms (Figure 3.6(c and d)), where it is clear that suramin-

resistance was expressed in both suramin-resistant lines. The data from within triplicate experiments was fairly repeatable (Table 3.5), although, as with the bloodstream EC_{50} values for the melaminophenyl arsenicals, the experiments tend to be noticeably less repeatable than the procyclic assays. The average EC_{50} values for 247 and 386 (Table 3.6) show that bloodstream culture forms are approximately 200-fold more sensitive to growth inhibition by suramin than their procyclic counterparts, with EC_{50} values of 0.97μM for 247 and 0.44μM for 386 compared to 190μM and 110μM for the procyclics respectively. Unlike the procyclics, suramin-resistance was expressed in bloodstream culture forms, with resistance factors of 20 for $247Sur^R$ compared to 247 and 150 for the equivalent comparison between 386 and $386Sur^R$.

Cross-Resistance between Suramin and MelCy

In the search for a suitable assay for the analysis of clones generated from a cross between a suramin-resistant line and a melCy-resistant line, it was necessary to show that the lack of cross-resistance between suramin and melCy found *in vivo* could also be shown *in vitro*. Cross-resistance between suramin and melCy in procyclics was not investigated due to the finding that suramin-resistance was not expressed in procyclics. Table 3.6 shows that both suramin-resistant lines in bloodstream culture had very similar levels of resistance to melCy (0.038μM and 0.039μM for $247Sur^R$ and $386Sur^R$ respectively) when compared to their sensitive counterparts (0.031μM and 0.046μM for 247 and 386 respectively). There was however some indication of slight cross-resistance between suramin and melCy when the melCy-resistant lines were assessed for their levels of suramin-resistance. A suramin concentration of 2.2μM was required for 50% growth inhibition of $247MelCy^R$, approximately twice as much required for 247, and $386MelCy^R$ required 5-fold more suramin than 386 to be inhibited to the same extent. Nevertheless, this apparent increase in suramin-

resistance in the melCy-resistant lines was substantially lower than the increase in the suramin-resistant lines.

3.4 DISCUSSION

This chapter examined the merits of a 24-hour growth inhibition assay using either procyclics or short-term bloodstream cultures to assess the levels of resistance to melaminophenyl arsenicals and suramin. The procyclic cultures showed relatively high mean population doubling times compared to previous findings (for example Brun and Schonenberger 1979). Nevertheless, there was an indication that the resistant lines grew more slowly in procyclic cultures compared to the unselected lines. This might have had important consequences during the co-transmission through tsetse flies of two lines with different growth rates.

The use of EC_{50} values as criteria for assessing the levels of drug-resistance was chosen as it provides a fairly rapid and reliable method that easily distinguishes between drug-resistant and sensitive clones and one that was also sufficiently sensitive to identify intermediate levels of resistance. Twenty-four and 48 hour drug incubation studies have been applied to different drugs for a number of different trypanosome species *in vitro*. However, these studies have not all expressed levels of drug-resistance in EC_{50} values. In one study, levels of resistance to a range of drugs was measured in terms of the maximum concentration that still allowed trypanosomes to grow at 100%, and the minimum concentration required to totally kill cultures after 24 hours (Zhang *et al.* 1992). During the earlier stages of the present study, a large number of assays were carried out *in vitro* using this latter method of measuring drug-resistance, namely the lowest drug concentration in which no motile trypanosomes could be seen after a 24 hour incubation. Although the use of

trypanosome death is undoubtedly a more attractive yard-stick to measure the effectiveness of a trypanocide than inhibition of growth, in practice it was extremely difficult to accurately assess the end-point of such an assay. On the whole, arsenical-resistant trypanosomes could be separated from sensitive trypanosomes in procyclic culture using this assay but the data from bloodstream cultures, particularly with suramin, was too variable to be used with any confidence and the method was subsequently discarded in favour of the growth inhibition assay. The method used to measure EC_{50} values in the present study was based on a previously published method (Kaminsky and Zweygarth 1989). This latter study calculated the EC_{50} values from the growth inhibition graphs using the minimum χ^2 method. Attempts at repeating this method were unsuccessful because insufficient information on the method of data analysis was given in the original publication (Kaminsky and Zweygarth 1989). Therefore, the data points between 10% and 90% growth were approximated to a straight line. Although the relationship between growth and drug concentration was undoubtedly not linear over the whole range of drug concentrations, it was acceptable to approximate the values between 90% and 10% growth to a straight line as can be seen by the r^2 values in tables 3.2, 3.5 and 3.7. A total of 104 assays were carried out, 55 of which had r^2 values greater than 0.9. Furthermore, the range of the EC_{50} values within triplicate repeats was found to be within one doubling dilution in the majority of cases. In experiments where the range was more marked, such as the 9-fold range seen in the combination of 386 and melCy in bloodstream culture, the highest EC_{50} value in the sensitive line (0.11μM) was still 10-fold lower than the lowest EC_{50} value in the resistant line (1.1μM). In this respect the growth inhibition assay with bloodstream trypanosomes was more than adequate in distinguishing between sensitive and resistant lines for both melCy-resistant lines and both suramin-resistant lines. The same could not be said for the procyclic assays, in which the suramin-resistant lines could not be distinguished from sensitive lines.

Apart from melB, arsenical-resistance was undoubtedly expressed in procyclic culture, although it was equally clear that suramin-resistance was not. Insect stage trypanosomes for *in vitro* drug-resistance assays have been used previously with conflicting results. Forty-eight hour growth inhibition assays have been carried with *T. congolense* for isometamidium chloride (samorin) and diminazene aceturate (berenil) using procyclic cultures using a similar method to the one described in the present study (Elrayah and Kaminsky 1991). For both drugs, differences between drug-resistant and sensitive lines that were detected *in vivo* were not seen with *in vitro* procyclic cultures. Another study comparing bloodstream and procyclic cultures of *T. congolense* isolates also showed a loss of expression of berenil-resistance and samorin-resistance in procyclic cultures (Brun and Rab 1991). This latter study used [^{3}H]hypoxanthine incorporation rather than growth inhibition to measure the levels of drug-resistance. It was possible that the *T. congolense* procyclics did not express samorin-resistance or berenil-resistance in the same way that suramin-resistance and melB-resistance was not expressed in *T. brucei* procyclics in the present study. However, the [^{3}H]hypoxanthine incorporation assay was also used in a similar study on the effect of samorin and berenil on bloodstream and procyclic cultures of *T. congolense* (Ross and Taylor 1990). In this study both samorin-resistance and berenil-resistance that was expressed *in vivo*, was also found to be expressed in bloodstream and procyclic cultures. However, on the whole, it would appear that the use of insect-stage cultures is not reliable enough for the routine screening of trypanosomes from a range of sources.

Bloodstream cultures appear to offer the best system with which to assess the levels of drug-resistance *in vitro*. The EC_{50} values from the *in vitro* bloodstream assays closely reflected the minimum curative doses from the *in vivo* assays with suramin and the melaminophenyl arsenicals, with the notable exception of the lack of expression of melB-resistance in culture. Thus, the

melCy-resistant lines expressed resistance to melW both *in vivo* and *in vitro*, and similarly, the suramin-resistant lines also expressed resistance in bloodstream trypanosomes both *in vivo* and *in vitro*. The almost total absence of cross-resistance between suramin and melCy seen *in vivo* was also found with the *in vitro* bloodstream cultures. The slight increase in suramin-resistance found in both 247MelCy^R and 386MelCy^R *in vitro*, was 2-fold and 5-fold respectively. This may be due to experimental variation, but it is interesting to note that a slight increase in suramin-resistance was also seen in the 386MelCy^R line *in vivo*. The *in vitro* bloodstream assay and the method used to calculate the EC_{50} values was therefore sufficiently accurate to be used in place of the *in vivo* assay for the assessment of melCy-resistance and suramin-resistance. Consequently, it was suitable to be used for the analysis of the inheritance of drug-resistance during a cross between melCy-resistant and suramin-resistant lines.

The EC_{50} values for melCy for the sensitive lines are similar to previously published values. One study with six *T. b. brucei* isolates had a range of EC_{50} values from 0.008μM to 0.054μM (Zweygarth and Kaminsky 1990), with the latter value coinciding with a minimum curative dose *in vivo* of 6.3mg/Kg. However, studies in other trypanosome species have given different values, such as those found in *T. simiae* of greater than 1μM (Zweygarth *et al.* 1993) and between 6.4nM and 17nM for five stocks of *T. evansi* (Zweygarth and Kaminsky 1990). However, the first of these two studies did not carry out *in vivo* comparisons and could have used an arsenical-resistant isolate. Similarly, the latter study was carried out with susceptible *T. evansi* isolates with minimum curative doses in mice of less than 0.4mg/Kg. A different study on *T. evansi* and *T. equiperdum* conducted experiments similar to the present study but expressed the results differently. However, from the data given, it can be estimated that for all eight *T. evansi* isolates and all three *T. equiperdum* isolates, EC_{50} values were approximately 0.01μM for melCy (Zhang *et al.* 1992). None of these isolates

expressed melCy-resistance *in vivo*. The same study also investigated levels of suramin-resistance, which again could be estimated to be between 0.1μM and 1μM, similar to the values for 247 and 386. A *T. evansi* isolate with suramin-resistance was found to have an EC_{50} value of 15μM, which was comparable to the $247Sur^R$ (19μM) and $386Sur^R$ (63μM) lines (Kaminsky and Zweygarth 1989). However, the same study showed that two lines of *T. brucei* that were sensitive to suramin in mice had very low EC_{50} values of 0.068μM and 0.017μM, which was over an order of magnitude lower than those for 247 and 386 (0.97μM and 0.44μM respectively). Unfortunately, only the fact that they were suramin-sensitive *in vivo* was given and not the minimum curative doses, which could have also been lower than those for 247 and 386. However, in conclusion, previously published values are not too far removed from the values for the six lines used in this study, thus providing further support for the methods used.

Selection of melCy-resistance *in vivo* could only be carried out to the maximum tolerated dose of 40mg/Kg, and similarly, the two melCy-resistant lines could only be assayed *in vivo* at a maximum of 40mg/Kg. However, there was no such constraint for the *in vitro* assays and it was interesting to note that in bloodstream culture, the resistance factor for $247MelCy^R$:247 for melCy was noticeably larger than for $386MelCy^R$:386. Although it is questionable whether the *in vitro* bloodstream assay was sensitive enough for this difference to be real and not just due to experimental variation, there may be some significance in the fact that *in vivo* 247 had a lower level of sensitivity to melCy (0.3mg/Kg) than 386 (2.0mg/Kg) prior to selection of drug-resistance. Consequently, it was reasonable to expect the selection of melCy-resistance *in vivo* to produce a $247MelCy^R$ line with a greater relative increase in resistance than a $386MelCy^R$ line, thus providing an explanation for the differences in resistance factors seen *in vitro*.

The resistance factors for the melaminophenyl arsenicals (Table 3.4) show the expected patterns of cross-resistance with the notable exception of melB. In both procyclic and bloodstream cultures, cross-resistance between melCy and melB is virtually absent. It is the generally held assumption that for all melaminophenyl arsenicals to be active, they must first be converted to melarsen oxide (Berger and Fairlamb 1994). It is this active melarsen oxide, that binds to intracellular dithiols such as trypanothione, that is responsible for the trypanocidal actions of the drugs and explains the existence of cross-resistance between the melaminophenyl arsenicals (for example see Fairlamb et al. 1992b; Pospichal *et al.* 1994). The lack of cross-resistance between melCy and melB *in vitro* was therefore unexpected. There are two possible explanations for this observation. Although melB-resistance is expressed *in vivo*, it is possible that the actual levels of melB-resistance were considerably lower than the levels of melCy and melW-resistance and it was the limitations of the *in vivo* assays, in which the mice could only tolerate maximum doses of arsenicals of 40mg/Kg, that made it impossible to detect the higher levels of melCy-resistance and melW-resistance compared to melB-resistance. Consequently, a situation may exist where the levels of melCy-resistance and melW-resistance *in vivo* were much higher than 40mg/Kg although it was not possible to measure these levels. There is some evidence for this limitation in the *in vivo* assay with the comparison of the *in vitro* data between 247MelCy^R and 386MelCy^R. The 247MelCy^R line had higher average EC_{50} values for the melaminophenyl arsenicals in five out of six cases in both procyclic and bloodstream cultures compared to the 386MelCy^R line, although the *in vivo* assay could not distinguish between these two lines. Therefore, the *in vitro* assay may simply be picking up a difference between melB and melCy that occurs *in vivo* but one that can not be detected in mice. An alternative explanation for the absence of melB-resistance *in vitro* lies with the different possible mechanisms of uptake of the melaminophenyl arsenicals. MelB is highly soluble in lipids compared to melCy

and melW which are both water soluble. It has recently been proposed that all melaminophenyl arsenicals are taken up by the trypanosomes by a common purine transporter (Carter and Fairlamb 1993). Furthermore, this same study found this adenosine transporter to be altered or down-regulated in some way in an arsenical-resistant line, thereby bringing about a decrease in drug uptake and accounting for the drug-resistance phenotype. The role of this transporter will be dealt with in more detail in **chapter 6**. Briefly, the melCy-resistant lines in this study may also possess altered purine transporters. Arsenical-resistance is expressed *in vivo* because the bloodstream trypanosomes are exposed to fairly high concentrations of arsenical (approximately 10μM) immediately after injection of the drug, which rapidly falls within less than one hour (Burri *et al.* 1993). It is over this short period after injection that the sensitive trypanosomes are killed, and during which the arsenical-resistant trypanosomes do not take up the drug in sufficient amounts to be killed. Similar concentrations that were found in the serum immediately after drug injections were capable of lysing the sensitive bloodstream trypanosomes *in vitro*. Arsenical-resistant trypanosomes were not lysed at these concentrations *in vitro* (Burri *et al.* 1993; Yarlett *et al.* 1991; **chapter 6**). However, the 24 hour incubations presented here with arsenicals in bloodstream culture were carried out at much lower concentrations than those required to lyse the trypanosomes. Although melCy, melW, melB and melOx may all be taken up by the adenosine transporter or some other active transport mechanism, the high lipid solubility of melB also allows it to slowly diffuse over the cell membrane. This diffusion is not rapid enough to have an effect during short exposures to melB, such as occurs *in vivo*. However, a 24 hour exposure is sufficiently long for the diffusion of melB to negate the effect of an altered uptake mechanism. Hence, the arsenical-resistant lines do not express melB-resistance over 24 hours *in vitro*. There are other possible explanations for this lack of melB-resistance, involving more than one route for arsenical uptake. This will be dealt with in **chapter 6**.

The other notable feature of the EC_{50} values was the extremely high concentrations of suramin required to inhibit procyclic growth (Table 3.8). These concentrations of above 0.1mM are undoubtedly not physiological and in many ways the procyclics can actually be considered to be totally insensitive to suramin. The simplest explanation of this observation lies with the procyclics energy metabolism. A recent study summarised data from different sources and found that a number of glycolytic enzymes from bloodstream trypanosomes were inhibited by suramin at physiological concentrations (Willson *et al.* 1993). Furthermore, suramin has been found to decrease glycolytic activity *in vivo* (Fairlamb and Bowman 1980a). A number of the glycolytic enzymes found in the glycosome were altered in procyclics (Hart *et al.* 1984), thus providing a possible explanation for the almost total lack of suramin-sensitivity in the procyclic cultures. A second possible explanation for the lack of suramin-resistance in procyclic culture could be that suramin simply does not enter procyclics whether they are resistant or not. Suramin uptake appears to be via receptor-mediated endocytosis (Fairlamb and Bowman 1980b; Vansterkenberg 1993). Since endocytosis occurs in bloodstream forms but not in procyclics, if suramin is solely reliant on endocytosis for entry into the trypanosome, it may not be surprising that suramin had virtually no activity against procyclics except at extremely high, non-physiological concentrations (>0.1mM).

Figure 3.1
Procyclic Cultures: Growth Curves

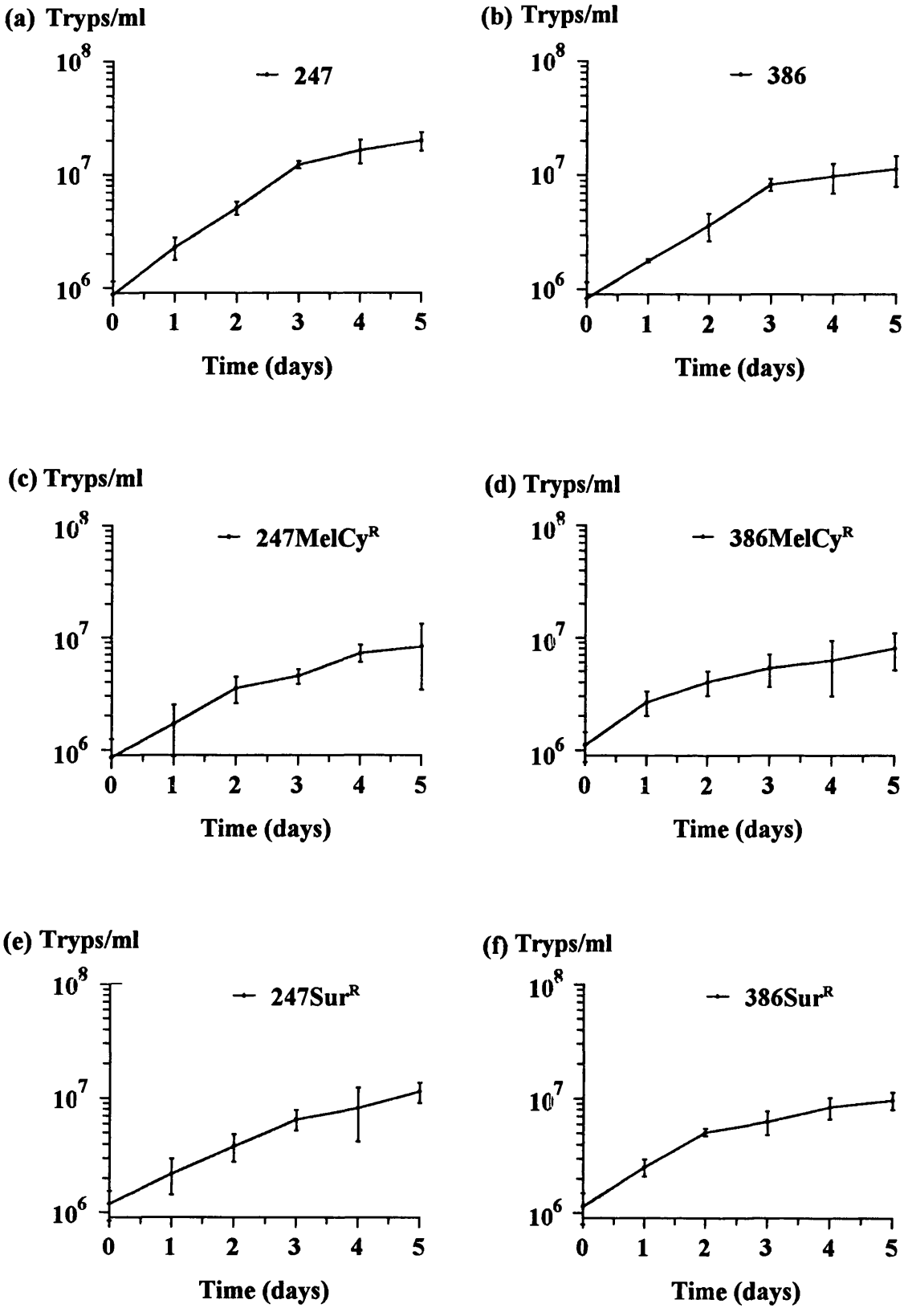
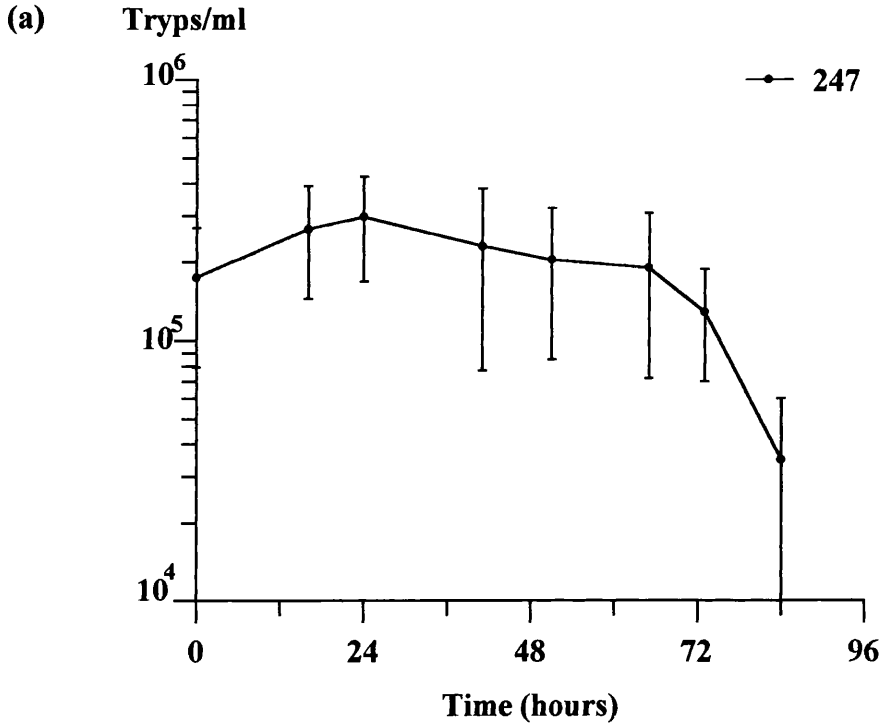


Figure 3.1
Procyclic Cultures: Growth Curves
(a) in 247
(b) in 386
(c) in 247MelCy^R
(d) in 386MelCy^R
(e) in 247Sur^R
(f) in 386Sur^R

Cultures were initiated in 96 well plates at starting trypanosome densities of 10^6/ml and counted in octuplicate every 24 hours for 5 days. The vertical axis represents the density of the trypanosome culture on a logarithmic scale, plotted against time (days). Vertical bars are ± 2 Standard Errors (Minitab Release 8 Extended).

Figure 3.2
Short-Term Bloodstream Cultures: Growth Curves



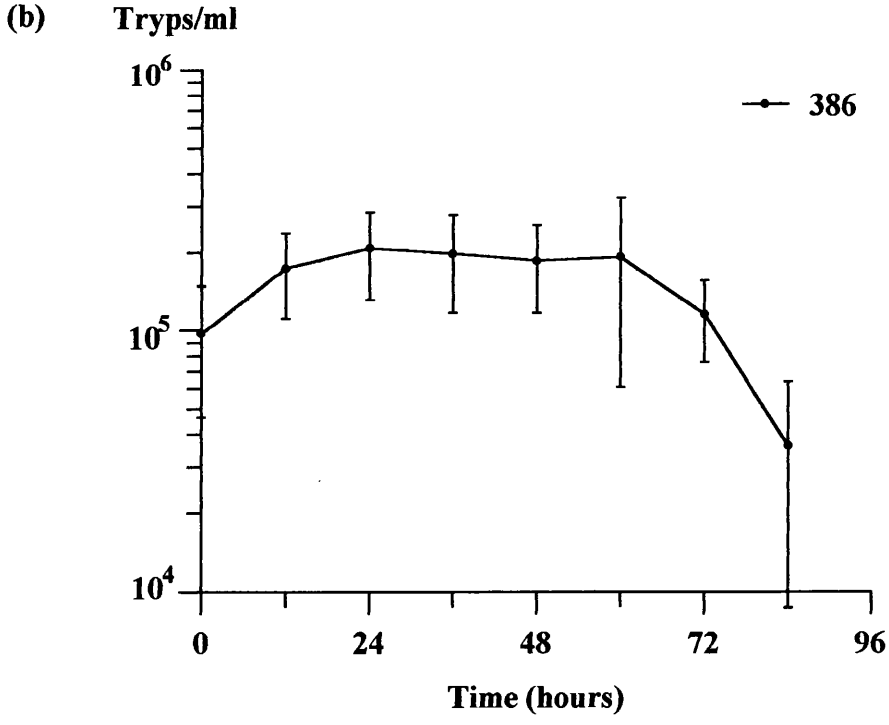
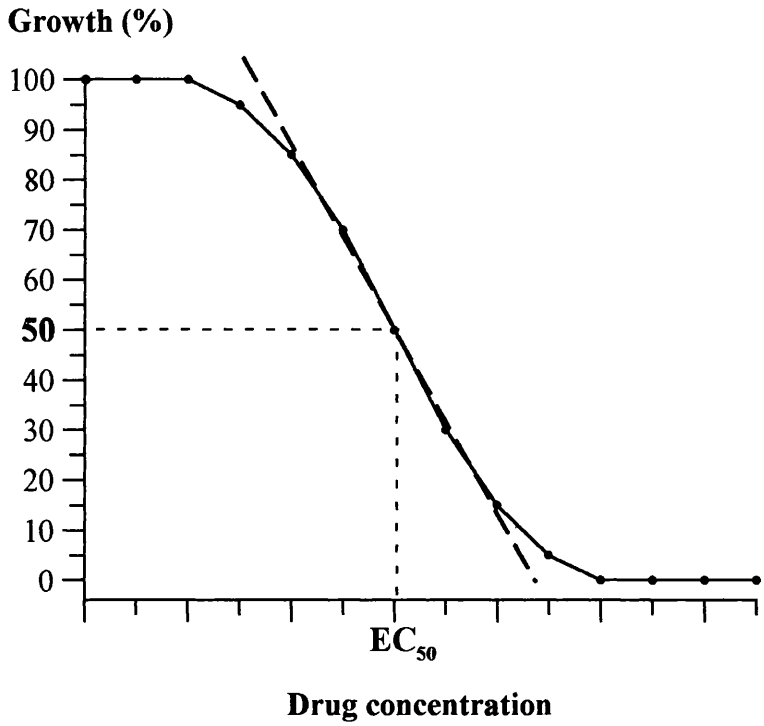


Figure 3.2
Short-Term Bloodstream Cultures: Growth Curves
(a) in 247
(b) in 386

Cultures were initiated in 96 well plates at starting trypanosome densities of 10^5/ml and counted in octuplicate approximately every 12 hours for 3½ days. The vertical axis represents the density of the trypanosome culture on a logarithmic scale, plotted against time (hours). Vertical bars are ± 2 Standard Errors (Minitab Release 8 Extended).

Figure 3.3
Calculation of EC_{50} Values by Linear Regressiom



Method used to calculate EC_{50} values from the growth inhibition profiles. The growth data (vertical axis) was plotted against increasing drug concentration, and the values between 10% and 90% growth were approximated to a straight line by linear regression. The drug concentration at which growth was inhibited by 50% was calculated from the regression equation.

Figure 3.4
Procyclic Cultures: Growth Inhibition Profiles of 247 and 247MelCy^R

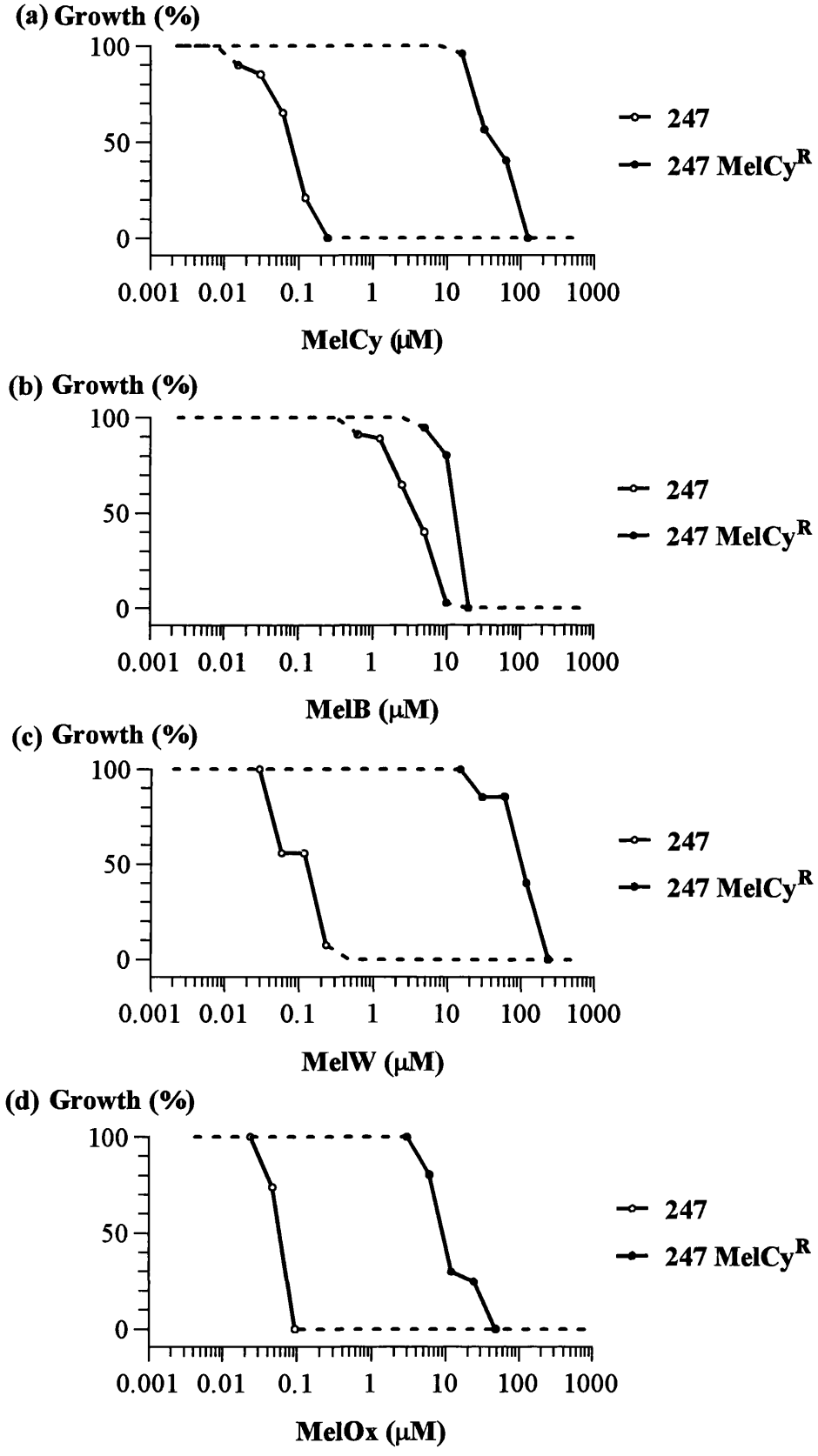
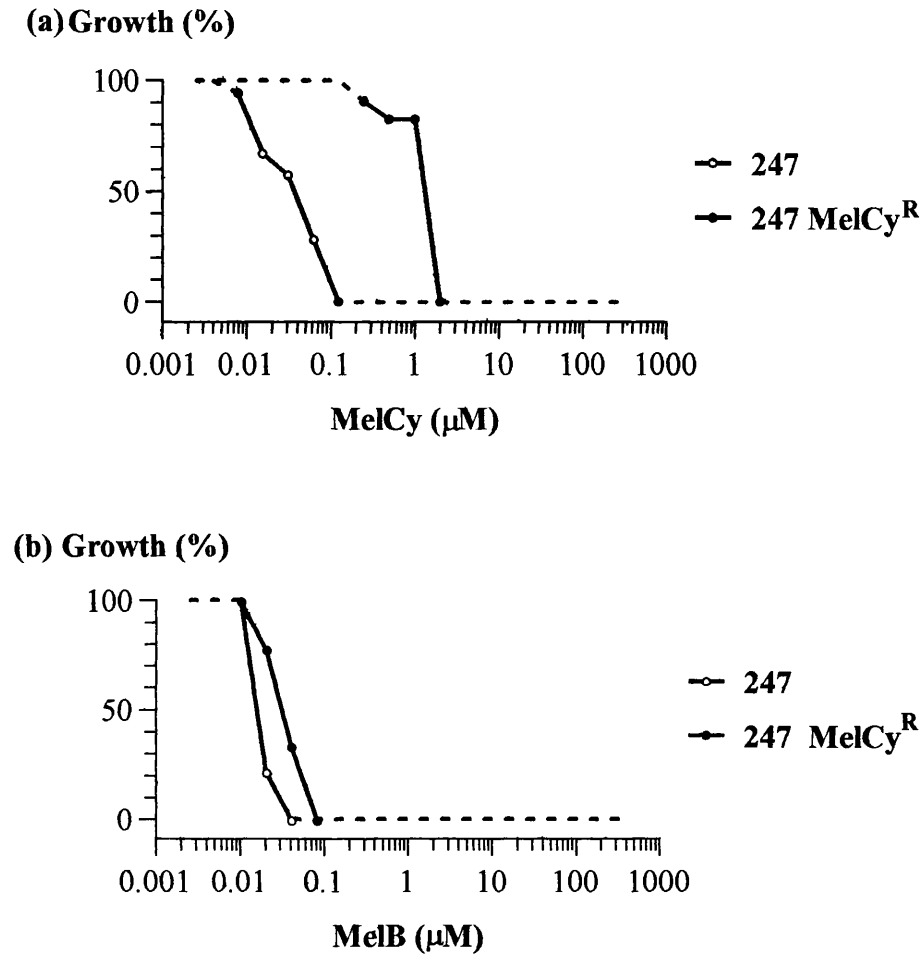


Figure 3.4
Procyclic Cultures: Growth Inhibition Profiles of 247 and 247MelCy^R
(a) with MelCy
(b) with MelB
(c) with MelW
(d) with MelOx

Percentage growth inhibition (vertical axis) in procyclic cultures, with increasing concentrations of melaminophenyl arsenicals, plotted on a logarithmic scale (horizontal axis). The differences in levels of drug-resistance can be seen between 247, shown as open circles (o), and 247MelCy^R, shown as closed circles (•).

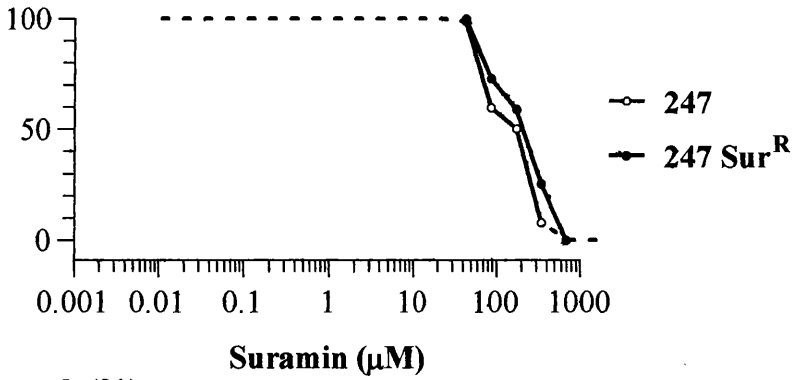
Figure 3.5
Bloodstream Cultures: Growth Inhibition Profiles of 247 and 247MelCy^R



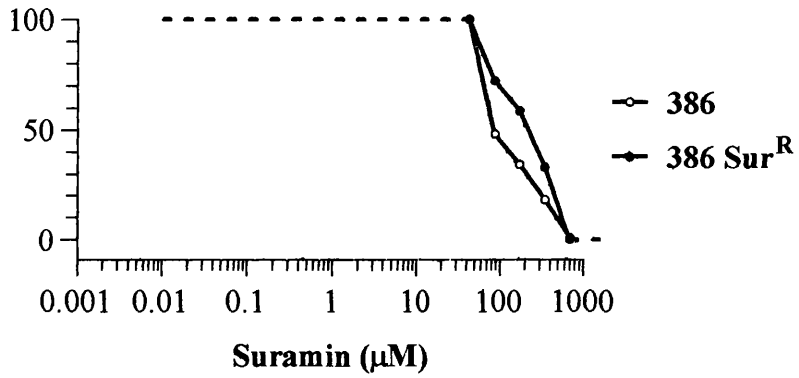
Pattern of growth inhibition (vertical axis) in bloodstream cultures, with increasing concentrations (logarithmic horizontal scale) of (a) melCy and (b) melB. The lack of expression of melB-resistance in bloodstream cultures can be seen with comparison between the unselected line and melCy-resistant line.

Figure 3.6
Procyclic Cultures: Growth Inhibition Profiles and Lack of Expression of Suramin-Resistance

(a) Growth (%)

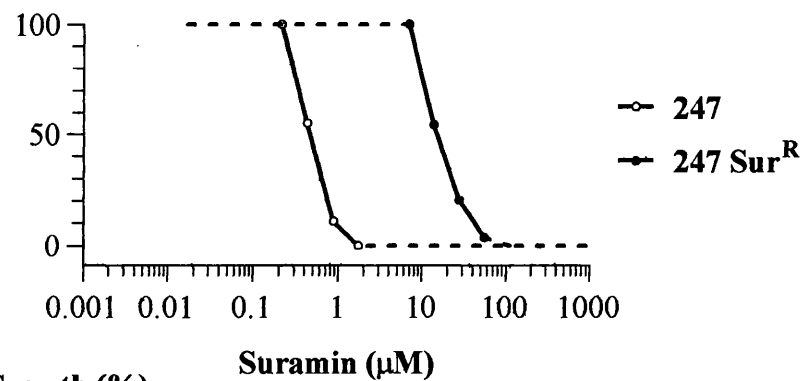


(b) Growth (%)



Bloodstream Cultures: Growth Inhibition Profiles and Expression of Suramin-Resistance

(c) Growth (%)



(d) Growth (%)

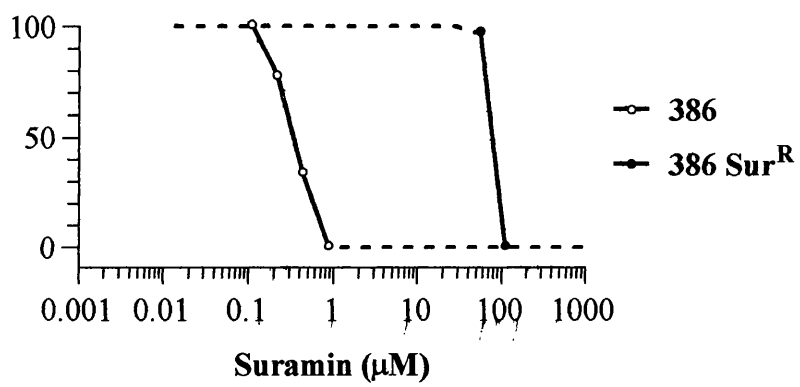


Figure 3.6
Procyclic Cultures: Growth Inhibition Profiles and Lack of Expression of Suramin-Resistance
(a) in 247 and 247Sur^R
(b) in 386 and 386Sur^R

Percentage growth inhibition (vertical axis) in procyclic cultures with increasing concentrations of suramin, plotted on a logarithmic scale (horizontal axis). The lack of expression of suramin-resistant lines, shown as closed circles (•), can be seen with comparison to the unselected lines, shown as open circles (o).

Bloodstream Cultures: Growth Inhibition Profiles and Expression of Suramin-Resistance
(c) in 247 and 247Sur^R
(d) in 386 and 386Sur^R

Percentage growth inhibition (vertical axis) in bloodstream cultures with increasing concentrations of suramin, plotted on a logarithmic scale (horizontal axis). The differences in the levels of suramin-resistance in the suramin-resistant lines, shown as closed circles (•), can be seen with comparison to the unselected lines, shown as open circles (o).

Table 3.1
Procyclic Cultures: Mean Population Doubling Times over the First 3 Days

Trypanosome Line	Mean population Doubling Time (hours)
247	15.7
386	24.7
247MelCy^R	43.8
386MelCy^R	34.7
247Sur^R	29.3
386Sur^R	28.2

The average population doubling times for the six cloned lines in procyclic culture were calculated from octuplicate counts taken every 24 hours over the first 3 days.

Table 3.2
Procyclic Cultures: EC_{50} and r^2 Values for Melaminophenyl Arsenicals

Trypanosome Line	EC_{50} and r^2 Values							
	MelCy		MelB		MelW		MelOx	
	(μM)	r^2	(μM)	r^2	(μM)	r^2	(μM)	r^2
247	0.062	0.935	4.8	0.977	0.18	0.862	0.096	0.866
	0.096	0.825	9.8	0.679	0.12	0.904	0.061	0.994
	0.057	0.788	6.5	0.804	0.098	0.925	0.051	0.932
386	0.039	0.953	1.1	0.960	0.14	0.912	0.12	0.979
	0.011	0.827	0.85	0.826	0.23	0.994	0.12	0.985
	0.034	0.934	0.93	0.854	0.21	0.859	0.19	0.940
247MelCy^R	19	0.772	9.8	0.837	60	0.952	17	0.750
	19	0.772	13	0.898	120	0.971	29	0.966
	66	0.937	24	0.919	126	0.896	13	0.895
386 MelCy^R	20	0.965	1.7	0.810	17	0.929	47	0.926
	14	0.633	0.25	0.870	15	0.959	38	0.835
	13	0.611	1.8	0.881	12	0.882	46	0.809

The EC_{50} and r^2 values of melCy, melB, melW and melOx for the two unselected lines (247 and 386) and the two melCy-resistant lines (247MelCy^R and 386MelCy^R) in procyclic culture.

Table 3.3
Procyclic Cultures: Average EC_{50} values for Melaminophenyl Arsenicals

Trypanosome Line	Average EC_{50} values			
	MelCy (μM)	MelB (μM)	MelW (μM)	MelOx (μM)
247	0.060	7.0	0.13	0.069
386	0.025	0.98	0.19	0.14
247MelCy^R	31	16	100	20
386MelCy^R	14	1.3	15	44

The average EC_{50} values of the four melaminophenyl arsenicals for the two unselected lines (247 and 386) and the two melCy-resistant lines (247MelCy^R and 386MelCy^R) in procyclic culture.

Table 3.4
Levels of Arsenical-Resistance Expressed in Different Assay Systems

Resistance Assay Employed	Drug	Resistance Factor	
		247 : 247MelCy^R	386 : 386MelCy^R
In Vitro Procyclic	MelCy	520	560
	MelB	2.3	1.3
	MelW	770	79
	MelOx	290	310
In Vitro Bloodstream	MelCy	87	24
	MelB	2.1	1.4
In Vivo	MelCy	>130	>20
	MelB	>125	>63
	MelW	>50	>13

The relative increases in drug-resistance of the melCy-resistant lines compared to the unselected lines from which they were generated are shown as resistance factors. The data for the *in vitro* assays were taken from Tables 3.3 and 3.6. *In vivo* data were taken from the results of the *in vivo* assays in the previous chapter (Table 2.2 (a and b)).

Table 3.5
Bloodstream Cultures: EC_{50} and r^2 Values for Melaminophenyl Arsenicals and Suramin

Trypanosome Line	EC_{50} and r^2 Values					
	MelCy		MelB		Sur	
	(μM)	r^2	(μM)	r^2	(μM)	r^2
247	0.017	0.989	0.13	0.803	0.67	0.755
	0.030	0.708	0.033	0.823	1.0	0.989
	0.048	0.913	0.020	0.767	1.2	0.55
386	0.018	0.990	0.021	0.842	0.15	0.715
	0.108	0.938	0.083	0.811	0.52	0.984
	0.012	1.000	0.15	0.902	0.54	0.996
247MelCy^R	3.0	0.825	0.093	0.888	3.5	0.932
	1.0	0.738	0.16	0.938	1.1	1.000
	4.0	0.951	0.13	0.811	2.2	0.998
386MelCy^R	1.1	0.766			2.5	1.000
	1.2	0.882	0.083	0.983	3.6	0.914
	1.1	0.787	0.16	0.928	0.38	0.899
247Sur^R					16	1.000
	0.040	0.979	ND	ND	17	0.998
	0.036	0.996			23	0.784
386Sur^R	0.032	0.990	ND	ND	84	1.000
	0.046	1.000			42	1.000

The EC_{50} and r^2 values of melCy, melB and suramin for the two unselected lines (247 and 386), the two melCy-resistant lines (247MelCy^R and 386MelCy^R) and the two suramin-resistant lines (247Sur^R and 386Sur^R) in bloodstream culture.

Table 3.6
Bloodstream Cultures: Average EC_{50} values for Melaminophenyl Arsenicals and Suramin

Trypanosome Line	Average EC_{50} Values		
	MelCy (μM)	MelB (μM)	Sur (μM)
247	0.031	0.061	0.97
386	0.046	0.085	0.44
247MelCy^R	2.7	0.13	2.2
386MelCy^R	1.1	0.12	2.2
247Sur^R	0.038	ND	19
386Sur^R	0.039	ND	63

The average EC_{50} values of melCy, melB and suramin for the two unselected lines (247 and 386), the two melCy-resistant lines (247MelCy^R and 386MelCy^R) and the two suramin-resistant lines (247Sur^R and 386Sur^R) in bloodstream culture.

Table 3.7
Procyclic Cultures: EC_{50} and r^2 Values for Suramin

Trypanosome Line	EC_{50} and r^2 Values	
	Suramin (μM)	r^2
247	140	0.557
	120	0.961
	64	0.932
386	190	0.842
	320	0.757
	69	0.970
247 Sur^R	91	0.958
	220	0.923
	130	0.958
386Sur^R	84	0.750
	84	0.686
	98	0.859

The EC_{50} and r^2 values of suramin for the two unselected lines (247 and 386) and the two suramin-resistant lines (247Sur^R and 386Sur^R) in procyclic culture.

Table 3.8
Procyclic Cultures: Average EC_{50} values for Suramin

Trypansome Line	Average EC_{50} Values
	Suramin (μM)
247	110
386	190
247Sur^R	150
386 Sur^R	89

The average EC_{50} values of suramin for the two unselected lines (247 and 386) and the two suramin-resistant lines (247Sur^R and 386Sur^R) in procyclic culture.

CHAPTER 4
GENERATION OF HYBRID TRYPANOSOMES EXPRESSING RESISTANCE TO CYMELARSAN AND SURAMIN

4.1 INTRODUCTION

Chapters 2 and 3 described the generation and characterisation of four cloned lines expressing resistance to either suramin or melCy. These drug-resistance phenotypes were shown to be stable in the absence of drug pressure after cyclical transmission through tsetse flies. The four drug-resistant lines were generated from 247 and 386 - lines known to be capable of undergoing non-obligatory genetic exchange during co-transmission through tsetse flies (Jenni *et al.* 1986). Previous co-transmission experiments with 247 and 386 generated cloned hybrid trypanosomes that had characteristic phenotypes (isoenzymes) and genotypes (RFLPs and karyotypes) indicating that genetic exchange had occurred (Jenni *et al.* 1986; Sternberg *et al.* 1988; Sternberg *et al.* 1989; Turner *et al.* 1990)

A recent study has exploited drug-resistance as selectable markers to identify hybrids (Gibson and Whittington 1993). In this study, procyclic culture forms of two trypanosome lines (KP2, *T. b. brucei* and 058, *T. b. rhodesiense*) were each transfected with a plasmid construct (*neo* and *hph*) conferring resistance to neomycin and hygromycin respectively. Those trypanosomes that had been successfully transformed were selected with G418 for neomycin-resistance and with hygromycin for hygromycin-resistance, and subsequently transmitted separately through tsetse flies to generate bloodstream clones. Co-transmission of these two cloned lines resulted in metacyclic infections containing hybrid trypanosomes that were identified due to their expression of resistance to both G418 and hygromycin. Southern hybridisation with probes for the *neo* and *hph* genes showed that the double drug-resistant clones contained both genes (Gibson

and Whittington 1993). This study demonstrated that selectable markers could be used successfully to identify hybrid trypanosomes produced during co-transmission experiments.

This chapter describes how drug-resistant lines of 247 and 386 were used in co-transmission experiments in order to show that the drug-resistant lines retained their "fertility" and were able to produce trypanosomes expressing the hybrid phenotype (i.e. double drug-resistant). The drug-resistant phenotypes of the transfected lines in the previous study were known to result from the insertion of the drug-resistant genes into one of a pair of homologous chromosomes (Gibson and Whittington 1993) whereas the exact natures of the genotypes in the melCy-resistant and suramin-resistant lines were unknown. However, the use of lines generated to genuine trypanocides, rather than antibiotics, allows both for the exploitation of drug-resistance as selectable markers as well as the simultaneous investigation into the inheritance of drug-resistance during genetic exchange. Furthermore, the two lines (247 and 386), from which the drug-resistant lines were generated, have been well characterised in terms of the inheritance of a wide variety of markers (reviewed in Tait *et al.* 1993).

4.1.1 Experimental Design

Figure 4.1 illustrates the procedure through which two drug-resistant cloned lines were used in co-transmission experiments, and the subsequent screening of the metacyclic populations to identify those flies producing double drug-resistant trypanosomes. Teneral tsetse flies, in batches of approximately 100, were given an initial infective feed containing a mixture of two trypanosome lines, each with a different drug-resistance phenotype (in this case 247Sur^R and 386MelCy^R). Infected flies over 18 days old were screened for the presence of salivary gland infections by allowing flies to probe and deposit saliva onto

microscope slides. In order to determine the nature of the metacyclic infections in terms of the drug-resistance phenotypes present, those flies producing metacyclic trypanosomes were each allowed to feed on a mouse. Trypanosomes from the first parasitaemic wave in infected mice were transferred to three mice, mouse 1 of which was treated with melCy only, mouse 2 with suramin only, and mouse 3 with both melCy and suramin (Figure 4.1). The drug doses chosen allowed drug-resistant trypanosomes to grow, but were sufficient to kill drug-sensitive trypanosomes. Consequently, by monitoring the infections in the mice after drug treatment, the drug-resistance phenotypes present in the metacyclic infections could be deduced, as shown in Figure 4.1. Trypanosomes growing in mouse 3, which was treated with both melCy and suramin, must therefore have a double drug-resistance phenotype, which would suggest they were the products of genetic exchange between 247Sur^R and 386MelCy^R. Depending on the genetic nature of drug-resistance, genetic exchange might also be expected to produce hybrid progeny that were sensitive to both drugs, although this screen would be unable to detect double drug-sensitive trypanosomes.

4.2 MATERIALS AND METHODS

4.2.1 Drugs

The two trypanocidal drugs suramin and melCy were used to screen for drug-resistant trypanosomes after cyclical transmission through tsetse flies. Both drugs were supplied and stored as described previously (**section 2.2.1**).

4.2.2 Trypanosome Stocks and Mice

A total of 1510 tsetse flies were used in 17 separate mixed transmission experiments in an attempt to generate hybrid trypanosomes. The two lines used (247Sur^R and 386MelCy^R) have been described previously (**chapters 2 and 3**).

Adult female Balb/C mice (approximately 20g body weight) were used for all aspects of the drug screening.

4.2.3 Assessment of the Rate of Clearance of Infections from Mice

An *in vivo* assessment was conducted to determine the speed with which the two trypanocides, melCy and suramin, were each able to clear trypanosome infections from mice, and the effect of the size of the trypanosome inoculation had on the rate of clearance. Two lines were assessed: 247MelCy^{R}ft (post fly-transmission (GUP 3650)) and 24Sur^{R}ft (GUP 3621). Trypanosomes from a rising parasitaemia were taken from an adult female TO Swiss mouse (immunosuppressed with cyclophosphamide 24 hours previously at a dose of 250mg/Kg by i.p. injection) and transferred to Balb/C mice (immunosuppressed with cyclophosphamide at a dose of 150mg/Kg by i.p. injection). Groups of six Balb/C mice each received different numbers of trypanosomes between 10^{1} and 10^{7}/mouse. Twenty four hours after inoculation with trypanosomes, each mouse then received drug treatment of either suramin at a dose of 5mg/Kg or melCy at a dose of 5mg/Kg by i.p. injection. The parasitaemias were monitored with the rapid matching technique (Herbert and Lumsden 1976) for up to 13 days post drug-treatment.

4.2.4 Infection of Tsetse Flies

Teneral tsetse flies were infected with either bloodstream trypanosomes or procyclic trypanosomes at a final trypanosome density of $1x10^{6}$ to $6.3x10^{7}$ trypanosomes/ml in batches of approximately 100. Batches and individual flies were given unique numbers. The life cycle stage (bloodstream or procyclic) and the source of these trypanosomes (bloodstream stabilate, bloodstream infections in mice or established procyclic cultures) used to infect the tsetse flies are

summarised in Table 4.1.

Batch No's 25700, 26100 & 27500

Flies in batch no.'s 25700 and 26100 were fed an initial infected feed of trypanosomes taken directly from two infected mice. The parasitaemias of each mouse were measured with a haemocytometer and the blood from the mice was mixed with citrated sheep's blood to give an infective feed containing the two trypanosome lines, 247Sur^R and 386MelCy^R, in a 1:1 ratio. This was then warmed on a tray at 37^{0}C and fed to teneral tsetse flies through a silicone membrane. Infected tsetse flies were maintained on citrated sheep's blood as described previously (Hajduk *et al.* 1981; Turner *et al.* 1990). Flies in batch no. 27500 were also given an infective feed of trypanosomes taken from mice but containing 247Sur^R and 386MelCy^R in a 1:2 ratio instead of the normal 1:1 ratio.

Batch No's 26300, 26400, 26500, 26600, 27000, 27100, 27200, 28000, 28100, 28200 & 28300

Flies in batch no.'s 26300 to 27200 were given bloodstream trypanosomes taken from cryopreserved samples (stabilated using 7% DMSO) containing a 1:1 mixture of the two stocks. The stabilates (either GUP 3599 or GUP 3606) were resuspended in citrated sheep's blood and fed to tsetse flies as described above. Flies in batch no.'s 28000 to 28300 were also fed trypanosomes taken from cryopreserved samples. These flies were fed in a similar manner to that just described except that the two drug-resistant lines were cryopreserved separately in 10% glycerol. The stabilates were then sent to Roger Barker in the Tsetse Research Laboratories, Bristol, where they were mixed in a 1:1 ratio before being fed to tsetse flies.

Batch No's 27700 & 27900

Flies in batch no.'s 27700 to 27900 were fed an infected feed of trypanosomes taken from established procyclic cultures. Complement-containing serum was removed from citrated sheep's blood by washing the blood twice in serum-free buffer. Each wash was carried out by mixing the blood with two volumes of sterile Hank's balanced salt solution containing heparin (50units/ml) and centrifuging at 200g for 10 minutes at 4^0C. The resulting pellet of red blood cells was then mixed with procyclics and heat-inactivated serum in a ratio of 1 part red blood cell "slurry" : 1 part procyclic culture (cell density > 10^7/ml) : 1 part heat-inactivated foetal calf serum (Gibco). This was then fed to tsetse flies as described above, with procyclics retaining their viability, as judged by motility, at 37^0C for at least 45 minutes.

4.2.5 Identification of Double Drug-Resistant Trypanosomes

Flies harbouring salivary gland infections were identified by allowing flies over 18 days old to probe onto microscope slides heated to 37^0C. The dried saliva was then examined under phase-contrast microscopy and scored for the presence of proventricular forms and phase-bright metacyclics. Flies with metacyclic infections were individually fed on Balb/C mice (anaesthetised with 1 part Hypnovel : 1 part Hypnorm : 2 parts $dddH_20$ - 0.1ml/20g mouse by i.p. injection). As many parasites as possible were taken from each subsequent infection by cardiac puncture, and were then transferred to 3 immunosuppressed (cyclophosphamide at a dose of 150mg/Kg by i.p. injection) Balb/C mice. After 24 hours, each mouse received a dose of trypanocide by i.p. injection as follows: mouse 1 was given 5mg/Kg suramin; mouse 2 was given 5mg/Kg melCy; mouse 3 was given both 5mg/Kg suramin and 5mg/Kg melCy. Tail blood from each mouse was monitored for a patent infection (above 2.5 x 10^5 trypanosomes/ml) for up to

14 days, using the rapid matching technique (Herbert and Lumsden 1976). This screen identified flies as having produced mature infections with either single, mixed or hybrid (double drug-resistant) phenotypes (Figure 4.1).

4.3 RESULTS

4.3.1 Rate of Clearance of Trypanosomes

The procedure used to screen the metacyclics produced from the co-transmission experiments meant that the size of the trypanosome inoculum into each of the three Balb/C mice varied depending on the degree of the parasitaemia in the donor mouse originally infected by the tsetse fly. Consequently, it was necessary to examine the effect that the size of the trypanosome inoculum had on both the rate of clearance of drug-sensitive lines and the rate of development of a patent infection with drug-resistant lines in drug treated mice.

Table 4.2 shows how rapidly patent infections of two fly-transmitted lines (247MelCy^{R}ft and 247Sur^{R}ft) appeared in the presence of the drugs to which they expressed resistance (melCy and suramin respectively). As expected, the larger the trypanosome inoculum, the sooner the infection became patent. The appearance of patent infections of 247MelCy^{R}ft occurred between 4 and 6 days after treatment with melCy at a dose of 5mg/Kg when the inoculum size was only 10^{1} trypanosomes/mouse, whereas the infections were patent the day after drug treatment for inoculum sizes of 10^{7} trypanosomes/mouse. Similar values were obtained with the suramin-resistant line in the presence of suramin at a dose of 5mg/Kg, with infections becoming patent the day after drug-treatment with an inoculum size of 10^{7} trypanosomes/mouse, and taking up to 12 days with and inoculum of 10^{1} trypanosomes/mouse.

MelCy appeared to clear the infections of melCy-sensitive (247Sur^{R}ft) trypanosomes very efficiently, with inoculum sizes up to and including 10^7 trypanosomes/mouse being cleared within the first 24 hours after drug-treatment (Table 4.3). This is not the case for infections of 247MelCy^{R}ft after treatment with suramin. Five out of 6 mice given 10^7 trypanosomes showed patent infections 24 hours after suramin treatment. These infections were cleared by the following day. Although a similar experiment was not carried out with the (fly-transmitted) melCy-resistant line used in the co-transmission experiments (386MelCy^{R}ft), it would be expected that the rate of clearance of infection with this line would be similar after suramin treatment, since the minimum curative doses of suramin for both melCy-resistant lines were similar (1.5mg/Kg and 2.5mg/Kg for 247MelCy^R and 386MelCy^R respectively, **chapter 2**).

4.3.2 Generation of Hybrid Trypanosomes

Only 9 of the 17 batches that were infected with 247Sur^R and 386MelCy^R produced metacyclic trypanosomes. Of these 9 batches, only 3 were identified as having produced trypanosomes with hybrid double drug-resistance phenotypes. The 14 batches that failed to produce hybrids will be dealt with briefly, followed by a more detailed account of the 3 successful co-transmissions. Table 4.1 summarises the results of the *in vivo* drug screens for all 17 batches. The process of feeding an infected tsetse fly on a mouse meant that only a sample of the metacyclic population could be screened, and it was possible that unidentified phenotypes were present in the fly that did not enter the mouse. Nevertheless, the drug-resistance phenotypes identified in the mouse screen gave an indication of the natures of the metacyclic populations in the tsetse flies. Single parental infections were identified when trypanosomes grew only in the presence of one drug in the mouse screen; mixed infections grew in both mice treated with a single drug but not in the mouse treated with both melCy and suramin; whereas hybrid infections

were identified as growing in the double drug treated mouse (Figure 4.1).

Unsuccessful Attempts at Generating Hybrids

No hybrids were identified in the first 9 batches, all of which were infected with bloodstream 247Sur^R and 386MelCy^R at a ratio of 1:1. However, the first batch (25700) produced three flies with mature infections all of which developed into mixed phenotype infections (both 386MelCy^R and 247Sur^R were present in the metacyclics). Three batches (27000, 27100 and 27200) produced mature infections consisting of only suramin-resistant trypanosomes (247Sur^R only). Of the 4 batches infected in Bristol with trypanosomes from stabilates, 2 failed to produce hybrids. Batch no. 28000 produced 7 flies all with the suramin-resistant parental phenotype. Batch no. 28100 also contained 7 flies producing only 247Sur^R by itself, along with one fly producing a mixed infection.

Flies in batch no.'s 27700 to 27900 were infected with procyclic 386MelCy^R and 247Sur^R at a ratio of 1:1. All 3 batches produced no mature infections.

Successful Attempts at Generating Hybrids

Flies in batch no. 27500 were infected with bloodstream 386MelCy^R and 247Sur^R at a ratio of 2:1. The flies in this batch produced infections with a variety of phenotypes. One fly produced only suramin-resistant trypanosomes (247Sur^R parental only), two produced mixed infections (both 247Sur^R and 386MelCy^R), and two flies produced a population of trypanosomes which was resistant to both suramin and melCy given together. Table 4.4 gives details of the course of the salivary gland infections in the 5 flies that were successfully screened in batch no. 27500. Fly 27514 was not identified as having produced a mature infection until

day 39, and was fed only once on a mouse, producing melCy-resistant trypanosomes (386MelCy^R only). The other four flies all produced mixed infections, with trypanosomes, expressing the hybrid phenotype, appearing in two flies at the same time as the appearance of a mixed infection (day 27 in fly 27543 and day 39 in fly 27568). The mouse screen identified double drug-resistant trypanosomes on four separate occasions. The previously described nomenclature (Sternberg *et al.* 1989), incorporating the fly number with the first two digits deleted together with the day post infection, has been used to refer to flies and trypanosomes on particular days. For example, trypanosomes from fly 27568 on day 39 post infection are referred to as 568/39; the fly on this day is referred to with an 'F' prefix, i.e. F568/39). The parasitaemias in the drug treated mice from the mouse screens are shown in Table 4.5. On all four occasions that hybrids were identified in batch no. 27500, the patent infections arising in mice treated with both drugs, were very transient, lasting only one or two days and at only barely patent levels (parasitaemias of 10^6/ml or less). However, with F543/27, /28 and /31, trypanosomes were identified in the double drug treated mice between up to 4 and 6 days after treatment with both melCy and suramin. This is in comparison to the results obtained with the rate of clearance of infection studies (**section 4.3.1**), in which suramin-resistant trypanosomes were cleared within 1 day by melCy, and similarly, melCy-resistant trypanosomes were cleared within 2 days by suramin (Table 4.3). The mouse screen also identified trypanosomes in the double drug-treated mouse infected with 568/39. However, these were only seen at barely patent levels the day after double drug treatment, similar to the situation observed with high levels of trypanosome inoculum of 247MelCy^{R}ft, in which a curative dose of suramin (5mg/Kg) was unable to clear the infection 1 day after drug treatment, but cleared the infection on the following day (Table 4.3).

Batch no. 28200 produced three flies with trypanosomes of suramin-resistance phenotype only, and three with mixed salivary gland infections, two of

which also produced infections that were double drug-resistant. The patterns of infections in these 6 flies are shown in Table 4.6. The results of the mouse screens for the two occasions on which double drug-resistant trypanosomes were identified, F201/43 and F205/43, are shown in Table 4.7. In both cases when double drug-resistant trypanosomes were identified, the parasitaemias were at barely patent levels ($2.5x10^5$ trypanosomes/ml), and trypanosomes were identified only one day after drug treatment. This compares to the parasitaemias observed in the melCy-only treated mice and in particular the suramin-only treated mice, in which relatively high levels of infection were observed 1 and 2 days after drug treatment.

Batch no. 28300 produced two flies with mature infections that survived long enough to be fed on mice (Table 4.8). One fly produced only suramin-resistant parental trypanosomes, whilst one fly showed evidence of having hybrid trypanosomes, with double drug-resistance being evident in F301/54 (Table 4.9), although this did not appear again during the subsequent 17 days of the flies life. Furthermore, no melCy-resistant trypanosomes were identified throughout the life of either of the two flies.

4.4 DISCUSSION

Double drug-resistant trypanosomes have been generated by co-transmitting through tsetse flies two "fertile" lines each expressing resistance to a single drug. These trypanosomes were identified, using the *in vivo* mouse screen described, by exploiting the drug-resistance phenotypes as selectable markers. $247Sur^R$ and $386MelCy^R$ were chosen as the two lines used to generate double drug-resistant trypanosomes. The other alternative combination of lines available at that time was $386Sur^R$ and $247MelCy^R$. This combination was not used due to the relative unsuitability of $386Sur^R$ on two counts - it was relatively difficult to

transform this line to procyclics and, possibly for the same reason, attempts at transmitting the cloned line through tsetse flies had so far proved unsuccessful. However, even with 247Sur^R and 386MelCy^R, a number of problems were encountered during the course of generating hybrids. In particular, it would appear (not surprisingly) that the nature of the initial infective feed had a very marked effect on the type of mature infection that the flies produced. The later attempts at generating hybrids were generally far more successful than earlier attempts, and therefore the changes made to the initial infective feeds will be discussed in chronological order.

The first two attempts at generating hybrids (batch no.'s 25700 and 26100) were fed with 247Sur^R and 386MelCy^R in a 1:1 ratio with trypanosomes taken directly from mice (Table 4.1). It was occasionally difficult to synchronise two different trypanosome infections in mice with each other and also with the hatching of tsetse flies, and consequently the use of pre-mixed stabilates was employed. The first stabilate (GUP 3599) was probably of poor quality, since it produced no mature infections in approximately 400 flies infected with it. The second stabilate (GUP 3606) appeared to be of better quality, although it favoured the production of 247Sur^R (batch no.'s 27000 to 27200).

It was assumed that the most important factor affecting the lack of success of hybrid production was the difficulty of transmitting 386MelCy^R through tsetse flies, and hence 386MelCy^R and 247Sur^R were not present together at the correct "fertile" stage of the life cycle. This can be seen clearly by examining the results from the mouse screens in all nine co-transmission experiments that produced mature infections (Table 4.1). A total of thirty-seven flies harbouring mature infections were successfully screened, with 24 of these producing 247Sur^R only and only 1 producing 386MelCy^R only. There are two events in the trypanosome's life cycle in the vector that are crucial for successful transmission to occur. The

first step is the successful establishment of a procyclic infection in the midgut from one, or more than one, short-stumpy form(s). The next step is the transformation to, and the successful migration of, one or more proventricular form trypanosomes to establish an infection in the salivary gland (Vickerman 1985). The relative difficulty of fly-transmitting both the drug-resistant lines generated from 386 occurs, therefore, because of a comparative low rate of success either in the establishment of a midgut infection or in the establishment of a salivary gland infection. Both of these factors are probably important. On purely anecdotal evidence, the *in vitro* transformation of 386MelCy^R bloodstream forms to procyclic culture forms appeared to be more difficult than the transformation of 247Sur^R, although this was as likely to be due to the difficulty of obtaining large numbers of short-stumpy forms of 386MelCy^R in mice, as it was to be due to a relative inability of these short-stumpy forms to transform to procyclics. However, it has also been found that a midgut infection of unselected 247 was more likely to give rise to a metacyclic infection than a midgut infection of unselected 386 (Maudlin and Welburn pers. comm.). Irrespective of whether mating occurs in the tsetse midgut, as proposed by Jenni *et al.* (1991) or in the salivary gland, as suggested by Gibson and Whittington (1993), the use of two lines with differences in fly-transmissibility will undoubtedly favour the production of metacyclic infections of one line. In an attempt to alleviate this problem, the next batch of flies (27500) was infected with 247Sur^R and 386MelCy^R at a ratio of 1:2 using trypanosomes taken from mice. The production of 4 flies with mixed infections, two of which also produced trypanosomes with the hybrid phenotype, seemed to support the use of an initial infective feed that was weighted, in terms of numbers, towards the line that transmitted less favourably through flies. However, although this may be the reason for the appearance of mixed and double drug-resistant infections, there may be a more important factor influencing the production of these mixed infections. 386MelCy^R trypanosomes for the infective feed were taken from a mouse on day 5 of infection. The parasitaemia was $2.5x10^8$/ml and had

peaked 2 days previously. However, 247Sur^R trypanosomes were taken from a mouse at a parasitaemia of $5x10^8$/ml on day 4 of an infection that had not yet peaked (or was at its peak). Therefore, the most significant factor concerning the infective feed was that 386MelCy^R was taken from mice when it consisted predominantly of short-stumpy form trypanosomes (as judged by sight on a wet blood film), and therefore it was far more capable of transforming to procyclics than in previous attempts, when trypanosomes had been taken from mice with rising parasitaemias (predominantly non-transforming long-slender forms). This may not be particularly important in co-transmission experiments with lines that are relatively easy to transmit (such as 247, 386, 247MelCy^R and 247Sur^R), but with more difficult lines it may have a considerable effect.

As a consequence of this finding, it was assumed that the use of procyclics for the infective feed would further enhance the prospects of generating hybrids. However, the next 3 batches (no.'s 27700 to 27900) infected with procyclics in a 1:1 ratio failed to produce mature infections. However, it was possible that this was due to insufficient washing of the red blood cells to remove the complement-containing serum during the preparation of the red blood cell 'slurry' for the infective feeds.

The final 4 batches (28000 to 28300) were infected with bloodstream trypanosomes taken from cryopreserved samples. The stabilates were prepared using trypanosomes taken from mice with predominantly short-stumpy infections. These 4 batches produced 5 flies with mixed infections, 3 of which also produced hybrids. However, 15 of the 22 mature infections that were tested were of a single phenotype only (247Sur^R). It would therefore appear that although the use of predominantly short-stumpy infections increases the chances of generating hybrids, it may also be valid to argue that weighting the infective feed in favour of 386MelCy^R is also an important factor. This could be achieved either simply in

terms of relative numbers or by disadvantaging 247Sur^R by not taking them from a predominantly stumpy population. Both these alternatives essentially amount to the same, namely that in terms of short-stumpy forms present in the infective feed, for lines that are more difficult to transmit, it appears to be better to have larger numbers.

The results of the *in vivo* screens that are summarised in Tables 4.5, 4.7 and 4.9 showed 7 separate occasions when the double drug-resistant trypanosomes were identified. Fly 27543 produced trypanosomes with double drug-resistance on 3 consecutive occasions, whilst the other 4 times that the double drug-resistant phenotype was identified occurred in 4 different flies on isolated occasions. It would appear that these 4 isolated occasions, on which trypanosomes with the hybrid (double drug-resistance) phenotype were found, were actually false positives. This conclusion has been arrived at for a number of reasons. Close examination of the actual parasitaemias in the mice that were given both drugs shows that for fly 27543, on all 3 occasions the parasitaemia was sub-patent for up to 4 days post drug treatment, prior to the emergence of a patent infection (Table 4.5; F543/27, /28, and /31). This shows categorically that the trypanosomes had survived treatment with both trypanocides, were multiplying in the mouse after this drug-treatment, and were therefore definitely double drug-resistant trypanosomes. On only one other occasion (F301/54) were trypanosomes found to have multiplied after treatment with both melCy and suramin (Table 4.9). However, for 2 weeks previous to this, and for over 2 weeks after this, the mouse screen identified only suramin-resistant trypanosomes, with no indication of melCy-resistant metacyclic trypanosomes being present at any time during the life of the fly (Table 4.8). Furthermore, the increase in parasitaemia was seen immediately after treatment with both drugs and not after a period of sub-patency. Finally, all attempts at rescreening these 'double drug-resistant' trypanosomes by transferring them to further mice failed to identify anything other than suramin-resistant

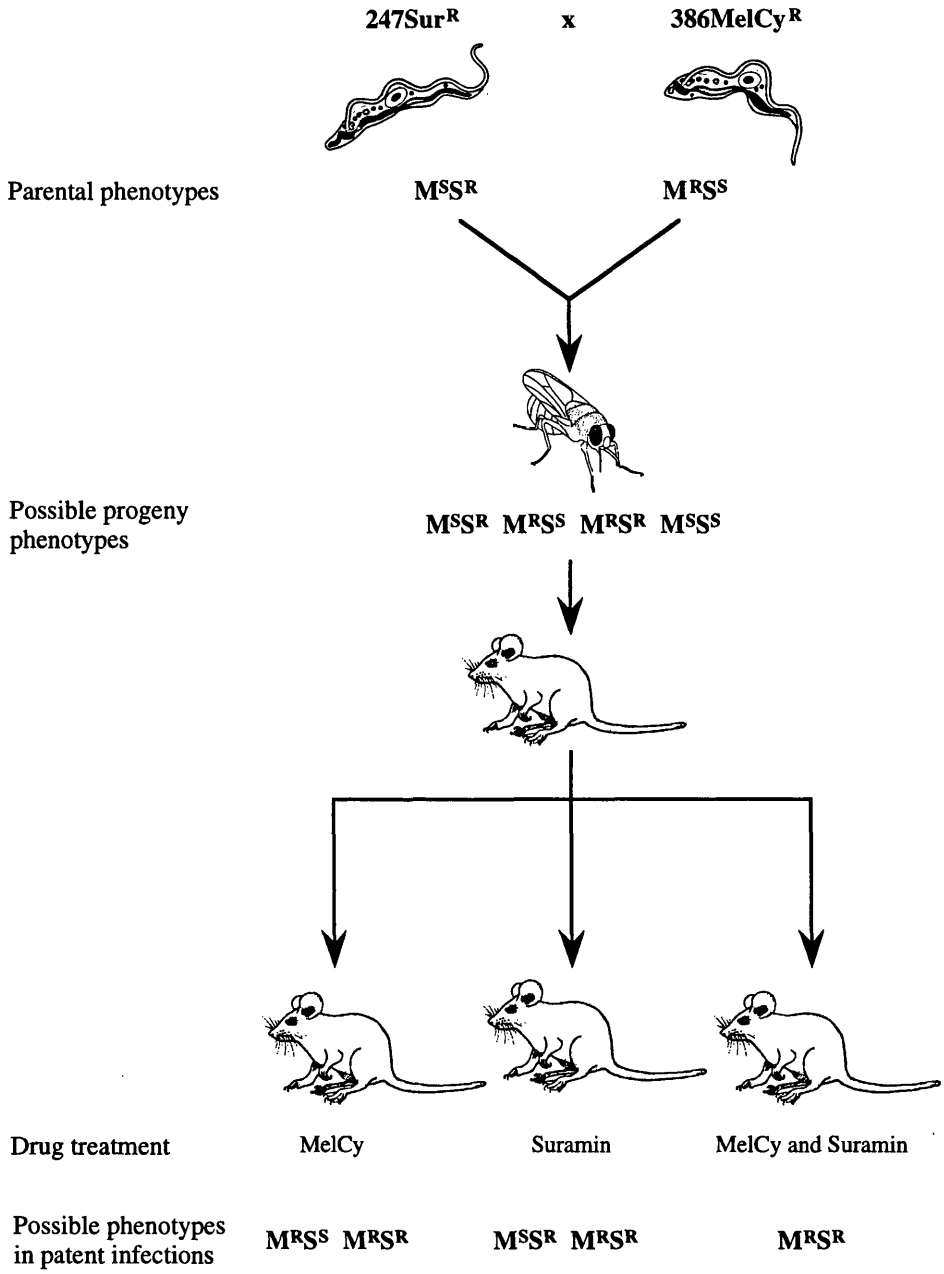
trypanosomes only. For these reasons, F301/54 almost certainly produced 247Sur^R trypanosomes only. On the 3 other occasions when putative double drug-resistant trypanosomes were identified (F568/39, F201/43 and F205/43) the parasitaemias were evident only on the day after drug-treatment (Tables 4.5 and 4.7). The studies on the effect of the size of the trypanosome inoculum on the rate of clearance of infection showed that although melCy was very efficient at clearing melCy-sensitive trypanosomes, mice infected with suramin-sensitive trypanosomes may still have patent infections the day after suramin treatment when infected with 10^6 trypanosomes or more (Table 4.3). Consequently, the 3 occasions on which trypanosomes were observed at barely patent levels the day after treatment with both drugs may in fact be due to melCy-resistant, suramin-sensitive trypanosomes that have not been fully cleared from the circulation by the action of suramin, and were not, therefore, double drug-resistant trypanosomes.

It would appear, therefore, that only one fly (Fly 27543) produced double drug-resistant trypanosomes, possibly as a result of genetic exchange between 247Sur^R and 386MelCy^R. Unfortunately, a number of attempts were made to rescreen the unselected populations (from the donor mice) that had been cryopreserved. No double drug-resistant trypanosomes could be identified in these rescreening attempts. Although this may indicate that the fly had not, in fact, produced double drug-resistant trypanosomes, it was more likely that the process of cryopreservation killed these trypanosomes. The low levels and transient nature of the parasitaemias observed in the double drug treated mice (F543/27, /28, and /31) indicate that the double drug-resistant trypanosomes were comparatively small in number, relative to trypanosomes expressing a single drug-resistance phenotype, and/or have a low growth rate compared to the trypanosomes with parental drug-resistance phenotypes. Consequently, the populations of trypanosomes that were cryopreserved from the unscreened donor mice would contain only a very small proportion of double drug-resistant trypanosomes. Cryopreservation, and the

subsequent thawing, renders over 99% of trypanosomes non-viable (Barry and Turner, unpublished results), and this process may have been sufficient to kill the few double drug-resistant trypanosomes that were present in the sample of blood used for cryopreservation. It is important therefore not to rely on cryopreservation to maintain the integrity of a trypanosome population with mixed phenotypes, and instead it would be better to passage double drug-resistant trypanosomes in order to obtain a sufficiently high parasitaemia from which clones can be generated.

In conclusion, the results of the co-transmission experiments indicate that the low transmissability of 386MelCy^R posed a problem for the generation of double drug-resistant trypanosomes. The use of infective feeds containing predominantly short-stumpy forms appeared to go some way towards alleviating this problem, as this would be expected to increase the probability of establishing a mixed infection in the tsetse midgut. With respect to the identification of flies producing double drug-resistant trypanosomes, consideration must be given to the fact that suramin may not clear all the trypanosomes within 24 hours, and only those trypanosomes that have multiplied after drug treatment should be considered drug-resistant. Furthermore, trypanosomes identified as double drug-resistant should be passaged and subsequently cloned in order to prove double drug-resistant trypanosomes are indeed products of genetic exchange.

Figure 4.1
Experimental Procedure for the Generation of Double Drug-Resistant Trypanososomes



This figure outlines the protocol used in the co-transmission experiments. The two drug-resistant trypanosome lines, 247Sur^R and 386MelCy^R, have the two phenotypes, M^{S}S^R and M^{R}S^S respectively. Flies producing mature infections were allowed to feed on a mouse, with the subsequent bloodstream infection being transferred to 3 mice. The parasitaemias emerging in these mice after drug treatment indicated the drug-resistance phenotypes present in the metacyclic populations.

M^{R}S^S MelCy-resistant and suramin-sensitive
M^{S}S^R MelCy-sensitive and suramin-resistant
M^{R}S^R MelCy-resistant and suramin-resistant
M^{S}S^S MelCy-sensitive and suramin-sensitive

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Table 4.1
Summary of Co-Transmission Experiments

Batch Number	Infective Feed	Source of Trypanosomes	Flies Producing Mature Infections	Phenotype of Trypanosomes in Mature Infections
25700	Bloodstream	Mice	3	3 x Mixed
26100	Bloodstream	Mice	0	-----
26300	Bloodstream	Stabilate[a]	0	-----
26400	Bloodstream	Stabilate[a]	0	-----
26500	Bloodstream	Stabilate[a]	0	-----
26600	Bloodstream	Stabilate[a]	0	-----
27000	Bloodstream	Stabilate[b]	5	2 x Sur^R
27100	Bloodstream	Stabilate[b]	3	2 x Sur^R
27200	Bloodstream	Stabilate[b]	4	2 x Sur^R
27500	Bloodstream	Mice	9	1 x $MelCy^R$; 2 x Mixed; 2 x Hybrid
27700	Procyclics	Culture	0	-----
27800	Procyclics	Culture	0	-----
27900	Procyclics	Culture	0	-----
28000	Bloodstream	Stabilate[c]	17	7 x Sur^R
28100	Bloodstream	Stabilate[c]	10	7 x Sur^R; 1 x Mixed
28200	Bloodstream	Stabilate[c]	7	3 x Sur^R; 1 x Mixed; 2 x Hybrid
28300	Bloodstream	Stabilate[c]	5	1 x Sur^R; 1 x Hybrid

This table summarises all 17 co-transmission experiments conducted with 247Sur^R and 386$MelCy^R$. Tsetse flies were fed either bloodstream (from stabilate or mice) or procyclic trypanosomes. Flies producing mature infections were identified and the number of flies producing metacyclic infections is shown. Not all mature infections were screened in mice because some flies died before being fed on a mouse. The drug-resistance phenotypes emerging in the mouse screen are shown.

[a] GUP 3599. Bloodstream trypanosomes that were pre-mixed and cryopreserved in a 1:1 ratio.

[b] GUP 3606. Bloodstream trypanosomes that were pre-mixed and cryopreserved in a 1:1 ratio. Different to GUP 3599.

[c] Bloodstream trypanosomes that were cryopreserved as seperate lines and mixed in a 1:1 ratio prior to being fed to flies.

MelCy^R Mouse screen identified trypanosomes expressing melCy-resistance only (i.e. 386MelCy^R).

Sur^R Mouse screen identified trypanosomes expressing suramin-resistance only (i.e. 247Sur^R).

Mixed Mouse screen identified trypanosomes expressing suramin-resistance only and melCy-resistance only (i.e. 247Sur^R and 386MelCy^R).

Hybrid Mouse screen identified trypanosomes expressing both suramin-resistance and melCy-resistance (i.e. 247Sur^R x 386MelCy^R putative hybrid).

Table 4.2
Rate of Development of Patent Infections in Drug Treated Mice

Trypanosome Line	Drug	No. of Trypanosomes/Mouse	No. of Mice Developing Patent Infections	Day Infection First Became Patent (range)[a]
247MelCy^{R}ft	MelCy	10^1	6	4-6
247MelCy^{R}ft	MelCy	10^2	6	3-4
247MelCy^{R}ft	MelCy	10^3	6	3-4
247MelCy^{R}ft	MelCy	10^4	6	2-3
247MelCy^{R}ft	MelCy	10^5	6	2
247MelCy^{R}ft	MelCy	10^6	6	1-2
247MelCy^{R}ft	MelCy	10^7	6	1
247Sur^{R}ft	Suramin	10^1	6	4-12
247Sur^{R}ft	Suramin	10^2	6	3-4
247Sur^{R}ft	Suramin	10^3	6	2-4
247Sur^{R}ft	Suramin	10^4	6	1-2
247Sur^{R}ft	Suramin	10^5	6	1-2
247Sur^{R}ft	Suramin	10^6	6	1
247Sur^{R}ft	Suramin	10^7	6	1

The effect of trypanosome inoculum size on the rate of develpment of a patent infection in drug-treated mice. Drug-resistant lines (247MelCy^R and 247Sur^R) were transmitted seperately through flies and subsequently used to infect Balb/C mice in groups of 6, each group of mice receiving 10^1 to 10^7 trypanosomes/mouse.

[a] The range (days) over which a patent infection appeared within a group of 6 mice. For example, a patent infection was detected between days 4 and 6 after melCy treatment in mice infected with 10^1 trypanosomes (247MelCy^{R}ft).

Table 4.3
Rate of Clearance of Infections in Drug Treated Mice

Trypanosome Line	Drug	No. of Trypanosomes/Mouse	No. of Mice in which Infection Cleared	Day of Clearance of Infection (range)[a]
247MelCy^{R}ft	Suramin	10^5	6	1
247MelCy^{R}ft	Suramin	10^6	6	1-2
247MelCy^{R}ft	Suramin	10^7	6	1-2
247Sur^{R}ft	MelCy	10^5	6	1
247Sur^{R}ft	MelCy	10^6	6	1
247Sur^{R}ft	MelCy	10^7	6	1

The effect of trypanosome inoculum size on the rate of clearance of an infection in drug-treated mice. Drug-resistant lines (247MelCy^{R}ft and 247Sur^{R}ft) were used to infect Balb/C mice in groups of 6, each group of mice receiving 10^5 to 10^7 trypanosomes/mouse.

[a] The range (days) over which a patent infection could no longer be detected within a group of 6 mice. For example, no patent infections were detected 1 day after suramin treatment in mice infected with 10^5 trypanosomes (247MelCy^{R}ft), whereas between 1 and 2 days were required for suramin to clear mice infected with 10^6 trypanosomes (247MelCy^{R}ft).

Table 4.4
Course of Salivary Gland Infection in Flies (Batch No. 27500)

Fly Number	Days post infection						
	27	28	31	33	35	39	40
27514						M^R	
27543	$S^R,M^R,\mathbf{D^R}$	$S^R,M^R,\mathbf{D^R}$	$M^R,\mathbf{D^R}$				
27556		S^R,M^R		S^R,M^R			S^R,M^R
27557	S^R		S^R,M^R	S^R,M^R			
27568					M^R	$S^R,M^R,\mathbf{D^{Ra}}$	

The results of the mouse screens are shown for 5 flies producing metacyclic infections in batch no. 27500. The unique fly numbers are shown, together with the day (after the fly was given an infective feed) on which the fly was fed on a mouse. The change in the drug-resistance phenotypes of the metacyclic populations, as defined by the mouse screen, are shown over the course of the life of the flies.

M^R Trypanosomes expressing melCy-resistance were present ($386MelCy^R$).
S^R Trypanosomes expressing suramin-resistance were present ($247Sur^R$).
$\mathbf{D^R}$ Trypanosomes expressing both melCy-resistance and suramin-resistance were present ($247Sur^R$ x $386MelCy^R$).
[a] Trypanosomes were present only for the first day after drug treatment in the double drug treated mouse, and at a barely patent level (antilog 5.4 trypanosomes/ml).

Table 4.5
Screening the Drug-Resistant Phenotypes present in Metacyclic Populations of Flies in Batch No. 27500

F514/39

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.6	8.4											
Suramin	0	0	0	0		0	0	0	0	0			
MelCy and Suramin	0	0	0	0		0	0	0	0	0			

F543/27

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.6		8.4	8.4	8.4								
Suramin	0		0	0	6.0	6.6	0						
MelCy and Suramin	0		5.4	0	5.4	5.4	0			0			

F543/28

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy		8.4	8.7										
Suramin		0	0	0	6.3	0			0				
MelCy and Suramin		0	0	5.4	0	0			0				

F543/31

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.3	7.5			8.4	8.4	8.1	8.1					
Suramin	0	0			0	0	0	0	0	0			0
MelCy and Suramin	0	0			5.7	0	0	0	0	0			0

F556/28

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy		5.4	7.2	8.1	8.1								
Suramin		8.4	8.7										
MelCy and Suramin		0	0	0	0	0			0	0			

F556/33

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	0			8.1	8.4							
Suramin	7.5	8.4											
MelCy and Suramin	0	0			0	0	0	0	0			0	

F556/40

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	5.4	7.5				8.4						
Suramin	7.2	7.8	8.7										
MelCy and Suramin	0	0	0			0	0	0					

F557/27

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0		0	0	0	0	0			0			
Suramin	7.5		8.7										
MelCy and Suramin	0		0	0	0	0	0			0			

F557/31

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	0	6.0	7.8	8.1								
Suramin	7.2	8.1	8.7										
MelCy and Suramin	0	0	0	0	0			0	0				

F557/33

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	0			8.4								
Suramin	5.4	7.8			8.7								
MelCy and Suramin	0	0			0		0	0	0				0

F568/35

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	0	0			8.1	7.8						
Suramin	0	0	0			0	0						
MelCy and Suramin	0	0	0			0	0						

F568/39

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.6	8.7											
Suramin	5.4	0	0	0		0	0	0					
MelCy and Suramin	5.4	0	0	0		0	0	0					

These tables show the results of the mouse screens used to identify the drug-resistance phenotypes present in the metacyclic populations of 5 flies in batch no. 27500. The numbers in the tables are the parasitaemias (antilog trypanosomes/ml) in the drug treated mice monitored for up to 13 days after drug treatment. The parasitaemias were measured using the rapid matching technique (Herbert and Lumsden 1976), which was unable to detect parasitaemias lower than 2.5×10^5 trypanosomes/ml.

Table 4.6
Course of Salivary Gland Infection in Flies (Batch No. 28200)

Fly Number	Days post infection									
	41	43	45	48	50	52	55	57	59	66
28201	S^R,M^R	S^R,M^R,D^{Ra}	S^R,M^R	S^R,M^R	S^R,M^R		S^R			
28202	S^R	S^R	S^R	S^R	S^R					
28203	S^R			S^R	S^R	S^R		S^R	S^R	
28204	S^R									
28205	S^R,M^R	S^R,M^R,D^{Ra}		S^R,M^R		S^R,M^R	S^R,M^R			S^R
28206			S^R,M^R							

The results of the mouse screens are shown for 6 flies producing metacyclic infections in batch no. 28200, showing the change in the drug-resistance phenotypes of the metacyclic populations, as defined by the mouse screens, over the course of the life of the flies.

M^R Trypanosomes expressing melCy-resistance were present ($386MelCy^R$).
S^R Trypanosomes expressing suramin-resistance were present ($247Sur^R$).
D^R Trypanosomes expressing both melCy-resistance and suramin-resistance were present ($247Sur^R$ x $386MelCy^R$).
[a] Trypanosomes were present only for the first day after drug treatment in the double drug treated mice, infected with 28201/43 and 28205/43. In both cases, the level of parasitaemia was antilog 5.4 trypanosomes/ml.

Table 4.7
Screening the Drug-Resistant Phenotypes present in Metacyclic Populations of Flies in Batch No. 28200

F201/43

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.3	7.8											
Suramin	7.8	8.7											
MelCy and Suramin	5.4	0	0	0	0	0	0		0	0			0

F205/43

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	6.6	7.8											
Suramin	8.7												
MelCy and Suramin	5.4	0	0	0	0	0	0	0	0			0	

These tables show the results of 2 mouse screens in which trypanosomes were identified in mice treated with both melCy and suramin. In both cases, infections in the double drug treated mice were just at patent levels and transient, lasting only one day. See legend for Table 4.5 for explanation

Table 4.8
Course of Salivary Gland Infection in Flies (Batch No. 28300)

Fly Number	Days post infection									
	40	42	45	49	54	59	62	64	69	71
28301		S^R	S^R	S^R	S^R,D^R	S^R	S^R	S^R	S^R	S^R
28302	S^R		S^R							

The results of the mouse screens are shown for 2 flies producing metacyclic infections in batch no. 28300, showing the change in the drug-resistance phenotypes of the metacyclic populations, as defined by the mouse screens, over the couse of the life of the flies.

S^R Trypanosomes expressing suramin-resistance were present ($247Sur^R$).
D^R Trypanosomes expressing both melCy-resistance and suramin-resistance were present ($247Sur^R$ x $386MelCy^R$).

Table 4.9
Screening the Drug-Resistant Phenotypes present in Metacyclic Populations of Flies in Batch No. 28300

F301/54

Drug	Days Post Drug Treatment												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MelCy	0	0	0			0	0	0	0	0			
Suramin	7.2	7.8											
MelCy and Suramin	6.0	7.8											

This table shows the results of the mouse screen to identify the drug-resistance phenotypes in the metacyclic population of F301/45. Trypanosomes were identified at high levels in the double drug treated mouse, and the parasitaemia increased. However, no trypanosomes were observed in the mouse treated with melCy only. See legend for Table 4.5 for explanation

CHAPTER 5
ANALYSIS OF CLONED PROGENY TO DETERMINE THE INHERITANCE OF DRUG-RESISTANCE

5.1 INTRODUCTION

The inheritance of a variety of phenotypic (isoenzyme) and genotypic (RFLP) markers has been investigated in a number of genetic crosses of *T. brucei* stocks (reviewed in Tait *et al.* 1993). Analysis of cloned progeny from these crosses have provided substantial evidence for the existence of a diploid Mendelian system of mating involving meiosis and recombination and segregation of alleles (Sternberg *et al.* 1989; Turner *et al.* 1990; Gibson *et al.* 1992). Karyotype analysis has also been used to investigate genetic exchange in *T. brucei*. The use of pulsed-field gel electrophoresis (PFGE) has allowed many of the details of the genome of *T. brucei* to be elucidated, and approximately 80% of the chromosomes have been identified using different running conditions to separate chromosomes ranging in size from approximately 150kb to 5.7Mb (Van der Ploeg *et al.* 1989; Gottesdiener *et al.* 1990). The chromosomes have been grouped into three different categories, according to their size and their pattern of inheritance during genetic exchange. The mini-chromosomes (50-150kb), of which there are approximately one hundred (Van der Ploeg *et al.* 1989), and the intermediate chromosomes (150kb-800kb) are not diploid and do not follow a Mendelian pattern of inheritance (Wells *et al.* 1987; Gibson 1989). However, most, if not all, the larger chromosomes (800kb to 5.7Mb) are thought to be diploid. Separation of these larger chromosomes, followed by probing of Southern blots with gene-specific probes, has shown that there are at least 14 large chromosomes, and that these chromosomes can be shown to belong to 7 homologous pairs (Gottesdiener *et al.* 1990). Analysis of the inheritance of the larger chromosomes has provided further evidence for them

being homologous pairs. Co-transmission of two stocks of *T. b. brucei* (196 and J10) through tsetse flies produced cloned hybrid trypanosomes with karyotypes different to either parent (Gibson and Garside 1991). Novel karyotypes have also been observed in hybrid progeny from a genetic cross between 247, *T. b. brucei*, and 386, *T. b. rhodesiense* (Tait *et al.* 1993), and between 058, *T. b. rhodesiense*, and KP2, *T. b. brucei*, (Gibson and Whittington 1993). Southern blotting and hybridisation with gene-specific probes showed that the homologous chromosomes in the parents from these genetic crosses had segregated, recombined and were inherited by the hybrid clones in a manner generally consistent with a Mendelian system of genetic exchange in a diploid organism. Such measurements that have been made on bloodstream and insect stages have shown that parental and progeny clones of *T. brucei* possess similar DNA contents (Wells *et al.* 1987; Kooy *et al.* 1989; Gibson *et al.* 1992; Tait *et al.* 1993). Further evidence for a mating system involving meiosis either before or after nuclear fusion has come from nuclear DNA measurements (Sternberg and Tait 1989). However, a number of anomalous results, inconsistent with Mendelian inheritance in a diploid organism, have also been observed. The DNA contents of some hybrid progeny have been found to be higher than those of the parents (Jenni *et al.* 1986; Gibson *et al.* 1992), and this has been explained in some cases by aberrant genetic exchange events involving the fusion of diploid and haploid nuclei to produce triploid progeny (Gibson *et al.* 1992). Furthermore, chromosome size changes have been observed in some diploid chromosomes (Tait *et al.* 1993), although this may not necessarily be a product of mating (Gibson *et al.* 1991). Despite these anomalies, karyotype analysis of a number of genetic crosses to date upholds a mating system in which the mini and intermediate chromosomes do not segregate in a diploid Mendelian manner, whereas, in the main, the larger chromosomes (>800kb) belong to homologous pairs, segregate and are inherited in a diploid Mendelian system involving meiosis and karyogamy.

The patterns of mating observed between different trypanosome stocks appear to indicate that there are no barriers to mating, or that mating is controlled by a mating type locus that is heterozygous in at least some stocks (Tait *et al.* 1993). Genetic exchange has been demonstrated between all pairwise combinations of three cloned stocks (i.e. 247 x 386, 247 x 927/4, and 386 x 927/4) (Turner *et al.* 1990), and although there has been no evidence for genetic exchange occurring during the cyclical transmission of single trypanosome stocks through tsetse flies (Tait *et al.* 1989), self-fertilisation (selfing) has been shown to occur during the co-transmission of two stocks that have undergone genetic exchange (Tait *et al.* 1993). Whether selfing can actually occur during single stock transmissions but has not yet been observed, or actually requires the presence of a second stock, perhaps to induce meiosis, still remains to be clarified. Nevertheless, due to the non-obligatory nature of genetic exchange, cloned progeny from co-transmission experiments can be considered to belong to one of three categories: "parental", the product of asexual propagation through the fly; "hybrid", the product of genetic exchange between two different stocks; or "selfer", the product of genetic exchange between trypanosomes of the same stock.

Parental clones have all the properties, in terms of karyotype and genotypic and phenotypic markers of one or other of the parental stocks used in the co-transmission experiments, and appear to be, in essence, clones of the parent. Hybrid progeny inherit some of the properties of both parental stocks, and can be identified from the inheritance of markers that are homozygous but different in the parents, in which case the hybrid clones exhibit heterozygous patterns (Figure 5.1(a)). Selfers are the products of self-fertilisation, and although they may, by chance, inherit all the properties of the parental stock, some will show homozygous patterns for markers in which the parent is heterozygous (Figure 5.1(b)).

5.1.1 Experimental Design

Hybrid formation has been demonstrated during the co-transmission of 247 and 386 (Sternberg *et al.* 1989; Turner *et al.* 1990) and further analysis of the cloned progeny has shown that selfing also occurred with 247 (Tait *et al.* 1993). Since not all selfers can be identified from the limited number of markers of genetic exchange available, the drug-resistance phenotypes of the lines described previously (**chapter 2**) have been used as selectable markers in order to identify selfers that have inherited both drug-resistance phenotypes. A three-way co-transmission experiment was conducted, involving the two drug-resistant lines of 247 (247MelCy^R and 247Sur^R), together with 386MelCy^R. It was intended that a successful three-way co-transmission of these lines through tsetse flies would generate both hybrid trypanosomes and products of self-fertilisation, as well as parental progeny. Hybrid trypanosomes would be generated from genetic exchange between 247MelCy^R and 386MelCy^R, or 247Sur^R and 386MelCy^R, whereas products of self-fertilisation would be generated from genetic exchange between 247MelCy^R and 247Sur^R. Identification of trypanosomes expressing double drug-resistance to both melCy and suramin would indicate that mating had occurred during cyclical transmission, and analysis of the inheritance of isoenzymes and karyotype in the cloned progeny would indicate whether these double drug-resistant trypanosomes were hybrids or selfers. Analysis of the pattern of inheritance of the drug-resistance phenotypes would also provide information on the genetic basis of melCy-resistance and suramin-resistance in terms of the number of loci and alleles controlling drug-resistance, and indicate whether the inheritance of drug-resistance is dominant or recessive. Depending on this exact genetic nature of drug-resistance in parental lines, other drug-resistance phenotypes would be expected to be present in the products of mating, such as double drug-sensitive progeny, although the screening procedure used was not designed to identify

these.

The design of this experiment was similar to that of previous co-transmissions. Flies infected with trypanosomes were screened for the presence of mature infections. The drug-resistance phenotypes in the metacyclic populations were determined by allowing metacyclic producing flies to feed on mice to produce bloodstream trypanosomes which were subsequently transferred to 3 mice that were given melCy, suramin, and melCy plus suramin as described previously (**4.2.5**). This screen would identify trypanosomes expressing resistance to both melCy and suramin, and therefore the progeny that could potentially be detected using the drug screening assays employed were 247Sur^R x 386MelCy^R hybrids and 247Sur^R x 247MelCy^R selfing products.

5.2 MATERIALS AND METHODS

5.2.1 Drugs

The two trypanocidal drugs melCy and suramin were used to screen for drug-resistant trypanosomes after the cyclical transmission of mixtures. MelCy and suramin were obtained and stored as described previously (**2.2.1**).

5.2.2 Trypanosome Stocks and Mice

Three trypanosome lines were used from established procyclic cultures These three lines (247MelCy^R, 247Sur^R and 386MelCy^R) have been described previously (**chapters 2 and 3**).

Adult female Balb/C mice (approximately 20g body weight) and adult female CFLP mice were used for screening the trypanosome populations after fly transmission.

5.2.3 Infection of Tsetse Flies

A single co-transmission experiment was conducted. A batch (No. 28400) of 101 teneral tsetse flies was infected with all three lines of procyclic trypanosomes in a ratio of 1:1:1 in a three-way mixed transmission experiment (247MelCy^R x 247Sur^R x 386MelCy^R). The flies were infected and given maintenance feeds as described in the previous chapter (**4.2.3**).

5.2.4 Identification of Double Drug-Resistant Trypanosomes

Flies harbouring mature infections were identified as described previously (**4.2.5**). The bloodstream trypanosomes from mice infected by flies harbouring metacyclic infections were screened for the presence of melCy-resistance, suramin-resistance and double drug-resistance to both melCy and suramin as described previously (**4.2.5**).

5.2.5 Generation of Cloned Trypanosomes

Cloned progeny were generated from two flies (F444 and F466) between 51 days and 63 days after the flies had been given the initial infective feed of 247MelCy^R, 247Sur^R and 386MelCy^R. A total of 23 clones were generated, some of which were isolated from drug-treated mice, and the remainder were isolated from unselected trypanosome populations. Bloodstream clones were isolated from infected tail blood suspended in approximately 100-200µl of guinea pig serum as described previously (**2.2.3**; McLintock 1990). A single metacyclic clone was isolated by allowing a metacyclic producing fly (F466/63) to feed directly from approximately 500µl guinea pig serum in a shallow well heated to 37^{0}C. This process resulted in the guinea pig serum containing some of the metacyclics from the fly's saliva. In order to increase the

density of the trypanosomes, the guinea pig serum containing metacyclics was centrifuged at 3000g for 2 minutes and most of the supernatant was removed to leave approximately 100µl volume of serum, in which the metacyclics were gently resuspended. Optical cloning of metacyclics was then carried out as for bloodstream trypanosomes as described previously (**2.2.3**).

5.2.6 Analysis of Isoenzymes

Isoenzyme analysis was conducted with the parental lines of trypanosomes and the cloned progeny from the co-transmission experiment. Four isoenzyme phenotypes were examined: three were analysed by starch gel electrophoresis (isocitrate dehydrogenase (ICD), alkaline phosphatase (AP), tyrosyl-tyrosyl-tyrosine peptidase (Tyr^3)) and one was analysed by isoelectric focusing (malic enzyme B (ME_B)). The buffers used to make the gels, the running conditions and the enzyme-specific stains used were as described previously (Tait *et al.* 1984). However, the procedure used to obtain trypanosome lysates was slightly different to that described previously. Trypanosomes were purified from infected blood by anion-exchange chromatography (Lanham and Godfrey 1970) and washed twice in PBSG (pH 7.4). Approximately 100µl (packed cell volume) of trypanosomes were stored in eppendorfs at -70^0C. Before use, these trypanosome pellets were allowed to thaw on ice, after which they were vortexed with an approximately equal volume of Trypanosome Lysis Buffer (TLB: 10mM Tris-HCl, 1mM EDTA, 2% (v/v) TRITON-X 100, 154µg/ml dithiothreitol, pH 7.5). A process of freeze-thawing was conducted twice to ensure the trypanosomes had lysed by freezing in liquid nitrogen, thawing on ice, and finally vortexing thoroughly, after which the lysates were centrifuged at 12000g for 10 minutes at 4^0C. The resulting supernatants were used to soak inserts of Whatman 3MM paper (ICD, AP and Tyr^3) or cellulose acetate (ME_B) for 10-15 minutes, after which the inserts were

applied to the gels in preparation for electrophoresis.

Analysis of the isoenzyme (ICD) phenotypes of unselected populations in procyclic culture (SDM-79 supplemented with 10% HIFCS) was also conducted in order to determine which trypanosome lines were present in the metacyclic populations. Bloodstream trypanosomes were obtained directly from mice infected by a fly and transformed to procyclic culture as described previously (**3.2.3**). Procyclic trypanosomes from established cultures were washed in PBSG, pH 7.4, and stored as pellets in eppendorfs at -70^{0}C. Isoenzyme electrophoresis was conducted on these uncloned populations as described above for the cloned progeny. This analysis would not distinguish between 247MelCy^R and 247Sur^R, nor would it identify the products of selfing between these two lines. However, it would be able to identify the presence of mixed infections of the 247-derived lines and 386MelCy^R, as well as the products of genetic exchange (hybrids) between either of the two 247-derived lines and 386MelCy^R.

5.2.7 Karyotype Analysis

Samples of DNA were prepared by lysing and deproteinising purified trypanosomes suspended in agarose blocks. Trypanosomes were purified by anion-exchange chromatography (Lanham and Godfrey 1970) after which they were washed and resuspended in PBSG, pH 7.4, to give a final trypanosome density of 1.5x10^9 trypanosomes/ml. A 1% (w/v) solution of Low Melting Point Agarose (LMA) (Difco) was prepared and cooled to 37^{0}C whilst the trypanosome suspension was warmed to 37^{0}C. Two volumes of LMA solution were mixed with 1 volume of trypanosome suspension to give a final trypanosome density of 5x10^8 trypanosomes/ml, which was pipetted into moulds and allowed to set for approximately 10 minutes at 4^{0}C to create

trypanosome-containing agarose blocks. The trypanosomes were lysed in situ by incubating the blocks in 5ml of 0.5M EDTA, pH 9.0, 1% (w/v) sodium lauryl sarcosine, 0.5mg/ml proteinase K for 20 minutes at room temperature followed by 48 hours at 50^{0}C. Digested protein was removed by washing the blocks three times in 50mM EDTA, pH 8.0. Blocks were stored in 50mM EDTA, pH 8.0, at 4^{0}C.

The DNA blocks were inserted into a 1% (w/v) agarose gel made with 1.1g electrophoresis grade agarose (BRL) dissolved in 110ml of TB(0.1)E (0.09M Tris-borate, 0.1mM EDTA, pH 8.0). Separation of chromosomes was conducted using an LKB Pulsaphor unit with a hexagonal electrode array with approximately 3 litres of TB(0.1)E as the running buffer. Two different running conditions were chosen, both using a voltage of 80V:

Condition	Phase 1		Phase 2		Phase 3		Phase 4		Phase 5		Phase 6	
	A	B	A	B	A	B	A	B	A	B	A	B
1	900	12	750	12	700	24	600	24	500	48	-	-
2	1200	24	1000	24	900	24	800	24	700	24	600	24

For each phase, column A shows the pulse time (seconds), while column B shows the duration of the phase (hours).

Gels were stained with ethidium bromide (0.5μg/ml in 0.5xTBE) for approximately 1 hour followed by destaining for approximately 1 hour in $dddH_2O$.

5.2.8 *In Vitro* Assessment of Drug-Resistance of Cloned Progeny

A number of the cloned progeny were analysed for the level of their

resistance to melCy and suramin. The method used was similar to that previously described (**3.2.4**). Briefly, trypanosomes were grown in immunosuppressed mice and transferred to a short-term bloodstream culture medium, in which they were exposed to a range of drug concentrations in order to assess the effective concentration (EC_{50} value) that inhibited growth over 24 hours by 50% (**3.3.2**).

5.3 RESULTS

5.3.1 Drug-Resistance Phenotypes of Metacyclic Populations

A total of 101 tsetse flies were infected in the three-way co-transmission experiment. Salivary gland infections of metacyclic trypanosomes were identified in 18 flies as defined by phase-contrast microscopy of dried saliva probes (**2.2.4**). Seven of these flies survived long enough to be fed on anaesthetised mice to produce bloodstream infections. Bloodstream infections were produced on a total of 30 separate occasions, and these were screened in the mouse assay (**5.2.5**) to determine the drug-resistance phenotypes of the metacyclic populations (Table 5.1). Unlike previous co-transmission experiments (**chapter 4**), the number of times each of the two drug-resistance phenotypes appeared was approximately equal, with melCy-resistance and suramin-resistance being observed in 24 and 21 samples of the metacyclic populations respectively. In 10 of the uncloned samples in which melCy-resistance was observed, the isoenzyme (ICD) phenotypes were analysed on starch gel electrophoresis (**5.2.7**). Table 5.1 shows the results of the isoenzyme analysis for some of the uncloned samples. On 10 occasions that melCy-resistance was observed, only the ICD phenotype of the 247-derived lines was observed, indicating that melCy-resistance observed in the mouse screen resulted from the presence of $247MelCy^R$ and not $386MelCy^R$. The previous co-transmission experiments of $247Sur^R$ and $386MelCy^R$ produced metacyclic

populations that were predominantly suramin-resistant resulting from the 247-derived line (**chapter 4**). However, it would appear from both the isoenzyme and drug screen data (Table 5.1) that the use of the two different drug-resistant lines of 247 (247MelCy^R and 247Sur^R) in the present co-transmission experiment produced mature infections expressing melCy-resistance at an approximately equal frequency to suramin-resistance.

Trypanosomes growing in the presence of both melCy and suramin were identified from the mouse screen in 10 samples of metacyclic populations (Table 5.1). However, on 7 of these occasions, a low level of infection (10^6 trypanosomes/ml or lower) was seen only one day after drug-treatment, and no patent infections were observed thereafter. Evidence of trypanosomes increasing in number after exposure to both drugs was seen on the other 3 occasions, 2 of which occurred with infections derived from the same fly (F466/51 and /59). Fly 28435 produced double drug-resistant trypanosomes on day 51 post infection, with trypanosomes appearing in a mouse 6 days after treatment with both melCy and suramin, following a period of 5 days of sub-patent parasitaemia. Similarly with F466/51, a period of sub-patency preceded the emergence of a patent infection in a double drug treated mouse. In the case of F466/59, one day after double drug treatment parasites were present at a density of $5x10^5$ trypanosomes/ml, after which a period of sub-patency lasting 8 days elapsed, followed by a period of rapidly rising parasitaemia up to $3.2x10^7$ trypanosomes/ml.

5.3.2 Generation of Unselected and Selected Cloned Progeny

Fly 28444

Figure 5.2 illustrates the generation of a total of 6 clones derived from fly 28444. Two of these clones (444/62 bcl1 and bcl2) were isolated from a

mouse infected by the fly on day 62 post infection, and were not exposed to drug prior to cloning (i.e. isolated from unselected populations). Clones 444/62S^R bcl1 and bcl2 were isolated from a suramin-resistant population, whereas clones 444/54M^RS^R bcl1 and bcl2 were isolated from a mouse that was initially treated with suramin, and the relapse infection was subsequently treated with melCy.

Fly 28466

A total of 17 clones were derived from fly 28466, one of which was a metacyclic clone, and the remainder were bloodstream clones (Figure 5.3). Ten of these clones were isolated from unselected populations in mice (466/59 bcl1-8, and 466/61 bcl1 and bcl2), and 6 were isolated from mice treated with both melCy and suramin. Bloodstream clones 466/51M^RS^R bcl1 and bcl2 were taken from a trypanosome population that had been selected twice with both drugs at doses of 5mg/Kg. The other 4 clones (466/61M^RS^R bcl1-4) were isolated from drug selected populations that had been exposed to melCy at a dose of 4.8mg/Kg and suramin at a dose of 24mg/Kg. These drug treatments represented doses that were 16-fold higher than the minimum curative doses of melCy and suramin in the unselected drug-sensitive line, 247.

5.3.3 Inheritance of Isoenzyme Phenotype in Cloned Progeny

The inheritance of 4 isoenzymes was analysed in the cloned progeny. Examples of the analyses conducted for each isoenzyme are shown in Figures 5.4, 5.5, 5.6 and 5.7 for ICD, AP, ME_B and Tyr^3 respectively. In each figure, a schematic diagram of the stained gel has been included, showing the isoenzyme bands and their interpretation in terms of the allelic variants present in the cloned progeny.

The isoenzyme data for all 4 phenotypes are summarised in Table 5.2, The data are expressed using the established method of identifying genotypic variants as numbers to represent the variant alleles present. Of the 23 clones isolated, 20 were analysed, 17 of which were identified, from the ICD and AP phenotypes, as possessing the phenotypic characteristics of 247 (genotypic variant 1). The other clone (466/59 bcl5) possessed the 386MelCy^R ICD phenotype (genotypic variant 2) and was presumed to be the parental 386MelCy^R line. Hybrid trypanosomes would have been identified at the ICD and AP loci, since both isoenzymes are homozygous but different in the parents, and hybrids would have been heterozygotes, and would have expressed multiple banding patterns (genotypic variant 1-2) (see Figure 5.1(a)). No hybrids were detected. Of the 17 clones identified with a 247 parental phenotype from their ICD and AP isoenzymes, 11 were analysed at the Tyr^3 locus. Products of selfing between 247MelCy^R and 247Sur^R would have a 50% probability of being homozygous (genotypic variants either 2 or 4), assuming random recombination of alleles, at a locus which was heterozygous in the parent, such as Tyr^3. Unfortunately, analysis of the Tyr^3 phenotype in all 11 clones showed that they all possessed the parental, heterozygous phenotype (genotypic variant 2-4). Therefore, there was no evidence for selfing having taken place based on isoenzyme analysis.

5.3.4 Inheritance of Karyotype in Cloned Progeny

In order to interpret the pulsed field gels, it is necessary to deal briefly with some of the details of the genome of the two cloned lines, 247 and 386, from which the drug-resistant lines were generated. A number of the large chromosomes have been identified, ranging in size from approximately 1-3Mb (Tait *et al.* 1993; Tait, Buchanan, Hide and Turner, unpublished data). Probing Southern blots with gene-specific probes has shown that these chromosomes

belong to 5 homologous pairs. Figure 5.8 is a composite of a number of separate experiments for these markers, showing the different bands that have been resolved and identified as belonging to the homologous chromosomes (A-E).

It is clear from Figure 5.8 that the chromosomes within a homologous pair are not always the same size as each other. For example, whereas chromosome A, in the 386-derived lines, consists of two similar sized homologues that cannot be resolved by PFGE (band 1), the comparable chromosome A in the 247-derived lines consists of 2 different sized homologues that are resolved as two bands (bands 1 and 2) (Figure 5.8). Assuming a system of mating involving meiosis and recombination, products of genetic exchange between one of the 247-derived lines and 386MelCy^R could be identified from the karyotype of cloned progeny that would possess a chromosome A consisting of one homologue the same size as band 1 of 386MelCy^R and the other homologue the same size as one or the other of bands 1 or 2 of the 247-derived line. Similarly, self-fertilisation between 247MelCy^R and 247Sur^R would produce some cloned progeny with an identical karyotype to the parent, and some cloned progeny that have inherited two homologues of the same size. This would result in the loss of one band, and the doubling of intensity (with ethidium-staining) of the other band, thereby identifying the products of selfing (Tait *et al.* 1993).

The two running conditions that were employed in PFGE experiments were able to separate the chromosome bands to different extents. Condition 1 was only able to separate chromosomes smaller than approximately 2Mb in size, with the remainder of the large chromosomes remaining unresolved either in the compression zone or in the application slot. This can be seen clearly in Figure 5.9. The schematic diagram (Figure 5.9(a)) focuses on 2 lanes (lanes 5 and 7),

which correspond to 386 and 247 respectively. It can be seen that only the lower size bands were resolved (bands 1-4 of 247, and bands 1-3 of 386). However, condition 2 was able to resolve the higher molecular weight bands, although this was at the expense of a decrease in the resolution of the smaller bands. Figure 5.10 (a) shows the separation of all seven bands of 247 (lane 13), although bands 6 and 7 were not easily distinguishable.

The karyotypes of 247 and the two drug-resistant lines, 247MelCy^R and 247Sur^R, were identical with respect to the large chromosomes (Figures 5.9(b) and 5.10(b)). However, whereas 386 and 386Sur^R possessed identical karyotypes, 386MelCy^R had a noticeably smaller chromosome at band no. 2 compared to 386. This reduction in size of chromosome band 2 could be crudely estimated to be 200Kb, from approximately 1.5Mb in 386 to approximately 1.3Mb in 386MelCy^R (Figure 5.9(c)).

Figure 5.10(c) shows examples of the karyotype analyses conducted on the cloned progeny using the running condition, 2. Taking into account the differences in sample concentration (with lower loadings causing faster migration), all these progeny clones possessed the 247 parental karyotype. Figure 5.11 shows a different set of clones analysed using condition 1. Although the majority of cloned progeny were shown to possess the 247 parental karyotype (Figure 5.11(a)), one particular clone (466/59 bcl5) possessed the distinct karyotype of 386MelCy^R (lane 7, Figure 5.11(b)). Comparison between this sample and that of 386Sur^R (lane 5) clearly shows the smaller chromosome band 2 of 466/59 bcl5, indicating that this clone possessed the same karyotype as 386MelCy^R.

The results of the karyotype analysis are summarised in Table 5.3. Eighteen progeny clones were found to possess the 247 parental karyotype,

with only 466/59 bcl5 possessing the 386melCy^R karyotype. Since condition 1 only separated the first 4 large chromosome bands in 247, this meant that the inheritance of only 2 homologous pairs (A and B) could be investigated. The use of condition 2 allowed the inheritance of a further homologous pair, E (bands 6 and 7), to be examined. With either condition, the 2 homologous pairs, C and D, could not be resolved (bands 5a and 5b). Consequently, there was a limited number of markers available, and unfortunately the karyotype analysis did not identify the products of selfing, even with the clones isolated from a double drug-resistant population.

5.3.5 Inheritance of Drug-Resistance in Cloned Progeny

Ten of the cloned progeny were analysed for their inheritance of the melCy-resistance and suramin-resistance phenotypes (Table 5.4). The results are expressed as EC_{50} values, and converted to the degrees of increase in drug-resistance compared to the drug-sensitive parents from which they were derived. Nine of the 10 progeny clones analysed possessed a 247 parental karyotype and isoenzyme phenotype. These clones all expressed melCy-resistance, with levels of resistance being between 27-fold and 190-fold greater than the unselected 247 line, compared to an 87-fold increase in melCy-resistance in the 247MelCy^R line. Most of the levels of melCy-resistance in the cloned progeny were approximately half the levels in the 247MelCy^R line. The one exception to this was seen in clone 466/59 bcl8, in which the EC_{50} value of 6.0μM was over twice that of the 247MelCy^R line (EC_{50} value of 2.7μM).

Suramin-resistance was not expressed in the clones derived from unselected populations, with EC_{50} values ranging between 0.38μM and 1.4μM, compared to 0.97μM in the unselected 247 line. However, there was a noticeable amount of suramin-resistance in the 4 clones isolated from a double

drug-resistant population (466/61S^RM^R bcl1-4). Levels of suramin-resistance were found to lie between 1.6-fold and 6.9-fold greater than the level of suramin-resistance in the unselected 247 line. However, this was in comparison to a 20-fold increase in suramin-resistance found in 247Sur^R and consequently, these 4 double drug-resistant clones appeared to express only partial resistance to suramin.

5.4 DISCUSSION

Examination of the drug-resistance phenotypes present in the mature infections in the flies' salivary glands showed that melCy-resistance and suramin-resistance were identified at approximately equal frequencies. Furthermore, these uncloned populations were examined for their ICD phenotypes, which can distinguish between infections consisting of only 247, only 386, mixed and hybrid infections. All the uncloned populations from the flies' salivary glands expressed the 247 parental ICD phenotype. This, therefore, indicates that the occurrence of melCy-resistance in the mature populations was largely a result of 247$MelCy^R$ and not 386$MelCy^R$. Previous co-transmission experiments with 247Sur^R and 386$MelCy^R$ produced mature infections that contained a majority of suramin-resistant trypanosomes, presumably because the 247Sur^R line transmitted more successfully than 386$MelCy^R$. The use of two different 247-derived drug-resistant lines in the present study, produced more balanced mature infections in terms of the drug-resistance phenotypes present, possibly since it would be predicted that both the 247-derived lines would be easier to transmit than the 386-derived lines.

The isoenzyme phenotypes and karyotypes of a total of 21 progeny clones were analysed, with all but one (466/59 bcl5) being identified as identical to the 247-parental clones. Assuming there was no selection pressure for the

products of selfing between 247MelCy^R and 247Sur^R, it should be possible to identify selfers at a number of loci. The Tyr^3 phenotype was heterozygous in the 247-derived lines, and consequently there was a 50% probability of the product of selfing being homozygous at this locus (Figure 5.1(b)). Eleven progeny clones were analysed at the Tyr^3 locus, none of which expressed a homozygous phenotype, although it was possible that these clones were not all different to each other, since they were generated on only 5 different occasions, and may therefore only represent 5 different clones. Furthermore, only 2 sets of clones were generated from double drug-resistant populations. Therefore, assuming these selected populations consisted of selfers, it was possible that only 2 different "selfer" clones were being analysed, each with a 50% chance of being homozygous at the Tyr^3 locus. Analysis of the karyotype of the cloned progeny was equally unsuccessful in identifying the products of selfing. Three sets of homologous pairs have been resolved (chromosomes A, B and E) in the 247 derived lines (Tait, Buchanan, Hide and Turner, unpublished data, Figure 5.8). The differences in size of the homologues means that novel karyotypes might result from selfing between 247MelCy^R and 247Sur^R (Tait *et al.* 1993; **5.3.4)** Double drug-resistant clones were isolated from three different drug selected populations, and on the pessimistic assumption that the clones generated on each of these occasions were the same, it therefore follows that only 3 different selfer clones expressing resistance to both melCy and suramin were analysed. However, separation of homologues would be expected to be identified in 50% of cases for each of the chromosome pairs. Two homologous pairs (chromosomes A and B) were analysed in the clones generated from 466/59M^{R}S^R, three homologous pairs (chromosomes A, B and C) were analysed in the clones generated from 466/61M^{R}S^R, and the same three homologous pairs were also analysed in the clones generated from 444/54M^{R}S^R. Consequently, 3 pairs of homologous chromosomes were analysed, together with 2 alleles at the Tyr^3 locus. This is essentially an analysis

of 4 loci for which 247 is heterozygous, and segregation of alleles was not observed at any of these loci..

Table 5.5 summarises all the isoenzyme, karyotype and drug screening analysis for the cloned progeny. Evidence of selfing having occurred was seen only from the drug-resistance phenotypes of the cloned progeny assessed *in vitro*, and the uncloned populations assessed *in vivo*. Four clones, 466/61M^RS^R bcl1-4, were isolated from a population of bloodstream trypanosomes that had been treated with melCy and suramin at doses that were 16-fold higher than the minimum curative doses for the unselected 247 line. The EC_{50} values for these clones indicated that they expressed resistance to both melCy and suramin. MelCy-resistance varied between approximately 50% and 90% of that found in the parental 247$MelCy^R$ line. Suramin-resistance, however, was expressed at lower levels, with EC_{50} values approximately 10-30% of those for the suramin-resistant parental line, 247Sur^R. Of the 6 other progeny clones that were assessed, all 5 clones that were identified, from isoenzyme and karyotype analysis, as being 247-derived expressed melCy-resistance, and appeared not to express suramin-resistance. This may indicate that they were in fact parental clones of 247$MelCy^R$, although the possibility that they were selfers can not be ruled out. One clone in particular, 466/59 bcl8, expressed a high level of melCy-resistance, with an EC_{50} value of 6.0μM, over double that of the 247$MelCy^R$ line. Although the genetic nature of melCy-resistance is not known, it may be predicted that some of the products of selfing between two 247$MelCy^R$ trypanosomes may express this high level of resistance to melCy. For example, if melCy-resistance is determined by a single dominant mutation in the parental 247$MelCy^R$ line, then one of the products of selfing would be a double melCy-resistant phenotype.

The appearance of a large deletion in one chromosome (band 2) of the

386MelCy^R line is particularly interesting. Chromosome size alterations have been observed previously and were considered as products of mitotic division (Gibson and Garside 1991). However, a recent study has demonstrated the stability of the 386 karyotype after multiple passages in mice, multiple passages in culture and cyclical transmission through tsetse flies (Tait *et al.* 1994). Although the possibility that the reduction in size may be due to mitosis cannot be ruled out, it is tempting to consider the deletion as a direct consequence of melCy-resistance. If this were the case, a genetic cross between 386MelCy^R and a melCy-sensitive line would generate progeny expressing melCy-resistance only if they also inherited the altered chromosome, thereby providing a powerful handle for direct studies into the genetic basis of melCy-resistance.

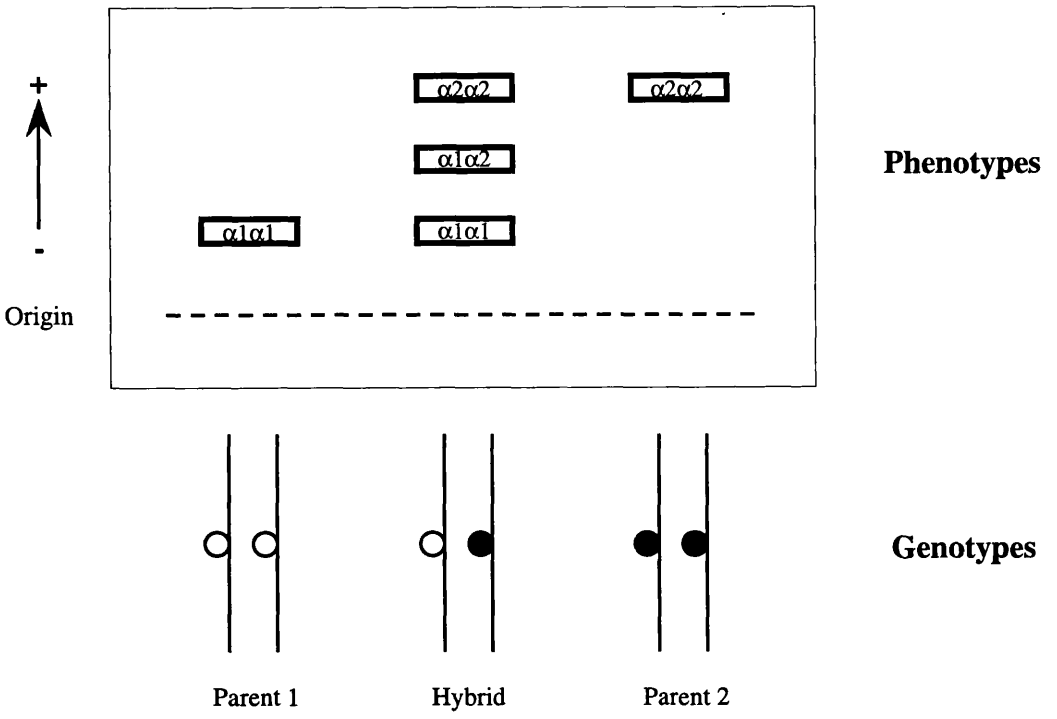
The relatively low level of expression of suramin-resistance in the cloned progeny could be explained in a number of ways. The possibility that suramin-resistance was generated during the initial *in vivo* screening in mice cannot be ruled out. Suramin treatment of 5mg/Kg represents only a 3-fold greater dose than the minimum curative dose for 247MelCy^R (**chapter 2**). Indeed, the initial stages of selection of the 247Sur^R line were conducted at a dose of 1mg/Kg followed by a dose of 5mg/Kg (**Figure 2.2(b)**). It is feasible, therefore, that the presence of double drug-resistant trypanosomes in the mouse screen could have resulted from a selection pressure of suramin at 5mg/Kg on parental infections of 247MelCy^R only. However, the four clones (466/61M^{R}S^R bcl1-4) were isolated from a mouse infected with the unselected trypanosome population, 466/61, and treated with melCy and suramin at drug doses 16-fold greater than the minimum curative drug doses, amounting to suramin at a dose of 24mg/Kg. In light of the selection history of 247Sur^R (**Figure 2.2(b)**) it would be extremely unlikely that a suramin-resistant mutant of 247MelCy^R would have increased from being sensitive to a minimum curative dose of 1.5mg/Kg up to greater than 24mg/Kg during what is essentially a single selection step.

Therefore, although the use of suramin at a dose of 5mg/Kg in the drug screen may be questionable, the four clones (466/61M^RS^R bcl1-4) are likely to be the genuine products of genetic exchange between 247MelCy^R and 247Sur^R. The low levels of suramin-resistance found in the *in vitro* drug screen, compared to the consistently higher relative levels of melCy-resistance, may indicate the existence of multiple alleles controlling suramin-resistance. The original selection procedure was certainly more arduous for suramin-resistance compared to melCy-resistance, and this may be an indication of a more incremental increase in suramin-resistance involving multiple mutations. Cloned progeny resulting from genetic exchange would therefore not necessarily be expected to inherit all the alleles conferring suramin-resistance, and this would result in a range of levels of suramin-resistance, lower than the parental 247Sur^R line, which possesses all the mutant alleles.

In conclusion, the evidence from the *in vitro* assays and the *in vivo* screens suggests that double drug-resistant trypanosomes were generated, presumably from mating between melCy-resistant and suramin-resistant lines. Furthermore, the ICD phenotypes of the uncloned populations, together with the isoenzyme phenotypes and karyotypes of the cloned progeny indicate that the double drug-resistant trypanosomes were produced from mating between 247MelCy^R and 247Sur^R, rather than 386MelCy^R and 247Sur^R. Unfortunately, the other markers of genetic exchange (isoenzymes and karyotype), did not identify any products of selfing, although the relatively limited number of markers available does not prove that selfing did not occur.

Figure 5.1
Identification of Products of Genetic Exchange by Isoenzyme Analysis

(a) Hybrids



(b) Selfers

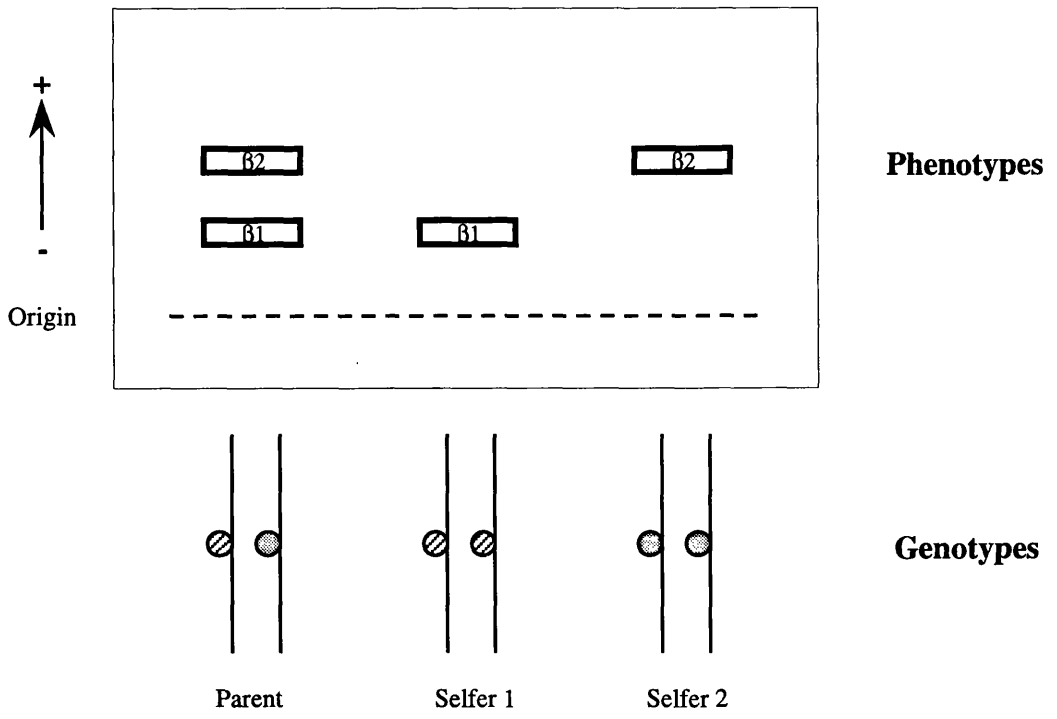


Figure 5.1
Identification of Products of Genetic Exchange by Isoenzyme Analysis

(a) Hybrids

Hybrid trypanosomes can be identified by the isoenzyme phenotype in which the parents are homozygous but different. Hybrids from genetic exchange between these parents will be heterozygous, which, for a dimeric enzyme such as isocitrate dehydrogenase as illustrated here, produces a triple-banded pattern.

(b) Selfers

The process of selfing in a parental line in which a given marker is heterozygous, would be expected to produce some progeny that are homozygous (as shown) and others that remain heterozygous (not shown).

Facing 144

Figure 5.2
Generation of Cloned Progeny from Fly 28444

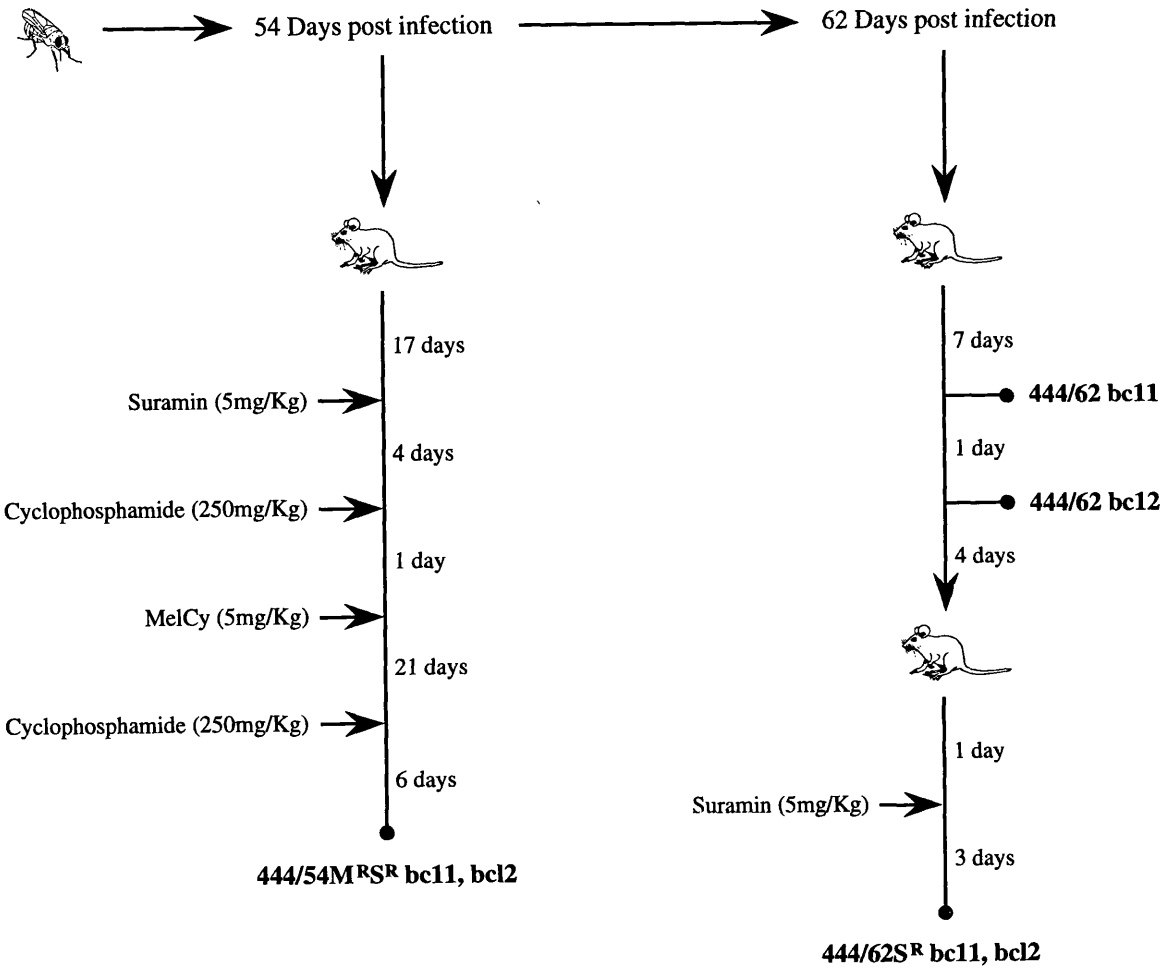


Figure 5.2
Generation of Cloned Progeny from Fly 28444

Fly 28444 was fed on mice on days 54 and 62 post infection. Clones were generated either before or after drug treatment as shown. Clones are shown in bold.

Figure 5.3
Generation of Cloned Progeny from Fly 28466

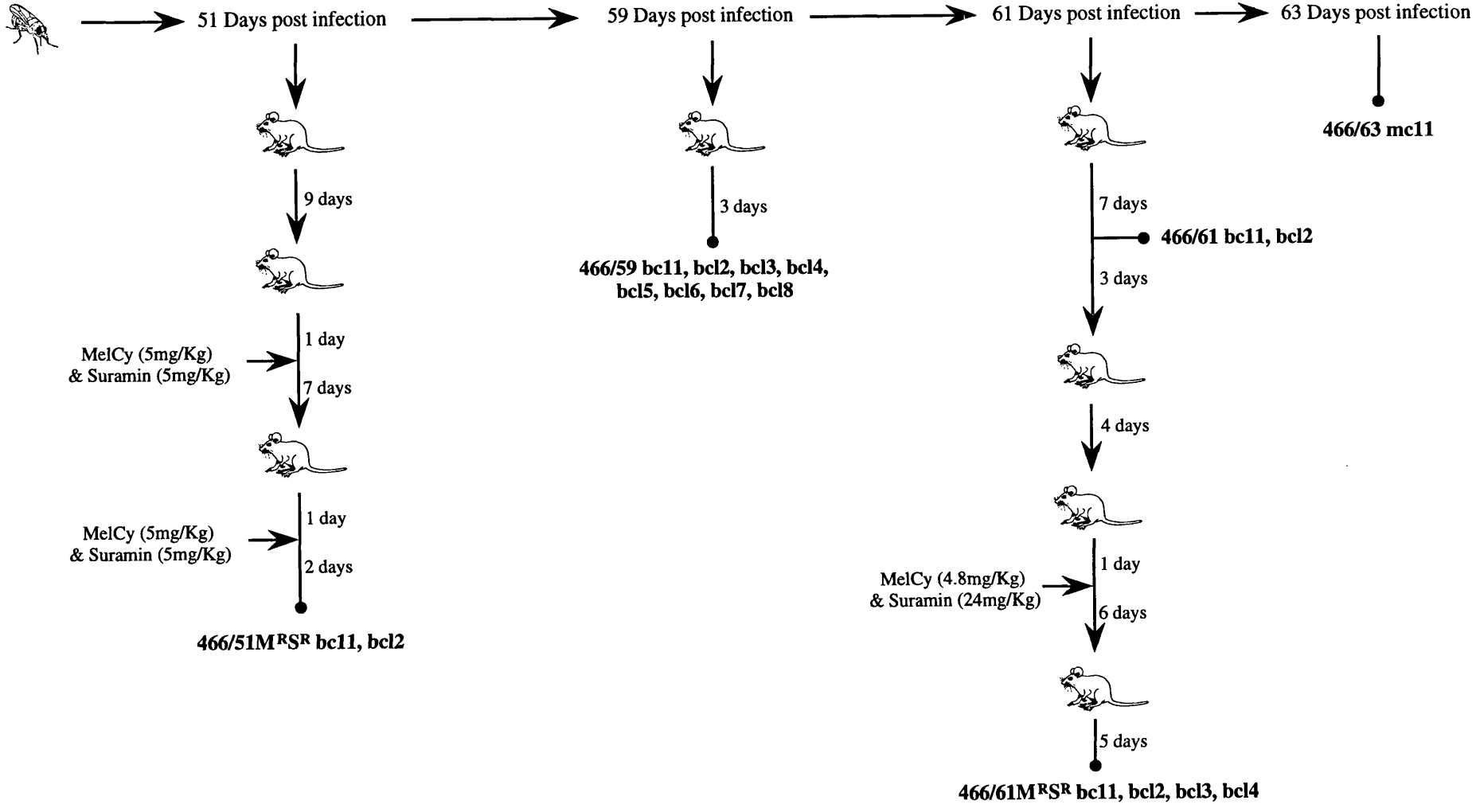
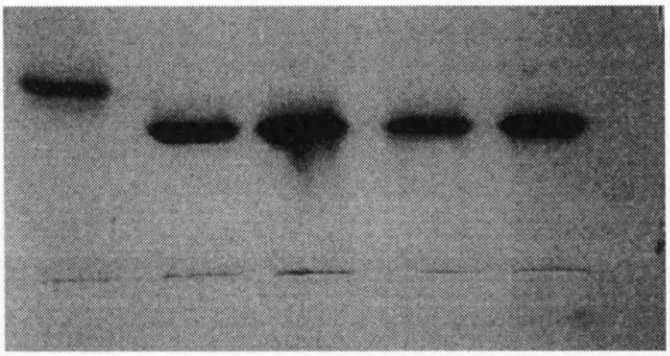
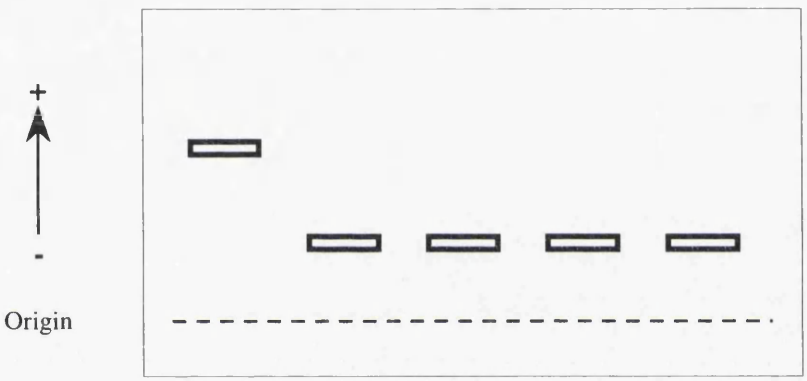


Figure 5.3
Generation of Cloned Progeny from Fly 28466

Fly 28466 was fed on mice on days 51, 59 and 61 post infection. Clones were generated either before or after drug treatment as shown. On day 63 post infection, a single metacyclic clone was also isolated. Clones are shown in bold.

Figure 5.4
Analysis of Isocitrate Dehydrogenase Phenotype in Cloned Progeny

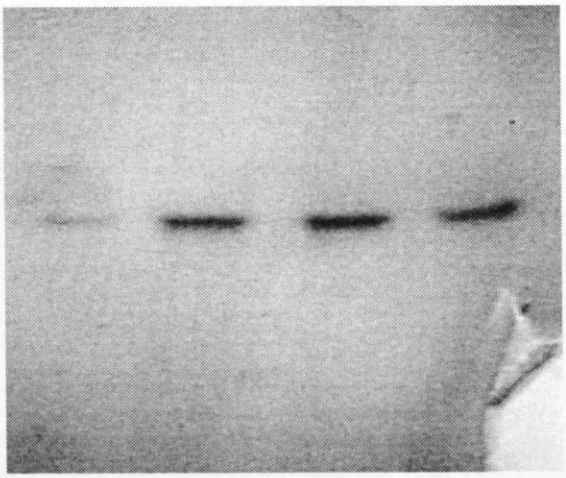


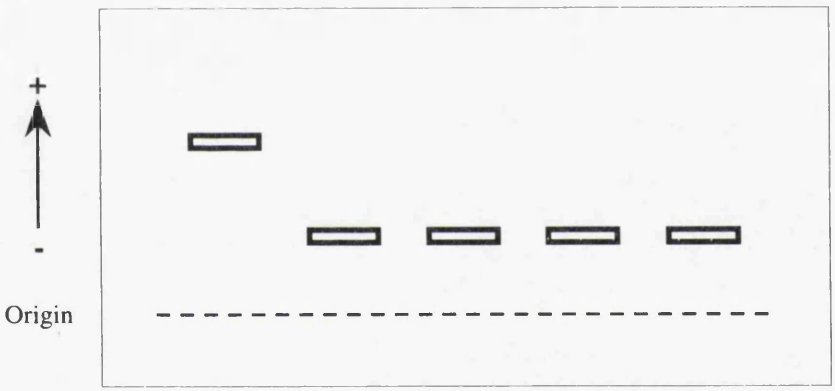


Genotypic Variants	2	1	1	1	1
Clones	386	247	466/59 bcl2	466/59 bcl4	466/59 bcl7

This figure shows an example of the results of analysing the ICD phenotype in the two unselected lines (386 (lane 1) and 247 (lane 2)) and three progeny clones (466/59 bcl2 (lane 3), 466/59 bcl4 (lane 4) and 466/59 bcl7 (lane 5)). A schematic interpretation of the gel is shown beneath the photograph, indicating the genotypic variants in each cloned line.

Figure 5.5
Analysis of Alkaline Phosphatase Phenotype in Cloned Progeny

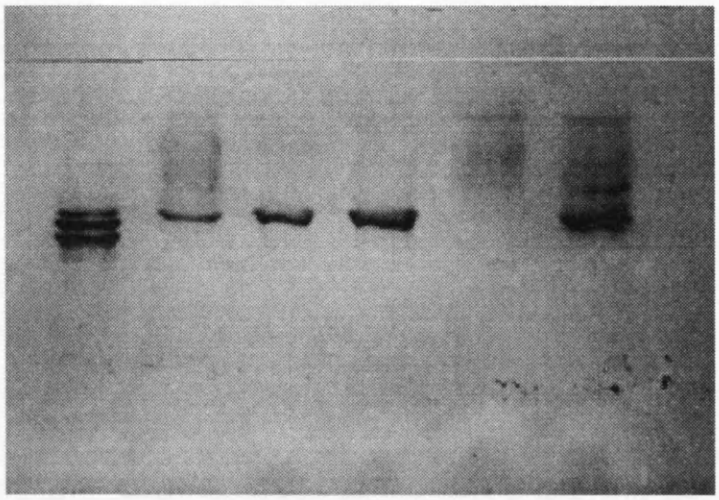


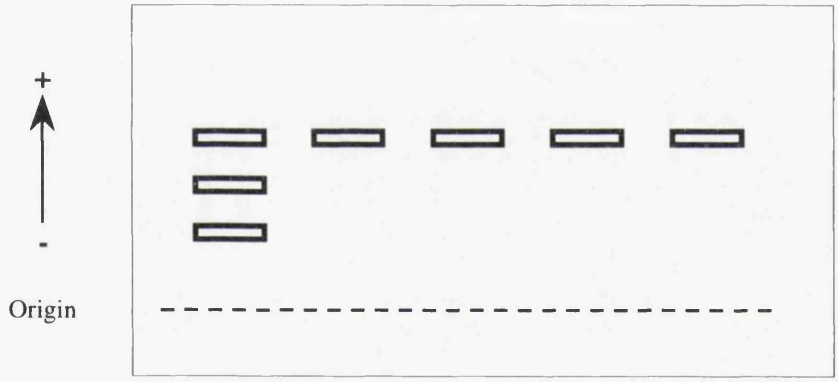


Genotypic Variants	2	1	1	1	1
Clones	386 (not shown)	247	466/59 bcl2	466/59 bcl4	466/59 bcl7

This figure shows an example of the results of analysing the AP phenotype in the two unselected lines (386 (lane 1) and 247 (lane 2)) and three progeny clones (466/59 bcl2 (lane 3), 466/59 bcl4 (lane 4) and 466/59 bcl7 (lane 5)). A schematic interpretation of the gel is shown beneath the photograph, indicating the genotypic variants in each cloned line. The photograph does not show the 386 sample, although it is included in the schematic diagram to show the difference between the parental lines.

Figure 5.6
Analysis of Malic Enzyme B Phenotype in Cloned Progeny

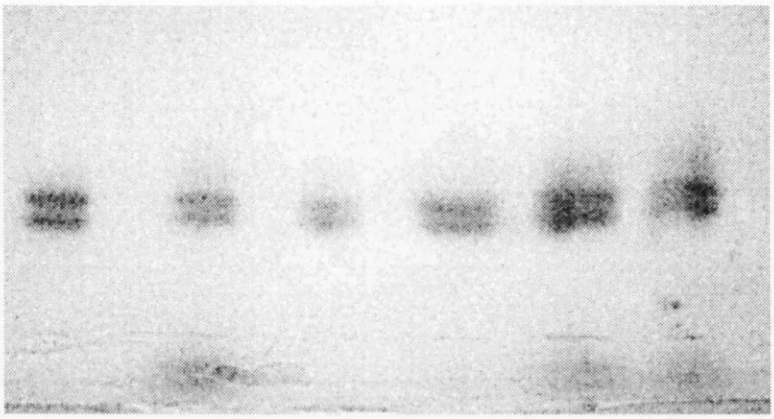


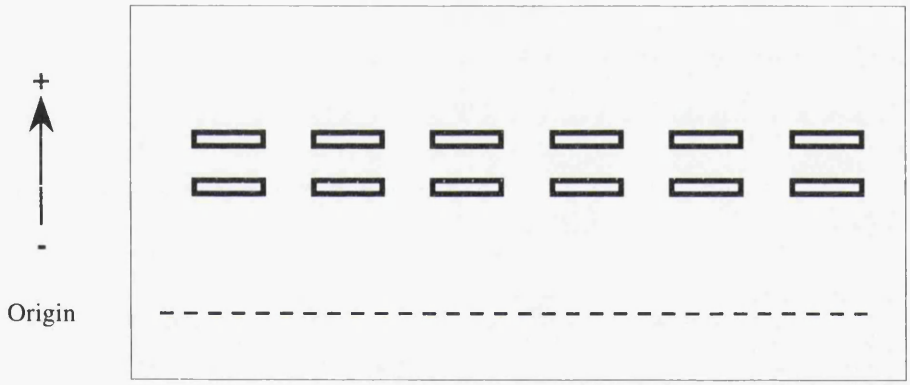


Genotypic Variants	2-3	3	3	3	3
Clones	386	247	466/51 M^RS^R bcl1	466/51 M^RS^R bcl2	444/54 M^RS^R bcl1

This figure shows an example of the results of analysing the ME_B phenotype in the two unselected lines (386 (lane 1) and 247 (lane 2)) and three progeny clones (466/51M^RS^R bcl1 (lane 3), 466/51M^RS^R bcl2(lane 4) and 444/54M^RS^R bcl1 (lane 5)). A schematic interpretation of the gel is shown beneath the photograph, indicating the genotypic variants in each cloned line.

Figure 5.7
Analysis of Tyrosyl-Tyrosyl-Tyrosine Peptidase Phenotype in Cloned Progeny

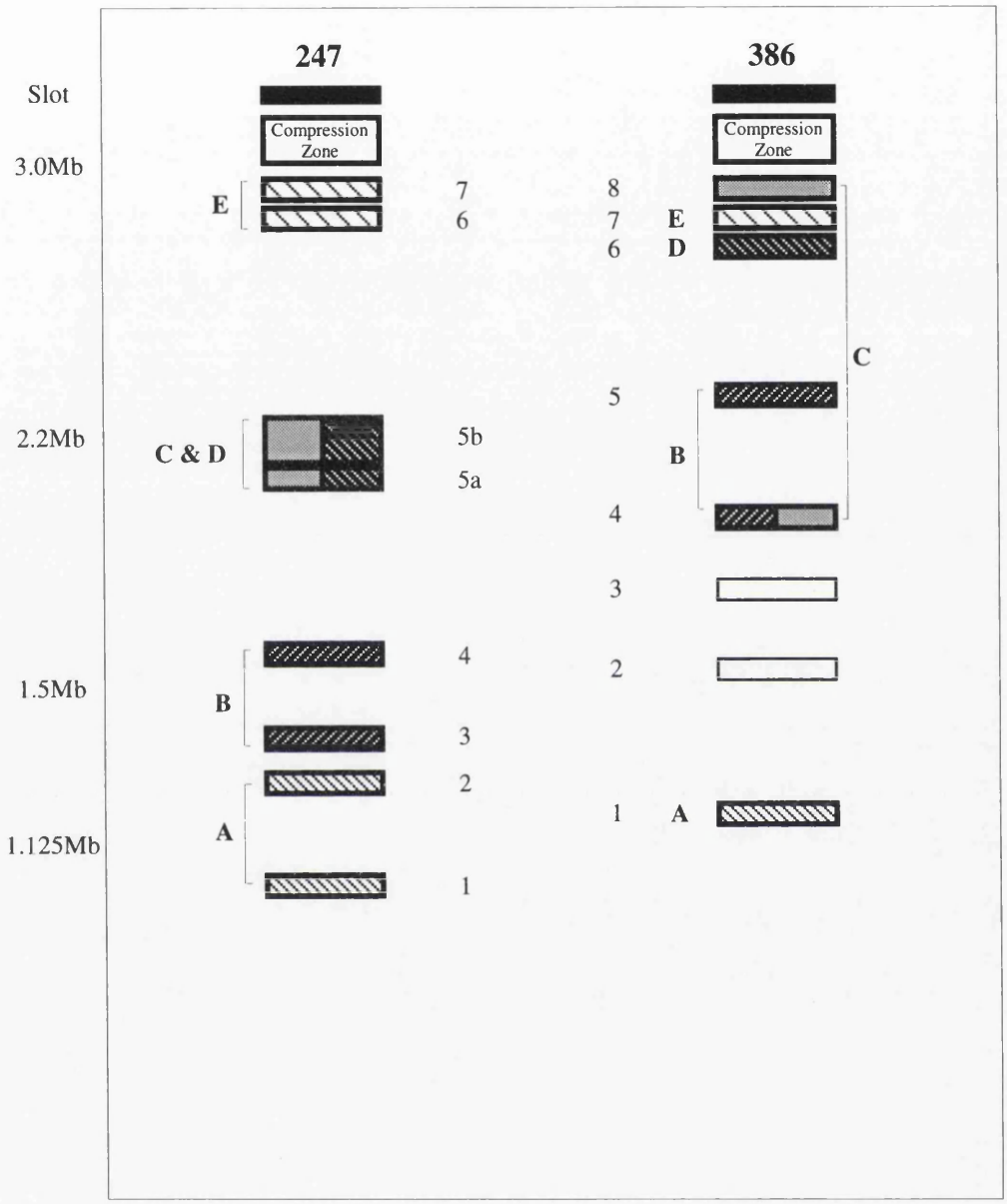




Genotypic Variants	2-4	2-4	2-4	2-4	2-4	2-4
Clones	386	247	466/51 M^RS^R bcl1	466/51 M^RS^R bcl2	444/62 bcl1	444/54 M^RS^R bcl1

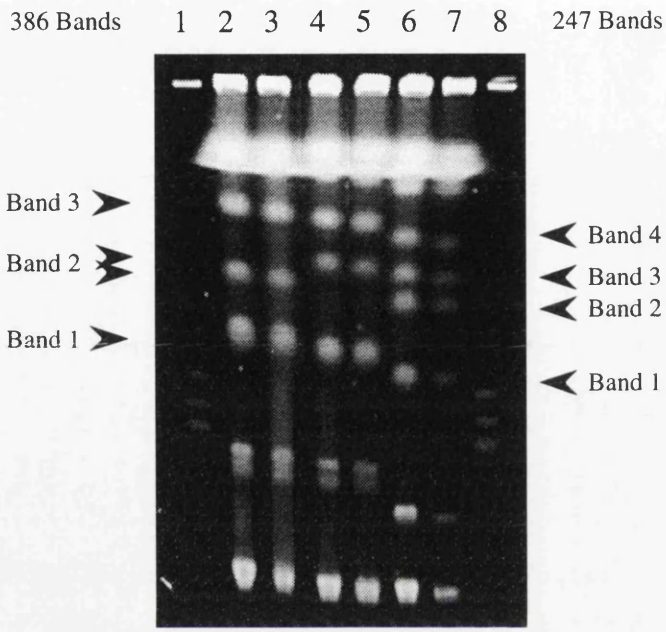
This figure shows an example of the results of analysing the Tyr^3 phenotype in the two unselected lines (386 (lane 1) and 247 (lane 2)) and four progeny clones (466/51M^RS^R bcl1 (lane 3), 466/51M^RS^R bcl2(lane 4), 444/62 bcl1 (lane 5) and 444/54M^RS^R bcl1 (lane 6)). A schematic interpretation of the gel is shown beneath the photograph, indicating the genotypic variants in each cloned line.

Figure 5.8
Separation of Large Chromosomes of 247 and 386 by PFGE



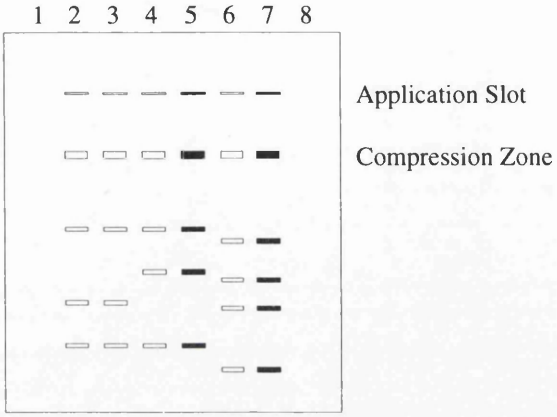
A schematic diagram of the separation and identification of some of the large chromosomes of the two unselected lines, 247 and 386, from which all the drug-resistant lines were generated. It is a composite illustration taken from results of ethidium stained gels and gene-specific probing of Southern blots (Tait, Buchanan, Hide and Turner, unpublished data). The letters indicate bands in which homologues have been identified. The numbers of the resolved bands that are referred to in the text are consistent with the scheme shown above. The different patterns of shading indicate which chromosomes are hybridised with specific single copy or closely linked multiple copy DNA probes.

Figure 5.9
Karyotype Analysis of Parental and Progeny Clones



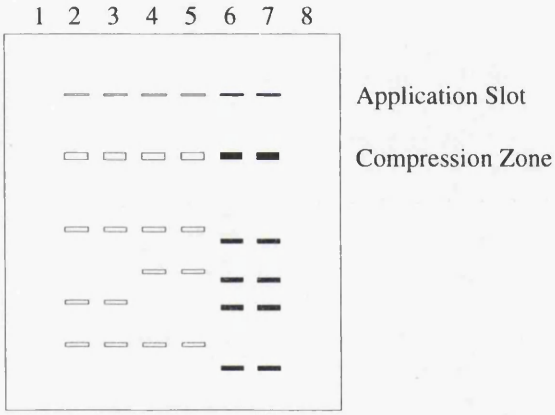
Karyotype analysis of 466/59 bcl5 (lane 2), 386MelCy^R (lane 3), 386Sur^R (lane 4), 386 (lane 5), 247Sur^R (lane 6) and 247 (lane 7) using running condition 1 (see text) to separate chromosomes of approximately 1-2Mb in size. Three diagrams of this ethidium stained gel are given for ease of comparison of different tracks. The band numbers refer to the resolved bands with reference to the two unselected parental lines, 247 and 386 (see Figure 5.8).

Figure 5.9
(a) Karyotype Analysis of 247 and 386



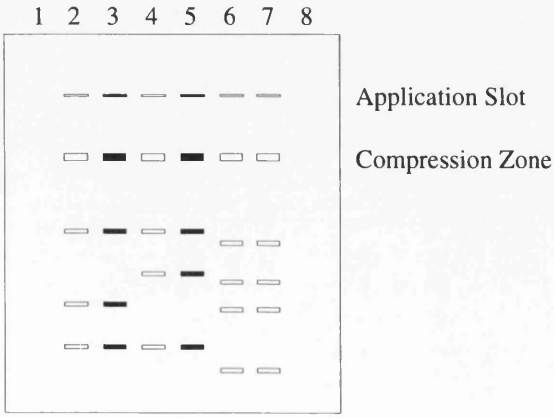
The separation of the large chromosomes below 2Mb in size are shown in bold for 247 (lane 7) and 386 (lane 5). Only the first 4 chromosome bands of 247 and the first 3 bands of 386 can be resolved using running condition 1. Chromosomes larger than 2Mb remain unresolved in the compression zone.

(b) Karyotype Analysis of 247 and 247Sur^R



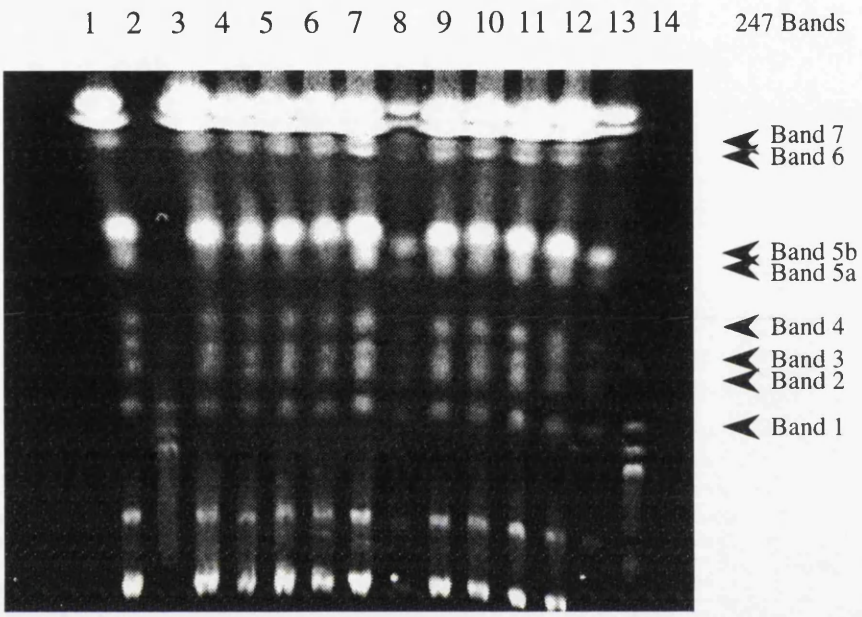
The karyotypes of 247 (lane 7) and 247Sur^R (lane 6) for the first 4 large chromosomes are shown in bold. The suramin-resistant line can be seen to have an identical karyotype to the unselected line, 247, from which it was generated.

(c) Karyotype Analysis of 386 and 386MelCy^R



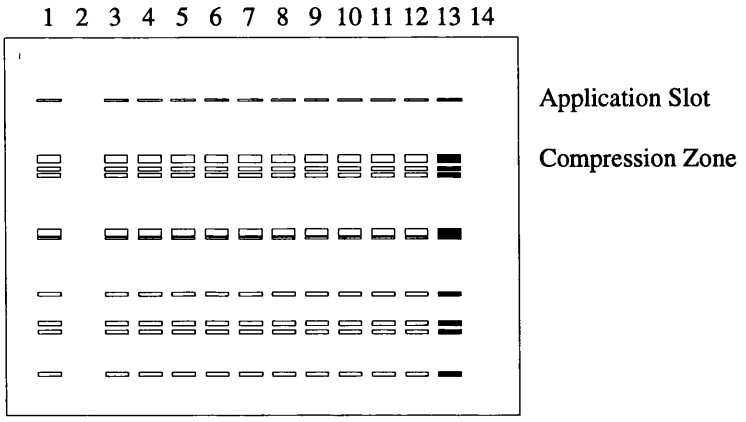
The karyotypes of 386 (lane 5) and 386MelCy^R (lane 3) for the first 3 large chromosomes are shown in bold. It is clear that chromosome 2 in the melCy-resistant line was smaller than that in the unselected line, 386.

Figure 5.10
Karyotype Analysis of Parental and Progeny Clones



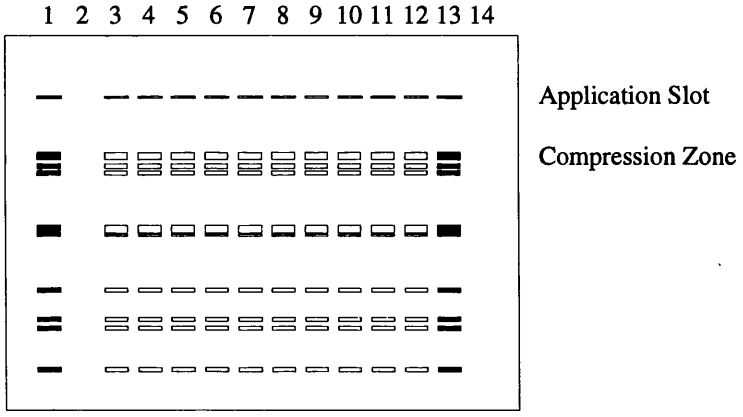
Karyotype analysis of 247MelCyR (lane 1), 444/62 bcl2 (lane 3), 444/ 62 bcl1 (lane4), 444/54M^RS^R bcl2 (lane 5), 444/54M^RS^R bcl1 (lane 6), 466/63 mcl1 (lane 7), 466/61 bcl2 (lane 8), 466/59 bcl7 (lane 9), 466/59 bcl4 (lane 10), 466/59 bcl3 (lane 11), 466/59 bcl2 (lane 12) and 247 (lane 13) using running condition 2 (see text) to separate chromosomes of approximately 1-3Mb in size. Three diagrams of this ethidium stained gel are given for ease of comparison of different tracks. The band numbers refer to the resolved bands with reference to the unselected parental line, 247 (see Figure 5.8).

Figure 5.10
(a) Karyotype Analysis of 247



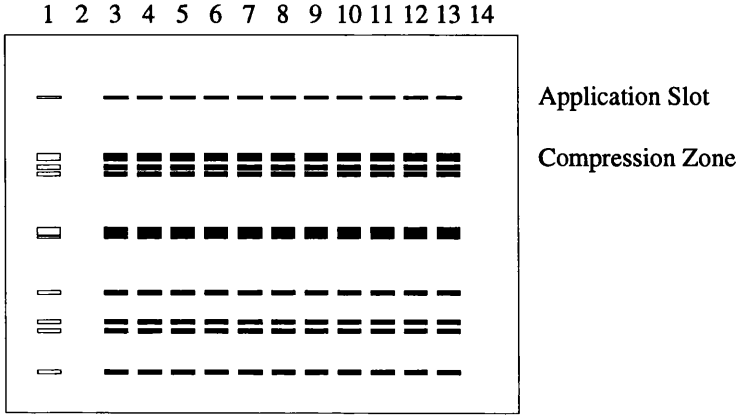
The separation of the large chromosomes of 247 (lane 13) up to 3Mb in size are shown in bold. A total of 8 chromosome bands can be resolved using running condition 2, although this is at the expense of compressing the lower molecular weight bands 1-4.

(b) Karyotype Analysis of 247 and 247MelCy^R



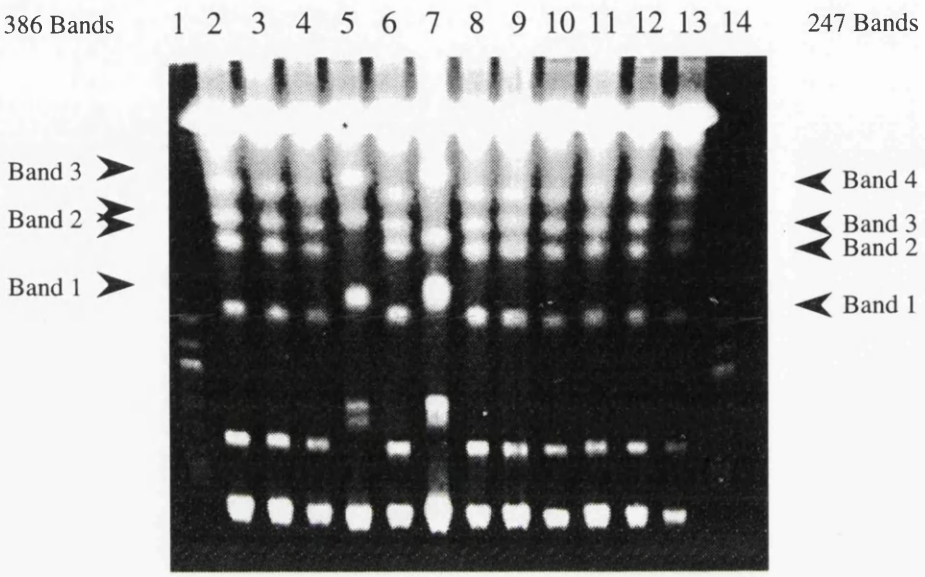
The karyotypes of 247 (lane 13) and 247MelCy^R (lane 1) are shown in bold. The melCy-resistant line can be seen to have an identical karyotype to the unselected line, 247, from which it was generated.

(c) Karyotype Analysis of Cloned Progeny



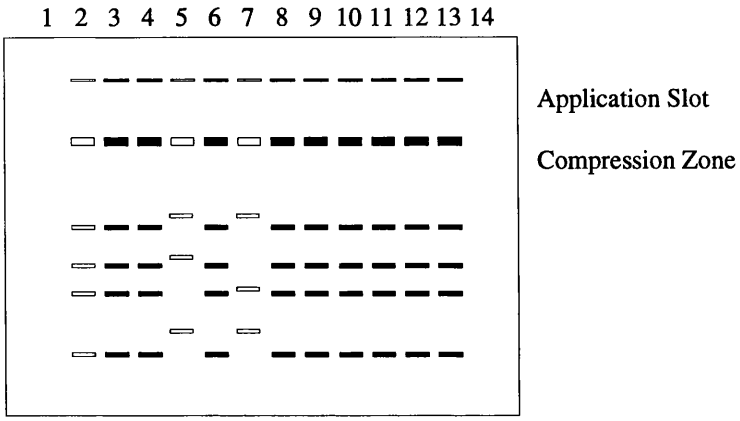
The karyotypes of 247 (lane 13) and a number of cloned progeny (lanes 3-12) are shown in bold. All the cloned progeny can be seen to have an identical karyotype to 247, and there was no evidence of selfing having occured. The lane numbers refer to the different clones as shown opposite.

Figure 5.11
Karyotype Analysis of Parental and Progeny Clones



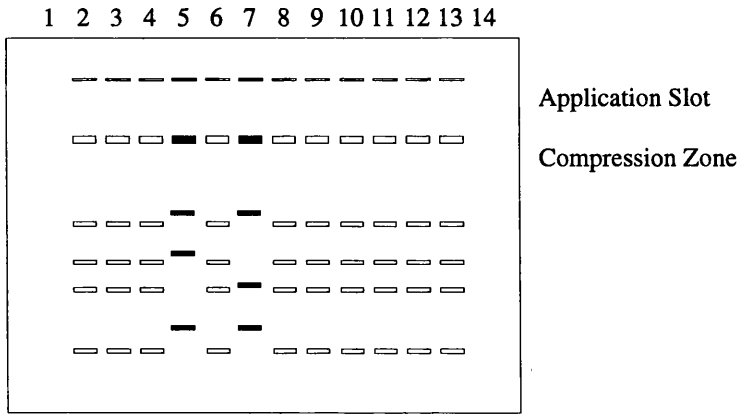
Karyotype analysis of 247Sur^R (lane 2), 444/54M^{R}S^R bcl2 (lane 3), 444/54M^{R}S^R bcl1 (lane 4), 386Sur^R (lane 5), 466/59 bcl7 (lane 6), 466/59 bcl5 (lane 7), 466/59 bcl4 (lane 8), 466/59 bcl2 (lane 9), 466/62S^R bcl1 (lane 10), 444/62 bcl2 (lane 11), 466/62 bcl1 (lane 12) and 247 (lane 13) using running condition 1 (see text) to separate chromosomes of approximately 1-2Mb in size. Two diagrams of this ethidium stained gel are given for ease of comparison of different tracks. The band numbers refer to the resolved bands with reference to the unselected parental lines, 247 and 386 (see Figure 5.8).

Figure 5.11
(a) Karyotype Analysis of Cloned Progeny



The karyotypes of 247 (lane 13) and 8 cloned progeny (lanes 2,3,6, and 8-12) are shown in bold. All these cloned progeny can be seen to have an identical karyotype to 247, and there was no evidence of selfing having occured. The lane numbers refer to the different clones as shown opposite.

(b) Karyotype Analysis of Clone 466/59 bcl5



The karyotypes of 386Sur^R (lane 5) and 466/59 bcl5 (lane 7) are shown in bold. The progeny clone 466/59 bcl5 can be seen to have the distinct karyotype of the melCy-resistant line, 386MelCy^R (see Figure 5.9(c)).

Table 5.1
Course of Salivary Gland Infection in Flies (Batch no. 28400)

Fly Number	Days post infection											
	36	39	43	46	48	51	54	59	61	62	64	67
28423		M^R			M^R	(247) M^R		(247) S^R, M^R	(247) M^R			S^R, M^R, $\mathbf{D^{Ra}}$
28435		(247) M^R	S^R, M^R		M^R	(247) M^R, $\mathbf{D^R}$	$\mathbf{D^R}$					
28444		(247) S^R		S^R		(247) S^R		(247) S^R		S^R, M^R, $\mathbf{D^{Ra}}$	(247) S^R	
28465		M^R		S^R, M^R, $\mathbf{D^{Ra}}$								
28466		M^R		S^R, M^R	M^R	(247) M^R, $\mathbf{D^R}$		(247) M^R, $\mathbf{D^R}$	$\mathbf{D^R}$			
28468	S^R											
28479		S^R, M^R, $\mathbf{D^{Ra}}$		S^R, M^R, $\mathbf{D^{Ra}}$		(247) S^R, M^R			(247) S^R, M^R, $\mathbf{D^{Ra}}$		(247) S^R, M^R	S^R, M^R, $\mathbf{D^{Ra}}$

The results of the mouse screens are shown for 7 flies producing metacyclic infections in batch no. 28400. The unique fly numbers are shown, together with the day (after the fly was given an infective feed) on which the fly was fed on a mouse. The change in the drug-resistance phenotypes of the metacyclic populations, as defined by the mouse screen, are shown over the course of the life of the flies.

M^R Trypanosomes expressing melCy-resistance were present (247MelCy^R and/or 386MelCy^R).

S^R Trypanosomes expressing suramin-resistance were present (247Sur^R).

$\mathbf{D^R}$ Trypanosomes expressing both melCy-resistance and suramin-resistance were present (247Sur^R x 386MelCy^R hybrids, and/or, 247Sur^R x 247MelCy^R selfers).

a Trypanosomes were present only for the first day after drug treatment in the double drug treated mouse, and at a low level of infection (lower than antilog 6.0 trypanosomes/ml).

(247) Uncloned trypanosome populations from fly-infected mice were characterised by isoenzyme electrophoresis at the ICD locus. All poulations that were analysed were identified as consisting of 247-derived lines only.

Table 5.2
Isoenzyme Analysis of Trypanosome Clones

Trypanosome Clone	ICD	AP	ME_B	Tyr^3
247	1	1	3	2-4
386	2	2	2-3	2-4
$247M^R$	1			
$386M^R$	2			
$247S^R$	1			
$386S^R$	2			
$466/51M^RS^R$ bcl 1	1		3	2-4
$466/51M^RS^R$ bcl 2	1		3	2-4
466/59 bcl 1				
466/59 bcl 2	1	1	3	2-4
466/59 bcl 3	1			
466/59 bcl 4	1	1	3	2-4
466/59 bcl 5	2			
466/59 bcl 6	1	1	3	2-4
466/59 bcl 7	1	1	3	2-4
466/59 bcl 8	1			
466/61 bcl 1	1			
466/61 bcl 2	1	1	3	2-4
$466/61M^RS^R$ bcl 1	1			
$466/61M^RS^R$ bcl 2				
$466/61M^RS^R$ bcl 3				
$466/61M^RS^R$ bcl 4				
466/63 mcl 1				
$444/54M^RS^R$ bcl 1	1	1	3	2-4
$444/54M^RS^R$ bcl 2	1	1	3	2-4
444/62 bcl 1	1	1	3	2-4
444/62 bcl 2	1	1	3	2-4
$444/62S^R$ bcl 1	1			
$444/62S^R$ bcl 2				

This table shows the isoenzyme phenotypes of each trypanosome clone. The numbers represent the genotypic variants, single numbers corresponding to homozygotes, and double numbers corresponding to heterozygotes. Clone 466/59 bcl 5 was identified as possessing the 386-type phenotype, and was the only clone to be identified that was not expressing a 247-type phenotype.

Table 5.3
Karyotype Analysis of Trypanosome Clones

Trypanosome Clone	Karyotype
247	247
386	386
$247M^R$	247
$386M^R$	$386M^R$
$247S^R$	247
$386S^R$	386
$466/51M^RS^R$ bcl 1	247[a]
$466/51M^RS^R$ bcl 2	247[a]
466/59 bcl 1	247
466/59 bcl 2	247
466/59 bcl 3	247[a]
466/59 bcl 4	247[a]
466/59 bcl 5	$386M^R$
466/59 bcl 6	247[a]
466/59 bcl 7	247
466/59 bcl 8	247[a]
466/61 bcl 1	ND
466/61 bcl 2	ND
$466/61M^RS^R$ bcl 1	247
$466/61M^RS^R$ bcl 2	ND
$466/61M^RS^R$ bcl 3	247
$466/61M^RS^R$ bcl 4	247
466/63 mcl 1	247
$444/54M^RS^R$ bcl 1	247[a]
$444/54M^RS^R$ bcl 2	247
444/62 bcl 1	247[a]
444/62 bcl 2	247
$444/62S^R$ bcl 1	247[a]
$444/62S^R$ bcl 2	ND

The karyotype of each trypanosome clone was determined by PFGE. Nineteen cloned progeny were analysed, 18 of which were found to possess the same karyotype as the 247 parental line (shown as 247). One clone possessed the distinct karyotype of the melCy-resistant line of 386 (shown as $386MelCy^R$).

[a] Only chromosomes below approximately 2.0Mb in size were analysed (i.e. chromosomes A and B).

Table 5.4
Inheritance of Drug-Resistance Phenotypes in Trypanosome Clones

	MelCy EC_{50} (μM)	Suramin EC_{50} (μM)	Resistance Phenotype[a]	
247	0.031	0.97	Mx1	Sx1
386	0.046	0.44	Mx1	Sx1
247M^R	2.7	2.2	Mx87	Sx2.3
247S^R	0.038	19	Mx1.2	Sx20
386M^R	1.1	2.2	Mx24	Sx5
386S^R	0.039	63	Mx0.85	Sx140
466/59 bcl 1	2.2	1.4	Mx71	Sx1.4
466/59 bcl 2	0.84	0.38	Mx27	Sx0.39
466/59 bcl 3	1.2	0.98	Mx39	Sx1.0
466/59 bcl 5	2.2	0.98	Mx48[a]	Sx2.2[a]
466/59 bcl 8	6.0	0.70	Mx190	Sx0.72
466/61S^{R}M^R bcl 1	2.4	1.6	Mx77	Sx1.6
466/61S^{R}M^R bcl 2	1.5	5.5	Mx48	Sx5.7
466/61S^{R}M^R bcl 3	1.5	4.1	Mx48	Sx4.2
466/61S^{R}M^R bcl 4	1.8	6.7	Mx58	Sx6.9
466/63 mcl 1	2.0	0.45	Mx65	Sx0.46

The levels of melCy-resistance and suramin-resistance are shown as EC_{50} values (μM), as determined in short-term bloodstream cultures.

[a] The drug-resistance phenotypes are expressed as the degree of increase in resistance compared to the sensitive parental line from which they were generated. The phenotypes of the clones were compared to the sensitive 247 line, except 466/59 bcl5, which was compared to the sensitive 386 line (since 466/59 bcl5 was identified as a 386-derived clone from isoenzyme and karyotype analysis).

Table 5.5
Summary of Isoenzyme, Karyotype and Drug Screening Analysis of Trypanosome Clones

Trypansosome Clone	Isoenzyme	Karyotype	Drug Screening
466/51M^{R}S^R bcl 1-2	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND
466/59 bcl 1	ND	247MelCy^R/247Sur^R	MelCy^R/Sur^S
466/59 bcl 2-3	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	MelCy^R/Sur^S
466/59 bcl 4	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND
466/59 bcl 5	386MelCy^R	386MelCy^R	MelCy^R/Sur^S
466/59 bcl 6-7	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND
466/59 bcl 8	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	MelCy^R/Sur^S
466/61 bcl 1-2	247MelCy^R/247Sur^R	ND	ND
466/61M^{R}S^R bcl 1	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	MelCy^R/Sur^S
466/61M^{R}S^R bcl 2	ND	ND	MelCy^R/Sur^R
466/61M^{R}S^R bcl 3-4	ND	247MelCy^R/247Sur^R	MelCy^R/Sur^R
466/63 mcl 1	ND	247MelCy^R/247Sur^R	MelCy^R/Sur^S
444/54M^{R}S^R bcl 1-2	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND
444/62 bcl 1-2	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND
444/62S^R bcl 1	247MelCy^R/247Sur^R	247MelCy^R/247Sur^R	ND

This table summarises the isoenzyme and karyotype analysis and the *in vitro* drug screening for the cloned progeny.

247MelCy^R/247Sur^R	The isoenzyme or karyotype analysis identified the clone as possessing the characteristics of the 247-derived lines.
386MelCy^R	The isoenzyme or karyotype analysis identified the clone as possessing the characteristics of the 386MelCy^R line.
MelCy^R/Sur^S	The drug screen identified the clone as expressing melCy-resistant, suramin-sensitive phenotypes.
MelCy^R/Sur^R	The drug screen identified the clone as expressing melCy-resistant, suramin-resistant phenotypes.

CHAPTER 6
PURINE TRANSPORT AND ARSENICAL UPTAKE

6.1 INTRODUCTION

The introduction of the melaminophenyl arsenicals by Friedheim in the 1940s provided the first opportunity for late-stage sleeping sickness to be cured. Melarsen, and its derivative melarsen oxide (melOx), were first synthesised in 1939, and were the first of the melaminophenyl arsenicals to be introduced (Friedheim 1948). Shortly after this, melB, a trivalent derivative of melOx, was introduced as a less toxic alternative (Friedheim 1949). Since then, trivalent water soluble derivatives of melOx have been synthesised. MelW was found to have no clear advantage over melB (Apted 1970). More recently melCy has been introduced and licensed for use in animal trypanosomiasis (Zelleke *et al.* 1989; Lun *et al.* 1991), but clinical trials with melCy in humans have not been conducted, leaving melB the only melaminophenyl arsenical in routine use for the treatment of sleeping sickness. There are very few alternatives to melB for treating late-stage sleeping sickness, although nifurtimox has proven effective against *T. b. gambiense* late-stage infections (Pepin *et al.* 1989) as has DFMO (McCann *et al.* 1981b). There has been little work conducted, however, on the effectiveness of nifurtimox against *T. b. rhodesiense*, and although treatment of experimental late-stage *T. b. rhodesiense* infections with DFMO has been found to be curative (McCann *et al.* 1981a), clinical trials have proved disappointing (Van Nieuwenhove 1992). Consequently, melB remains arguably the most important drug in use to cure late-stage sleeping sickness, almost 50 years after it was first introduced. It follows therefore, that the occurrence of melB-resistant cases of both *T. b. rhodesiense* and *T. b. gambiense* infections poses a serious problem, and necessitates the need for further investigations into both the mode of action of the different melaminophenyl arsenicals and the underlying biochemical mechanisms of arsenical-resistance.

It is a widely held belief that the melaminophenyl arsenicals melB, melW and melCy interact with the same targets through the active form of the drugs - trivalent melarsen oxide (Berger and Fairlamb 1994). All three compounds consist of a trivalent melarsen oxide moiety bound to a dithiol-containing group (or two identical monothiol-containing groups in melCy). Since melCy, melB and melW must each dissociate to become active compounds, it would be reasonable to assume that the ease with which dissociation occurs may influence their trypanocidal activity. In melCy, melOx is bound to two cysteamine molecules, whereas in melB and melW, melOx is bound to a single molecule of either 2,3-dimercaptopropanol or 2,3-dimercaptosuccinate respectively. Each of these bound thiols has a different affinity for melarsen oxide. The association constants K_a and the equilibria are shown below:

MelB $\Leftrightarrow$ 2,3-dimercaptopropanol + melOx $\quad K_a = 7.93 \times 10^{10} M^{-1}$

MelW $\Leftrightarrow$ 2,3-dimercaptosuccinate + melOx $\quad K_a = 4.50 \times 10^{10} M^{-1}$

Data from Fairlamb *et al.* (1989)

The situation with melCy is slightly different. MelCy is unstable in aqueous solution, immediately forming a mixture of melCy (43%), melCy with only one cysteamine moiety (24%), melOx (33%) and free cysteamine (Berger and Fairlamb 1994). However, no association constant has been determined for melCy, as the method used to measure the relative amounts of the products of dissociation was reported to be unsuitable to calculate an accurate association constant.

An important intracellular target for melOx has been identified as trypanothione ($Try[SH]_2$) with melOx binding to the sulphydryl groups of

trypanothione to form melT (Fairlamb *et al.* 1989). This study also calculated the association constant for melOx and trypanothione:

$$MelT \Leftrightarrow Try[SH]2 + melOx \qquad K_a = 1.21 \times 10^7 M^{-1}$$

MelB, melW and melCy are thought to interact with trypanothione in a similar way, with the formation of melT being brought about through the binding of the trivalent melarsen oxide moiety with trypanothione. In the case of melOx, this is brought about directly. In the cases of melB and melW, this occurs in conjunction with the dissociation of either 2,3-dimercaptopropanol or 2,3-dimercaptosuccinate respectively. The affinity of trypanothione for melOx is much lower than the affinity of the dithiols for melOx, with the K_a for melT being three orders of magnitude lower than that of melB and melW. However, the formation of melT from trypanothione and melOx is maintained due to the fact that both 2,3-dimercaptopropanol and 2,3-dimercaptosuccinate can readily diffuse out of the cell, thereby increasing the dissociation of melB and melW to release free melOx (Fairlamb *et al.* 1989). In the case of melCy, although direct evidence is not available, it is assumed that the reaction is essentially the same, with the dissociation of melCy releasing cysteamine instead of a dithiol (Berger and Fairlamb 1994). At this point it is important to recognise that trypanothione (with the formation of melT) is not the only potential intracellular target of the arsenical drugs and their primary mode of action still remains to be clarified. Pyruvate kinase has been shown to be inhibited by arsenicals (Flynn and Bowman 1974) and Ehrlich noted in the early stages of trypanocide development that arsenicals can interact with a number of sulphur containing compounds (Voegtlin *et al.* 1923). More recently, another major intracellular dithiol, dihydrolipoamide, was found to bind to melarsen oxide with an association constant of $5.47 \times 10^9 M^{-1}$, about 500-fold more stable than the association of melOx with trypanothione (Fairlamb *et al.* 1992a). However, although not all the

important targets for melaminophenyl arsenicals may yet be known, the view that they interact with the targets in the same way, via melarsen oxide, remains the most widely accepted assumption (Berger and Fairlamb 1994).

This chapter is concerned mainly with the mode of uptake of the melaminophenyl arsenicals melB, melOx and melCy. Recent studies have identified a purine transporter as being responsible for melB and melOx uptake, and furthermore, it has been proposed that alteration of this transporter accounts for a decrease in drug-uptake and consequently brings about arsenical-resistance in *T. brucei* (Carter and Fairlamb 1993). In a broader context, altered drug accumulation has been found to be responsible for drug-resistance in a number of parasites for a wide variety of drugs. Pentamidine, for example, has been shown to be taken up by a specific active transport system in *T. b brucei* (Damper and Patton 1975). Differences in drug-uptake were seen to correlate with pentamidine-resistance in strains of both *T. b. brucei* and *T. b. rhodesiense*, and a similar correlation between uptake and pentamidine-resistance was seen when procyclics were compared to bloodstream forms (Damper and Patton 1976). Another study, employing flow cytometric analysis with multidrug-resistant *T. b. brucei* and *T. b. rhodesiense*, showed that the resistant lines accumulated two diamidines (DAPI and Hoechst 33342) less than the sensitive lines (Frommel and Balber 1987). The explanation for this observation was that some alteration in the trypanosome membrane reduced the amount of drug that accumulated in the trypanosome. A study with nifurtimox-resistant strains of *T. cruzi*, also suggested that reduced accumulation of the drug rather than metabolic alterations were responsible for drug-resistance (Tsuhako *et al.* 1991). Reduced accumulation of isometamidium chloride was given also given as the explanation for drug-resistance in lines of *T. congolense* (Sutherland *et al.* 1991), and later studies indicated that an increase in drug-efflux as well as decreased drug-influx were involved in drug-resistance (Sutherland 1992). Increased drug-efflux rather

than decreased drug-influx has been found to be responsible for decreased drug accumulation in multidrug-resistant cancer cells (Gottesman and Pastan 1988). Verapamil has proven to be a very useful tool in determining whether reduced drug-accumulation is a consequence of decreased drug-influx or increased drug-efflux. Verapamil was shown to be effective in blocking extrusion pumps (P-glycoproteins), and in some cases of multi-drug-resistant cells, such as the cancer cells just mentioned, verapamil was found to reverse drug-resistance (Gottesman and Pastan 1988). Furthermore, verapamil, has been shown to reverse nifurtimox-resistance in *T. cruzi in vitro*, again suggesting that the mechanism of resistance was through increased drug-efflux rather than decreased influx (Neal *et al.* 1989). However, verapamil was unable to reverse resistance to samorin and berenil in a study with multidrug-resistant *T. b. brucei* suggesting that increased drug efflux was not responsible for drug-resistance in this case (Kaminsky and Zweygarth 1991).

A recent study on arsenical-resistance in *T. b. brucei* provided evidence that the melaminophenyl arsenicals were taken up by an adenosine transporter, and that this transporter was altered or down regulated in an arsenical-resistant line (Carter and Fairlamb 1993). The bloodstream form of the sensitive line had a total adenosine uptake of 10.7 ± 1.1 pmol/10^8cells/sec whereas the resistant line had a rate of uptake of only 1.9 ± 0.3 pmol/10^8cells/sec. The rapid melOx-induced lysis of the sensitive line could be blocked by adenine and adenosine but not inosine. It was concluded that two specific adenosine transporters existed (P1 and P2), and that these could be distinguished by their specificity for uptake of inosine (P1) and adenine (P2) respectively. Furthermore, it was the P2 transporter that was responsible for melOx and melB uptake, and this P2 transport was virtually absent in the arsenical-resistant line. It was found that the arsenical-resistant line also expressed cross-resistance to the diamidines (Fairlamb *et al.* 1992b), consistent with a number of previous reports

(Williamson and Rollo 1959; Frommel and Balber 1987). Since the diamidines also blocked melOx-induced lysis, it has been suggested that the P2 adenosine transporter was responsible for the uptake of berenil and pentamidine, as well as the melaminophenyl arsenicals (Fairlamb *et al.* 1992b). This chapter investigates the validity of these proposals using the arsenical-sensitive line (247) and the independently generated arsenical-resistant line (247MelCy^R).

6.2 MATERIALS AND METHODS

6.2.1 Drugs and Chemicals

MelCy, melB, and melOx were supplied and stored as described previously (**section 3.2.1**). Pentamidine (pentamidine isethionate), and silicone oil (75 centistokes, 1.05g/ml) were kind gifts from Prof. Alan Fairlamb, London School of Hygiene and Tropical Medicine. Berenil (diminazene aceturate), phenyl arsenoxide, adenine, adenosine and inosine were supplied by Sigma. [^{3}H]-adenosine (56Ci/mmol) was supplied by Amersham. Picofluor 40 scintillation fluid was supplied by Canberra Packard. Pentamidine and adenosine were stored desiccated at 4^{0}C. Berenil, phenyl arsenoxide, inosine and adenine were stored at room temperature.

6.2.2 Trypanosomes Stocks, Mice and Rats

Two cloned lines of trypanosomes were used in this study. 247 was taken from the original stabilate (GUP 3536) that was used to generate the drug-resistant lines of 247, and 247MelCy^R was taken from the original cloned stabilate, GUP 3611. Adult female Balb/C mice were used in the *in vivo* drug-resistance assays. Adult female CFLP mice and adult male and female Sprague-Dawley rats were used to grow trypanosomes.

6.2.3 *In Vivo* Assay to Determine Drug Resistance

The *in vivo* assays were conducted as described previously (**chapter 2**). The drug-sensitive stock (247) and the drug-resistant stock (247MelCy^R) were assessed for the levels of their resistance to pentamidine and berenil. Both drugs were desolved in dddH_2O immediately before use. Drugs were administered by i.p. injection.

6.2.4 *In Vitro* Lysis of Trypanosomes with Arsenical Drugs

Lysis of Bloodstream Trypanosomes

To grow trypanosomes for lysis studies, four adult female mice were immunosuppressed (cyclophosphamide at a dose of 250mg/Kg by i.p. injection 24 hours prior to trypanosome inoculation), infected with trypanosomes, and parasites from populations in exponential growth were combined to create new stabilates, cryopreserved in 0.5ml aliquots. One aliquot was then used to infect an immunosuppressed (cyclophosphamide at a dose of 50mg/Kg by i.p. injection 24 hours prior to trypanosome inoculation) adult Sprague-Dawley rat. The resulting parasites were transferred to six immunosuppressed rats, from which the trypanosomes for the lysis studies were taken. Trypanosomes were purified from infected blood by anion-exchange chromatography (Lanham and Godfrey 1970) and collected into 100ml Carter's Balanced Salt Solution on ice (CBSS: 25mM Hepes, 120mM NaCl, 5.4mM KCl, 0.55mM $CaCl_2$, 0.4mM $MgSO_4$, 5.6mM Na_2HPO_4, 11.1mM glucose, 5mg/l phenol red, pH 7.4: Fairlamb *et al.* 1992b). The trypanosome suspension was centrifuged at 1000g for 10 minutes at 4^0C, resuspended in CBSS at a trypanosome density of 10^8/ml and kept on ice. Arsenical-induced lysis at 37^0C was measured on a spectrophotometer (Yarlett *et al.* 1991; Fairlamb *et al.* 1992b). Briefly, as the trypanosomes lysed in the presence of arsenical, so more light was transmitted, and this decrease in

absorbance was measured at a wavelength of 750nm. By this method, arsenical-induced lysis can be followed spectrophotometrically for up to 1 hour. Four arsenical drugs were used: melCy (dissolved in $dddH_2O$), melOx, melB and phenyl arsenoxide (all dissolved in dimethyl formamide). The sensitivity of 247 and 247MelCy^R to lysis by each of these arsenicals was assessed at a final trypanosome density of 10^7/ml over 30 minutes in a range of drug concentrations. The effect of addition of adenine, adenosine, inosine, berenil and pentamidine on the arsenical-induced lysis of the drug-sensitive line (247) was also investigated as described previously (Carter and Fairlamb 1993). All OD_{750} measurements were made using buffer (CBSS) as a blank, and each experiment contained a control sample containing only trypanosomes in CBSS and solvents.

Lysis of Procyclic Trypanosomes

Procyclic trypanosomes (247 only) were grown in complete SDM-79. Arsenical-induced lysis was measured as for bloodstream trypanosomes at trypanosome densities of 10^7/ml. However, the lysis of procyclic trypanosomes was carried out over 1 hour in two different media at either 26^0C or 37^0C. Trypanosome cultures in logarithmic growth were spun at 1000g for 10 minutes at 4^0C and resuspended at a trypanosome density of 10^8/ml. Depending on the medium used to carry out the lysis experiment, the trypanosomes were resuspended in either SDM-79 (without adenosine) or CBSS and stored on ice.

6.2.5 Adenosine Uptake in Bloodstream Trypanosomes

Adenosine uptake studies were carried out on bloodstream trypanosomes using tritiated adenosine as described previously (Carter and Fairlamb 1993). Two separate studies were carried out to investigate the nature of the purine transporters and their role in arsenical resistance.

Rate of Adenosine Uptake in 247 and 247MelCy^R

The rate of adenosine uptake was measured over the first seven seconds after the addition of [^{3}H]-adenosine, in both 247 and 247MelCy^R. Purified trypanosomes were obtained as for the lysis studies, and resuspended at a trypanosome density of 10^8/ml in CBSS containing 1% BSA. CBSS (with 1% BSA) containing a final concentration of total adenosine (labelled and unlabelled) of 2μM and 1μM NaOH was overlaid on 100μl of silicone oil in a series of eppendorfs pre-warmed to 25^{0}C. 100μl of pre-warmed cells were pipetted onto the radiolabelled solutions at one second intervals. The trypanosomes mixed sufficiently with the radiolabelled solution due to the small volumes involved and the turbulent act of pipetting the trypanosomes onto the radiolabelled solutions. Adenosine uptake was stopped by centrifuging at high speed on an eppendorf microfuge for 2 minutes. This rapidly pelleted the trypanosomes through the oil leaving any radiolabel that had not been taken up in the aqueous supernatant. The resulting supernatants of oil and aqueous layer were removed by first removing the aqueous layer and washing the eppendorf sides twice in cold PBS to remove all traces of any radiolabel that had not been taken up by the trypanosomes, and then carefully removing the oil layer. Each pellet was then vortexed in 100μl of 2% TRITON-X scintillation grade (made up in dddH_2O) and left overnight at 4^{0}C. The radioactivity present in each pellet was then measured with 1ml Picofluor 40 scintillation fluid in a Beckman LS 6000LL. The amount of total adenosine taken up by the trypanosomes was calculated for each time point between 2 and 7 seconds. The rate of uptake of total adenosine (pmol/10^8cells/sec) was then calculated by linear regression. The adenosine uptake experiments were carried out in duplicate, and the means were calculated.

Characterisation of Adenosine Transport

The purine transporters responsible for adenosine uptake in 247 and 247MelCy^R were characterised with respect to uptake of adenine and inosine. The procedure employed was similar to that used to measure the overall rate of adenosine uptake. However, only the amount of adenosine uptake over 5 seconds was measured. The inhibition of [^{3}H]-adenosine uptake by adenine and inosine was determined by including a range of concentrations of each in the solutions containing the radiolabel. The results were expressed in terms of the extent by which adenine and inosine inhibited adenosine uptake compared to control samples. Each experiment for 247 was carried out in duplicate, and means were calculated. Duplicate experiments were not conducted with 247MelCy^R.

6.3 RESULTS

6.3.1 Pentamidine and Berenil Resistance *In Vivo*

The results of the *in vivo* assays for 247 and 247MelCy^R with berenil and pentamidine are shown in Table 6.1. The results are expressed as minimum curative doses i.e. the lowest dose required to completely clear the infection from all 6 mice in a group (**section 2.3.2**). This table also shows the resistance factors for each drug calculated as a ratio of the level of resistance in the melCy-resistant line (247MelCy^R) compared to the unselected drug-sensitive line (247). It can be seen that there is a marked degree of cross-resistance between the arsenicals and both berenil and pentamidine. There is a lesser amount of cross-resistance between melCy and berenil (resistance factor of 16) than there is between melCy and pentamidine (resistance factor of greater than 63). 247MelCy^R was resistant to a dose of pentamidine of 100mg/Kg, the maximum tolerated dose in mice (Carter pers. comm.).

6.3.2 Arsenical Induced Lysis

Bloodstream Trypanosomes

The lysis data for 247 is shown graphically in Figures 6.1 to 6.6, showing the decrease in absorbance at 750nm over 30 minutes after addition of arsenical drug. The susceptibility of 247 to arsenical-induced lysis to all four arsenicals can be seen in figures 6.1(a-d). Comparison of these four graphs shows that approximately equivalent rates of lysis occurred with markedly different concentrations of the four arsenicals. MelOx brought about rapid lysis of 247 at concentrations of 5μM and above (Figure 6.1(a)). The rate of lysis was markedly increased with same concentration of melCy (Figure 6.1(b)), and indeed, only 1μM melCy was necessary to bring about lysis at a roughly comparable rate to that brought about by 5μM melOx. The differences in potency of the melaminophenyl arsenicals was even more noticeable with melB (Figure 6.1(c)), which required approximately a 25-fold greater concentration to bring about lysis similar to that seen with 1μM melCy. Phenyl arsenoxide, which is essentially melOx without the melamine ring, lysed the trypanosomes in less than 5 minutes at concentrations of 0.1μM and greater, and was undoubtedly more potent than all the melaminophenyl arsenicals (Figure 6.1(d)). Consequently, within the melaminophenyl arsenicals, melCy was noticeably the most potent, requiring a concentration 5 times lower than melOx, and 25 times lower than melB to bring about similar degrees of lysis. Alternatively, a comparison of the effects of 1μM of the different melaminophenyl arsenicals showed that at this concentration, melCy was able to bring about much quicker lysis than the same concentration of melOx, while 1μM of melB was incapable of lysing the trypanosomes after 30 minutes.

In a previous study, the uptake mechanisms for the arsenical drugs were characterised by investigating which compounds blocked lysis, with the inference

being that lysis was reduced or blocked by those compounds that competed for transport with the arsenical (Carter and Fairlamb 1993). In the present study, the effect of adenine, adenosine, inosine, pentamidine and berenil on arsenical induced lysis was investigated. The maximum amount of excess of these compounds was different for each of the arsenicals used, due to the different arsenical concentrations that were employed to bring about similar degrees of lysis. In the unselected line, 247, addition of adenosine or adenine to the lysis medium protected the trypanosomes, in a concentration dependant manner, from lysis by melOx and melCy (Figures 6.2(a and b) and 6.3(a and b)). In these instances, a 40-fold excess of either adenosine or adenine brought about total protection over 30 minutes at 37^0C. For melB-induced lysis, however, a similar 40-fold excess of adenosine or adenine had no measurable effect on lysis (Figures 6.2(c) and 6.3(c)). A similar situation occurs with phenyl arsenoxide, in which even 1000-fold excesses of adenosine and adenine were unable to bring about any noticeable decrease in the rate of lysis (Figures 6.2(d) and 6.3(d)). Inosine had no measurable effect on lysis, regardless of the arsenical employed. At inosine concentrations of 100-fold greater than melOx and melCy (Figures 6.4(a) and 6.4(b)), 40-fold greater than melB (Figure 6.4(c)) and 1000-fold greater than phenyl arsenoxide (Figure 6.4(d)) the decreases in absorbance were maintained at the same rates as those in samples with no inosine.

It has been proposed that the melaminophenyl arsenicals share a common uptake mechanism with the diamidines (Fairlamb *et al.* 1992b). Like adenosine and adenine, increasing amounts of berenil and pentamidine protected 247 from the lysis caused by melOx and melCy, although full protection was not seen even with diamidine concentrations of 100-fold more than the arsenicals (Figures 6.5(a and b) and 6.6(a and b)). Interestingly, although neither adenosine, adenine or inosine could block melB-induced lysis, both berenil and pentamidine were able to partially protect the trypanosomes from melB (Figures 6.5(c) and 6.6(c)).

At 40-fold excess, berenil and pentamidine protected the trypanosomes to an approximately equal extent, whether the arsenical was melB, melOx or melCy. One further interesting result was found with phenyl arsenoxide. Berenil, like adenosine, adenine and inosine, could not protect the trypanosomes from lysis by phenyl arsenoxide even when they were present at concentrations of 0.1mM, 1000-fold greater than the concentration of phenyl arsenoxide (0.1μM) (Figures 6.2(d), 6.3(d), 6.4(d) and 6.5(d)). However, 1000-fold excess of pentamidine was able to prevent some cells lysing in the presence of 0.1μM phenyl arsenoxide, after an initial period of rapid lysis (Figure 6.6(d)).

Figure 6.7(a-d) shows the lysis of the resistant line (247MelCy^R) by the four arsenicals. Arsenical-resistance was expressed in the lysis assay to all four arsenicals, although to different extents. All concentrations of melOx, up to and including 100μM, had no noticeable effect on 247MelCy^R (figure 6.7a). On the other hand, 10μM melCy was unable to lyse the cells over 30 minutes, although there was a slight degree of lysis at 50μM melCy (Figure 6.7(b)) Similarly, with melB, 10μM had no noticeable effect on lysis, whereas 50μM melB brought about partial lysis (Figure 6.7(c)). In comparison to the melaminophenyl arsenicals, the trypanosomes expressed a much lower level of resistance to phenyl arsenoxide, with 0.1μM proving insufficient to cause lysis, and concentrations above 1μM bringing about rapid lysis. Of the three melaminophenyl arsenicals, melB was by far the most effective at lysing the resistant line (Figure 6.7(c)), the opposite of the situation that was found with the sensitive line, where melB was the least effective. At concentrations of 100μM, melOx caused no lysis, melCy caused very slight lysis, whereas, melB caused a much more marked degree of lysis compared to the other two drugs. Although phenyl arsenoxide was capable of lysing cells very rapidly and at much lower concentrations than those required for the melaminophenyl arsenicals, the resistant line did express a marked degree of resistance to phenyl arsenoxide

(Figure 6.7(d)). Whereas 247 was susceptible to lysis in less than 5 minutes by 0.1μM phenyl arsenoxide, greater than 100μM was required to have the same effect on 247MelCy^R (Figures 6.1(d) and 6.7(d)).

Figure 6.8(a and b) illustrates a different observation concerning the susceptibility of trypanosomes to arsenical-induced lysis. All the results described above used trypanosomes from rats only 3 passages away from the original cloned stabilates. However, equivalent experiments were also conducted using 247 that was syringe passaged in rats for approximately 2-3 months, involving at least 15 passages. This passaging resulted in the development of a 247 line that was less susceptible to lysis by all four arsenicals. Figure 6.8(a) shows the melOx-induced lysis of 247 before and after more than 15 passages. This reduction in the susceptibility to lysis was seen as a gradual change, with the degree of arsenical-induced lysis decreasing as the number of passages increased. As the number of times the line was passaged increased over a period of time, so the rate of lysis at a given concentration of arsenical drug was gradually reduced. This resulted in the need for increased concentrations of arsenical in order to bring about similar rates of lysis that were seen in the line prior to it being passaged. This same pattern was seen with all the arsenicals, a further example of which can be seen in figure 6.8(b). This shows melCy-induced lysis at a concentration of 1μM for 247 before and after more than 15 passages. In the case of phenyl arsenoxide, it was necessary to increase the concentration by 100-fold to bring about a similar rate of lysis in the passaged line to that observed with 0.1μM of phenyl arsenoxide in 247 before the passages.

Procyclic Trypanosomes

The unselected drug-sensitive line, 247, was assessed in the lysis assay with procyclic culture forms. Upon resuspension of the procyclics in CBSS, the

trypanosomes remained motile when stored on ice, but as soon as they were brought up to room temperature, upon examination under a microscope they were seen to lose their motility within 5-10 minutes. Nevertheless, lysis of 247 procyclics in CBSS was measured over one hour with frequent stirring of the samples to avoid the non-motile trypanosomes from settling. The trypanosomes remained highly motile when resuspended in SDM-79 (without adenosine). Figures 6.9(a-d) show no clear indication of lysis in either medium at either 26^0C or 37^0C with any of the arsenicals tested. All the arsenicals were present at high concentrations, capable of effecting lysis in 247 bloodstream forms. As a result of this finding, there was no point in examining lysis in the drug-resistant line, 247MelCy^R.

6.3.3 Adenosine Uptake

Alteration in the Rate of Adenosine Uptake

The rate of adenosine uptake for 247 was found to be almost 10-fold greater than for its arsenical-resistant counterpart, 247MelCy^R (Table 6.2) The sensitive line, 247, took up adenosine at a rate of 48pmol/10^8cells/sec, compared to 5.0pmol/10^8cells/sec for 247MelCy^R.

Characterisation of Adenosine Transport

Adenosine uptake in 247 bloodstream forms could be partially blocked by both adenine and inosine (Tables 6.3 and 6.4). Adenine in the absence of inosine, inhibited adenosine uptake in a concentration dependant manner, to a maximum inhibition of approximately 50%, which occurred at adenine concentrations above 10μM (Table 6.3). Similarly, inosine in the absence of adenine, was found to inhibit adenosine uptake up to a maximum of approximately 45% at and above concentrations of inosine of 10μM (Table 6.4).

In the presence of 500μM inosine the addition of adenine brought about a further increase in the inhibition of adenosine uptake above the level of 45% brought about by inosine alone. The reciprocal experiment with excess adenine and increasing amounts of inosine also showed the 50% inhibition that was brought about by adenine alone to be increased with the addition of inosine in a concentration-dependant manner. In both cases, excess amounts of inosine and adenine together inhibited adenosine uptake by approximately 70 to 75%.

Table 6.5 shows the results of the uptake studies carried out on 247MelCy^R. Like 247, increasing amounts of adenine inhibited adenosine uptake in the resistant line. However, the maximum amount of inhibition reached was approximately 35%, a value lower than that observed in 247. At excess concentrations of both adenine and inosine, the adenosine uptake could be inhibited to a maximum level of approximately 75%, similar to the level of inhibition observed in the unselected line, 247.

6.4 DISCUSSION

Trypanothione has been identified as the primary intracellular target for arsenical drugs (Fairlamb *et al.* 1989). However, alterations in trypanothione metabolism have not been found to be a mechanism through which trypanosomes express arsenical-resistance. Two recent studies have provided evidence for arsenical-resistance being brought about through a reduction in the uptake of melaminophenyl arsenicals (Yarlett *et al.* 1991; Carter and Fairlamb 1993). One of these recent studies (Carter and Fairlamb 1993) compared a line of T. b. brucei (S427) with an arsenical-resistant cloned line (S427 cRU15) that had been derived from S427 by exposing the unselected line to gradually increasing sub-curative doses of sodium melarsen, up to a maximum of 160mg/Kg (Fairlamb *et al.* 1992b). The results of lysis and radiolabelled uptake experiments, provided

evidence for the existence of an adenosine transporter (termed P2) that was responsible for the uptake of the melaminophenyl arsenicals. Based on the results of these studies, it was concluded that an alteration in the P2 adenosine transporter was responsible for the expression of resistance to the melaminophenyl arsenicals (Carter and Fairlamb 1993). It was the aim of the present study to conduct similar comparable experiments with 247 and 247MelCy^R in order to determine if the mechanism of arsenical-resistance in S427 cRU15 was also the mechanism of arsenical-resistance in 247MelCy^R. The aims were therefore, to determine whether or not an adenosine transporter was responsible for the uptake of melaminophenyl arsenicals, and if so, whether or not an alteration in adenosine transport could account for the expression of arsenical-resistance.

Reduced intracellular accumulation of drugs has been shown to be a frequent mechanism through which drug-resistance is expressed. For example, a DFMO-resistant line of *T. b. brucei* showed a decrease in the incorporation of DFMO compared to the sensitive line (Phillips and Wang 1987). However, reduced drug accumulation within the trypanosome may be the result of either decreased drug-uptake or increased drug-efflux, as well as possible alterations in the metabolism of the drug. Verapamil has been shown to inhibit the function of P-glycoproteins, which are found on the cell membrane and have the ability to transport lipophilic drugs out of the cell (Gottesman and Pastan 1988). Drug-resistance has been shown to be reversible by verapamil in a number of cases, such as the reversal of some cases of chloroquine-resistance in *Plasmodium falciparum* (Martin *et al.* 1987) and the reversal of sodium stibogluconate-resistance in *Leishmania donovani* (Neal *et al.* 1989). However, verapamil was not able to reverse drug-resistance in African trypanosomes (Kaminsky and Zweygarth 1991). A number of calcium-channel antagonists, including verapamil, were unable to reverse melOx-resistance in isolates of *T. b.*

rhodesiense (Yarlett *et al.* 1991), and current evidence would therefore suggest that resistance to melaminophenyl arsenicals is not due to increased drug-efflux. However, decreased drug accumulation has been shown to be involved in atoxyl-resistance in *T. brucei* (Yorke *et al.* 1931), and since alterations in trypanothione metabolism that could account for arsenical-resistance have not yet been found (Yarlett *et al.* 1991; Fairlamb *et al.* 1992b), it would therefore be reasonable to expect alterations in drug-uptake to be involved in drug-resistance.

The reduction in drug-uptake and the existence of a common transport mechanism has recently been offered as an explanation for the patterns of cross-resistance between melaminophenyl arsenicals and the diamidines (Carter and Fairlamb 1993). The existence of cross-resistance between arsenicals and diamidines has been reported in a number of independent studies (Williamson and Rollo 1951; Frommel and Balber 1987). The results presented here also show a marked degree of cross-resistance between melaminophenyl arsenicals and the two diamidines, berenil and pentamidine. The resistance factors for berenil and pentamidine were 16, and greater than 63 respectively (Table 6.1). An explanation for these patterns of cross-resistance has recently been offered based on the existence of a common transport mechanism, involving a specific transporter, for all the melaminophenyl arsenicals and the diamidines (Fairlamb *et al.* 1992b). The two benzamidine rings in the diamidines and the melamine and benzene rings in the melaminophenyl arsenicals have been proposed as the similar structures that are recognised by the transporter, thereby accounting for the patterns of cross-resistance. Furthermore, the interatomic distances between the centres of the two rings has been found to correlate with the degree of cross-resistance between melarsen oxide and four diamidines (Fairlamb *et al.* 1992b). As the distance between rings increased, so the degree of cross-resistance decreased, with only a very slight amount of cross-resistance existing between melarsen oxide and pentamidine, the latter having the greatest distance between

rings compared to the other three diamidines. However, for the present study, this hypothesis would have predicted 247MelCy^R to have a much greater degree of cross-resistance to berenil than pentamidine. This is clearly not the case, and although the existence of a common transport mechanism may indeed be the case, the results presented here are not in agreement with the proposal that inter-ring distances determine the specificity of the transporter, and therefore the relative degrees of cross-resistance.

It has been proposed that the common transport of the melaminophenyl arsenicals and diamidines occurs via a specific adenosine transporter (Carter and Fairlamb 1993). *In vitro* melOx-induced lysis of trypanosomes was found to be blocked by adenosine, which was consistent with adenosine competing with melOx for uptake, thereby resulting in a lower intracellular concentration of melOx. These lysis experiments were carried out on a line of *T. b. brucei* (S427), using melOx and phenylarsine oxide, which is highly lipid soluble and lacks the melaminyl moiety of melOx. The results presented in the current study were from experiments carried out on 247, in an attempt to establish whether the proposal of an adenosine transporter being involved in the uptake of melaminophenyl arsenicals was a phenomenon common to other trypanosome lines. The lysis studies with 247 were carried out using melCy and melB as well as melOx and phenylarsine oxide. It was clear that the sensitive line was highly susceptible to melCy, and that melB was the least trypanolytic agent of the arsenicals. The fact that phenylarsine oxide, which can rapidly enter the trypanosome by diffusion, was able to bring about rapid lysis at much lower concentrations than melOx is consistent with the proposal that the trypanosome's membrane acts as a permeability barrier for the melaminophenyl arsenicals and that a selective uptake mechanism exists. However, if all the melaminophenyl arsenicals were taken up by the same route and at the same rate, it would be expected that melOx would be the most potent as it represents the active form of

the drugs. Since melCy and not melOx was the most potent, and assuming they share the same target, this suggests that they are not taken up at the same rate. It therefore follows that either a single transporter distinguishes between different melaminophenyl arsenicals (and thereby providing further evidence against the influence of the inter-ring distances in drug recognition by the transporter) or that the different melaminophenyl arsenicals can be taken up by different routes.

The results of the lysis studies with 247 presented in the present study for melOx are consistent with the previous findings (Carter and Fairlamb 1993) that melOx is taken up via an adenosine transporter. Similar to the situation with S427, both adenosine and adenine, as well as berenil and pentamidine were able to block melOx-induced lysis in a dose dependant manner. Evidence has been presented for the existence of two adenosine transporters in the sensitive S427 line (Carter and Fairlamb 1993). One (termed P1) was responsible for the uptake of adenosine and inosine but not melOx, and the other (P2) transported adenosine, adenine as well as melOx and melB. The results presented here for 247 agree with the proposal that melOx uptake is blocked by adenosine and adenine (P2 transport) but not by inosine (P1 transport). Similarly, phenylarsine oxide, lacking the melaminyl moiety and being highly lipid soluble, entered the cell by diffusion and lysis could not be blocked by adenine, adenosine or inosine, in agreement with the proposal that the melaminyl moiety is important in recognition by the transporter. Although, melCy was not used in the previously published study with S427, melCy behaved similar to melOx for 247 in that adenosine, adenine, berenil and pentamidine blocked lysis whereas inosine did not. MelCy, therefore, also appears to enter the cell via the P2 adenosine transporter. However, with S427, melB was found to competitively inhibit adenosine transport by the P2 transporter with a K_i of 0.28μM (Carter and Fairlamb 1993). In the present study, melB-induced lysis was not blocked by adenosine or adenine although it was by berenil and pentamidine. It would

therefore appear on this evidence that melB was not taken up by an adenosine transporter. However, the fact that berenil and pentamidine blocked lysis to all three melaminophenyl arsenicals, including melB, may indicate that melOx, melCy, melB, berenil and pentamidine do indeed share a common transport system, although if this were the case, this would have to be a non-adenosine transporter since adenosine does not block melB-induced lysis. It is, therefore, impossible to explain the lysis data purely in terms of a single adenosine transporter that is responsible for the uptake of all melaminophenyl arsenicals and the diamidines. It may be that more than one route exists for drug entry into the trypanosome. The relatively high lipid solubility of melB may allow it to diffuse into the cell, bypassing the adenosine transporter, whereas melCy and melOx are reliant on a more specific mechanism of uptake. Alternatively, an as yet unidentified transporter may be responsible for melB uptake. However this does not take into account the fact that the diamidines can block lysis to all the melaminophenyl arsenicals, since if they block lysis by competing for a common transporter, they must compete not only for the melB transporter but also the melOx and melCy transporter, and consequently have two possible routes into the cell.

The fact that berenil and pentamidine block lysis by the melaminophenyl arsenicals, together with the existence of cross-resistance between the melaminophenyl arsenicals and diamidines seen here and elsewhere (Williamson and Rollo 1959; Fairlamb *et al.* 1992b) could be explained simply by the existence of similar modes of action for the two families of drugs rather than common uptake mechanisms. For example, assuming trypanothione is the primary target for arsenicals (Fairlamb *et al.* 1989), it may be that the primary targets for berenil and pentamidine may also be closely involved in maintaining the intracellular redox balance and detoxifying H_2O_2. A situation can be envisaged where alterations in trypanothione metabolism could bring about

resistance to both melaminophenyl arsenicals and diamidines. Consequently, to give an alternative explanation for the lysis results with 247; melOx and melCy may enter the cell via the adenosine transporter, explaining the blocking of lysis by adenosine and adenine. MelB may enter either by diffusion or via an as yet unidentified non-adenosine transporter, explaining why adenosine and adenine could not block melB-induced lysis. MelOx, melCy and melB may share a common target with the diamidines, explaining both the existence of cross-resistance between the diamidines and the melaminophenyl arsenicals, and also the reason for the diamidines blocking arsenical-induced lysis, assuming the diamidines are less potent at the molecular level and in some way interfere with the interaction of the arsenicals with their target.

Examination of the lysis data with the resistant line, 247MelCy^R provides further evidence that an alteration in adenosine transport cannot solely be responsible for arsenical-resistance, particularly for melB. 247MelCy^R showed a marked degree of resistance to melCy and melOx in the lysis assay. However, where melB was the least effective melaminophenyl arsenical for the lysis of 247, it was the most potent for 247MelCy^R. 100μM melOx was incapable of causing any noticeable lysis and 100μM melCy was capable of bringing about a slow, partial lysis over 30 minutes, whereas 100μM melB was noticeably a more effective trypanolytic agent, although some amount of melB-resistance was undoubtedly expressed in 247MelCy^R. Significantly, there was also a very marked increase in resistance to phenylarsine oxide. An increase in phenylarsine oxide-resistance was also seen in the melarsen-resistant line of S427, although this was largely ignored (Fairlamb *et al.* 1992b). This undoubtedly indicates that the mechanism(s) of arsenical-resistance involves at least one alteration that is not involved in drug uptake. Indeed, the proposal that melB can diffuse through the outer membrane or enters the trypanosome by a non-adenosine transporter, is in agreement with the lysis data for 247MelCy^R and the growth inhibition data

(**chapter 3**). Therefore, the existence of at least two alterations in 247MelCy^R, one at the adenosine transporter (inhibiting melOx and melCy uptake), and another at an intracellular target, would seem to be the most reasonable explanation for these results until more information is obtained. The distinct possibility that melB is not taken up by the same mechanism as melOx and melCy has important implications for previous and future investigations into the mechanism of melB-resistance that use arsenical-resistant lines that were not generated using melB. S427 cRU18 was selected from S427 in mice using increasing sub-curative drug doses of sodium melarsen up to a maximum tolerated dose of 160mg/Kg (Fairlamb *et al.* 1992b). Similarly, 247MelCy^R was selected with melCy up to a maximum tolerated dose of 40mg/Kg. Consequently, neither S427 cRU18 nor 247MelCy^R was selected using melB. Furthermore, these inconsistencies between melOx, melCy and melB bring into question the use of melB only, and not melCy, for the treatment of sleeping sickness.

One unexpected finding that arose from preliminary lysis studies was that the 247 line showed a gradual decrease in susceptibility to arsenical-induced lysis as it was passaged through rats. This was seen with all three melaminophenyl arsenicals as well as phenylarsine oxide. A possible explanation for this phenomenon is as follows: The trypanosomes were passaged in adult rats which were often simultaneously treated with cyclophosphamide at a dose of 50mg/Kg when they were infected with trypanosomes, both by i.p. injection. The toxic breakdown products of cyclophosphamide act on a number of different cell types by stopping cell division. It is thought that most cells that divide are affected by cyclophosphamide, including trypanosomes, although its effect on trypanosomes must only be slight. Nevertheless, it may be that cyclophosphamide exerts some sort of selective pressure on the trypanosome population in the same way as a sub-curative drug dose, with the subsequent development of "cyclophosphamide-

resistant" trypanosomes. The mechanism through which the trypanosomes become "resistant" to cyclophosphamide may also bring about cross-resistance to arsenicals, although how this might occur remains unclear. The possibility that the actual process of passaging itself may have brought about an increase in arsenical-resistance can be largely discounted since the suramin-resistant lines, $247Sur^R$ and $386Sur^R$, did not show any increase in resistance to arsenicals and where themselves passaged to similar extents, but in the absence of the breakdown products of cyclophosphamide (**chapter 2**). Importantly, during the selections for drug-resistance, mice were infected with trypanosomes 24 hours after they had received cyclophosphamide, allowing sufficient time for the toxic breakdown products of cyclophosphamide to be removed from the circulation, whereas the rats were often infected and given cyclophosphamide at the same time.

Procyclic trypanosomes were susceptible to melCy when incubated in complete SDM-79 for up to one day (results not shown), and indeed melCy-resistant trypanosomes can be distinguished from unselected lines in procyclics *in vitro* (**chapter 5**). However, for periods up to one hour, procyclics had an extremely low susceptibility to lysis by arsenicals. Lysis studies were carried out on 247 resuspended in either CBSS or SDM-79 without adenosine at either 26^0C or 37^0C in the presence of arsenicals at the maximum possible concentrations. Although CBSS is a buffered salt solution specifically designed for bloodstream trypanosomes (Fairlamb *et al.* 1992b), it was used to give a direct comparison with bloodstream trypanosomes. Since it contained glucose and no amino acids, the procyclic trypanosomes had no energy source, thus explaining the rapid loss in motility upon resuspension in CBSS. Active transport would be reduced due to this lack of energy substrate, and this may appear to provide a reason for the lack of susceptibility to lysis. However, even 1mM phenylarsine oxide could not lyse the procyclics over 1 hour, thereby discounting

the lack of active uptake as the reason for there being no lysis. Furthermore, if the absence of an energy substrate was important, then lysis would have been expected in SDM-79 without adenosine. Again, no definite signs of lysis were seen with any of the four arsenicals over one hour. Ionised calcium, at a concentration above approximately 0.3mM, has been shown to be necessary for lysis *in vitro* (Clarkson and Amole 1982), but this also could not explain the lack of lysis since both CBSS and SDM-79 contained sufficient calcium (0.55mM and 1.6mM respectively). Consequently, it appears that the differences in metabolism between procyclics and bloodstream trypanosomes may account for the lack of susceptibility to lysis of the procyclics. Glycolysis and glucose catabolism is replaced with amino acid catabolism as the source of energy in procyclics. However, despite the fact that it has been shown that arsenical drugs inhibit pyruvate kinase (Flynn and Bowman 1974), inhibition of glycolysis is not the reason for cell lysis (Van Schaftingen *et al.* 1987). Further investigations are required to explain the procyclic's resistance to arsenical-induced lysis.

Despite the fact that melB does not appear to be taken up by an adenosine transporter, melOx-resistance and melCy-resistance may, at least in part, be involved with alterations in adenosine transport. Radiolabelled adenosine was used to characterise the adenosine transporters in 247 and 247MelCy^R in order for a comparison to be made with previous findings. Two adenosine transporters were identified in S427. In this line, the P1-transporter was responsible for adenosine and inosine transport only, accounting for 60-70% of total adenosine uptake. The P2-transporter was the only other adenosine transporter identified, accounting for 30-40% of total adenosine uptake and being responsible for the uptake of adenine, melOx and melB (Carter and Fairlamb 1993). The situation with adenosine transport in 247 was slightly different. Adenine clearly inhibited adenosine uptake in a dose-dependant manner, which was saturable between 45-55% inhibition (Table 6.3). Similarly,

inosine inhibited adenosine uptake by up to 40-45% (Table 6.4). However, in S427, inosine and adenine transport were mutually exclusive. This was clearly not the case with 247, since if it were, then saturable concentrations of adenine and inosine together should have brought about inhibition of adenosine uptake by between 85-100%. In fact, the maximum inhibition of adenosine uptake, brought about by the saturable concentrations of both inosine and adenine was found to be between 65 and 75% (Tables 6.3 and 6.4). This therefore indicates that 247 differs from S427, in that inosine and adenine transport in 247 appear to share a common transporter, at least in part. Since saturable concentrations of both inosine and adenine together inhibited adenosine uptake between 65 and 75%, there remains approximately 30% of adenosine that was transported via a transporter(s) that was not blocked by either adenine or inosine. Therefore, this residual adenosine transport that occurred in the presence of excess inosine and adenine indicates a further adenosine transporter that does not transport adenine or inosine. Figure 6.8 illustrates one possible situation in 247 that would explain the results of both the transport studies and the lysis experiments. A situation could be envisaged in which 247 had at least four different adenosine transporters. In this situation P2 transport could again be responsible for melOx uptake, as in S427 (Carter and Fairlamb 1993). These differences in adenosine transport between different strains was not entirely unexpected, since a recent study on nucleoside transporters in *Leishmania major* found inosine transporters to be invariantly expressed in different strains, whereas adenosine transporters were extremely different in their inhibition profiles (Baer *et al.* 1992).

The two arsenical-resistant lines (S427 cRU18 and 247MelCy^{R}) showed altered rates of adenosine uptake compared to the unselected line from which they were generated (Table 6.2; Carter and Fairlamb 1993). In the sensitive line of S427, the rate of total adenosine uptake was 10.7pmol/10^{8}cells/sec compared to 48.0pmol/10^{8}cells/sec in 247. Thus, differences in adenosine uptake existed

between the two unselected lines. Since 247 had a higher rate of adenosine uptake than S427, then it may be expected to also have a higher rate of uptake of melOx, assuming melOx is taken up via an adenosine transporter. It would therefore also be reasonable to expect 247 to be more susceptible to melOx-induced lysis compared S427. However, the opposite was the case, with 5μM melOx being required to bring about a similar rate of lysis in 247 that was brought about by 0.5μM melOx in S427 (Fairlamb *et al.* 1992b). The sodium melarsen-resistant line of S427 cRU18 had a total rate of adenosine uptake of 1.9pmol/10^8cells/sec, all of which was accounted for by the P1-transporter. The resistant line, 247MelCy^R, also showed a decrease in total adenosine transport to 4.8pmol/10^8cells/sec. However, whereas P2-transport had totally disappeared in S427 cRU18, it had only decreased by approximately 10% in 247MelCy^R (Table 6.5). This suggests that the decreased rate of adenosine uptake in 247MelCy^R, which was approximately 10-fold lower than in 247, was due to alterations in one or more other adenosine transporters, as well as in the P2 transporter. This provides further evidence for the existence, in 247, of more than the two adenosine transporters that were identified in S427. In conclusion therefore, assuming melOx and melCy are indeed transported via the P2-transporter, then this reduction in P2 adenosine transport could account for a lower rate of uptake of melOx and melCy. However, even if this is the case, the lysis data certainly shows that altered uptake alone can not explain the patterns of drug-resistance seen, particularly with melB, the drug in clinical use.

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Figure 6.1

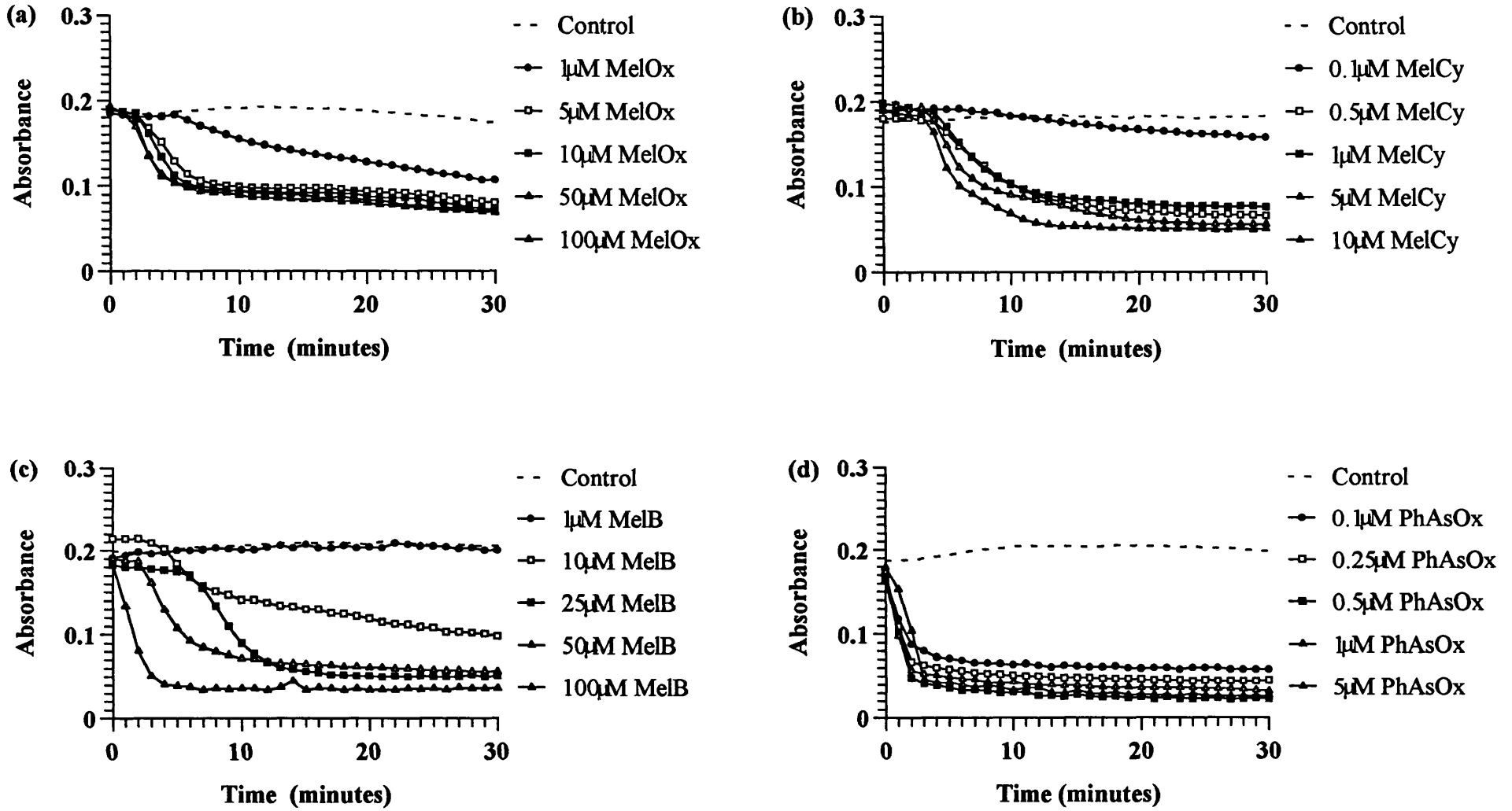

Figure 6.1
Arsenical-Induced Lysis in 247
(a) with MelOx
(b) with MelCy
(c) with MelB
(d) with Phenyl Arsenoxide

The effects of increasing concentrations of arsenical on the lysis of 247 are shown, measured spectrophotometrically at a wavelength of 750nm. Control samples, containing only trypanosomes in solvents and buffer are shown (---).

Figure 6.2

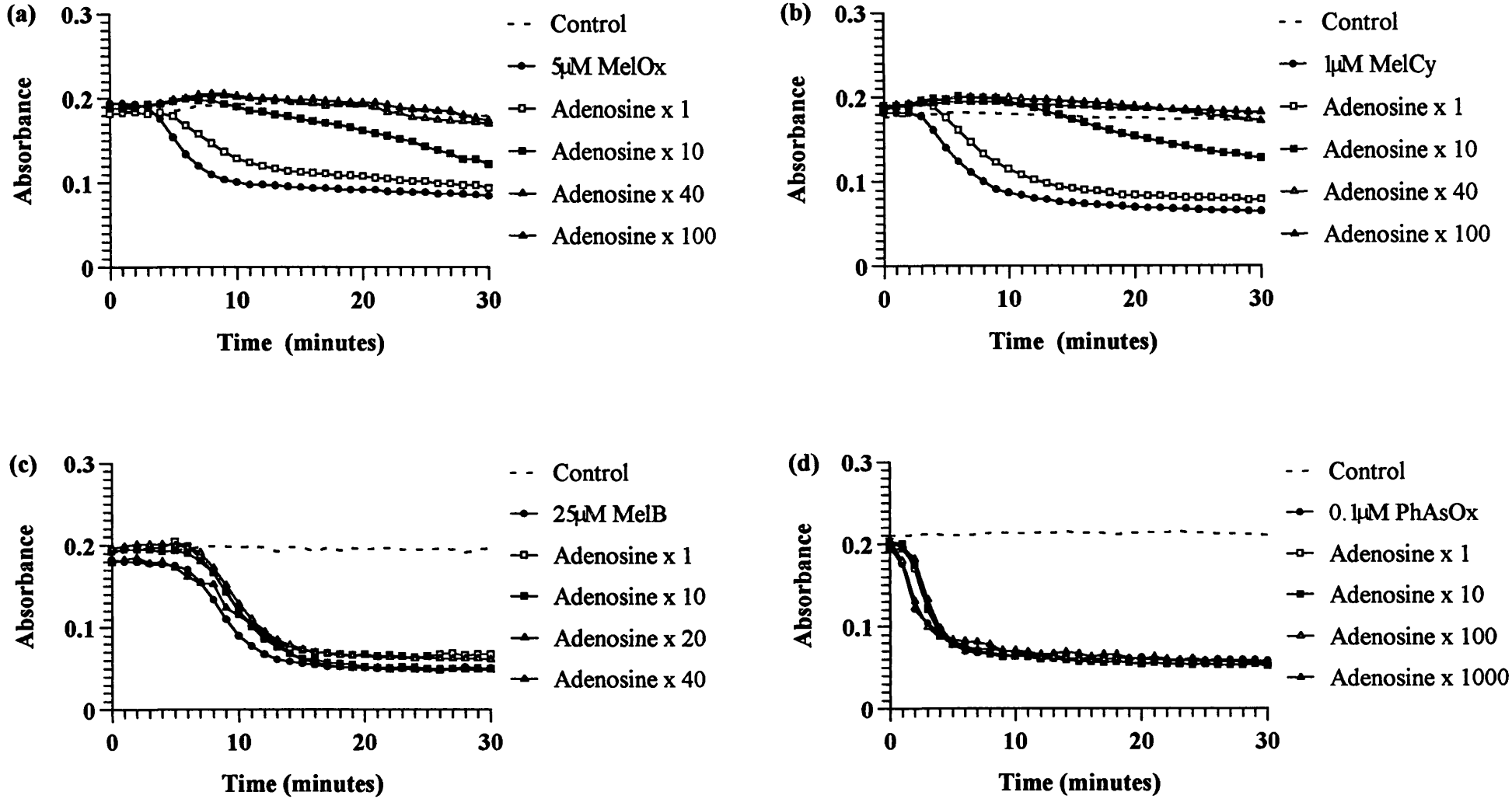
(a)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
5μM MelOx
Adenosine x 1
Adenosine x 10
Adenosine x 40
Adenosine x 100
(b)
Absorbance
Time (minutes)
Control
1μM MelCy
Adenosine x 1
Adenosine x 10
Adenosine x 40
Adenosine x 100
(c)
Absorbance
Time (minutes)
Control
25μM MelB
Adenosine x 1
Adenosine x 10
Adenosine x 20
Adenosine x 40
(d)
Absorbance
Time (minutes)
Control
0.1μM PhAsOx
Adenosine x 1
Adenosine x 10
Adenosine x 100
Adenosine x 1000

Figure 6.2
The Effect of Adenosine on the Arsenical-Induced Lysis of 247
(a) with 5μM MelOx
(b) with 1μM MelCy
(c) with 25μM MelB
(d) with 0.1μM Phenyl Arsenoxide

The effects of increasing concentrations of adenosine on the arsenical-induced lysis of 247 are shown. The concentrations of adenosine are indicated in terms of the relative concentrations compared to the arsenical (i.e. adenosine x 1 means equimolar concentrations of both arsenical and adenosine).

Facing 188

Figure 6.3

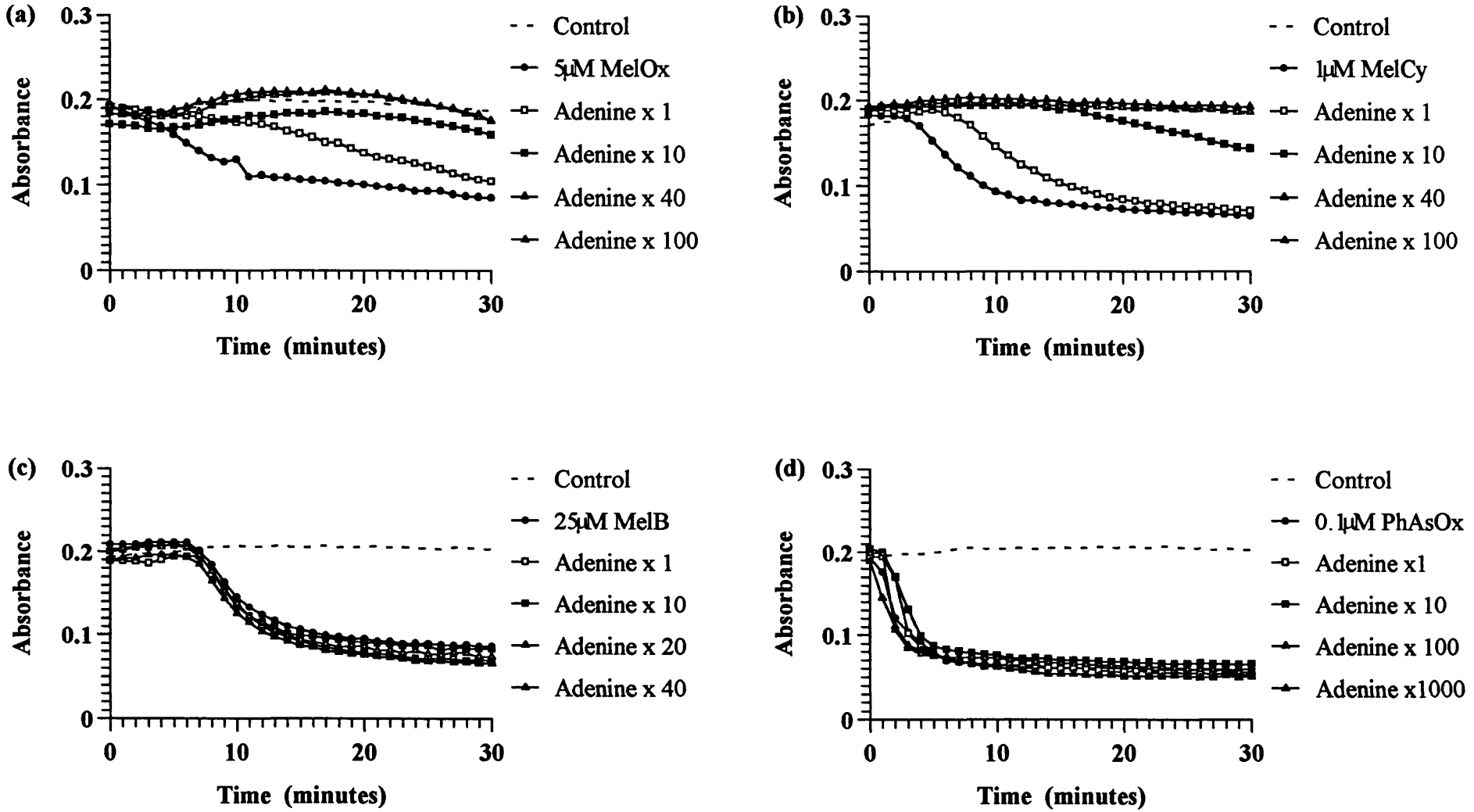

Figure 6.3
The Effect of Adenine on the Arsenical-Induced Lysis of 247
(a) with 5μM MelOx
(b) with 1μM MelCy
(c) with 25μM MelB
(d) with 0.1μM Phenyl Arsenoxide

The effects of increasing concentrations of adenine on the arsenical-induced lysis of 247 are shown. See legend for Figure 6.2 for explanation.

Figure 6.4

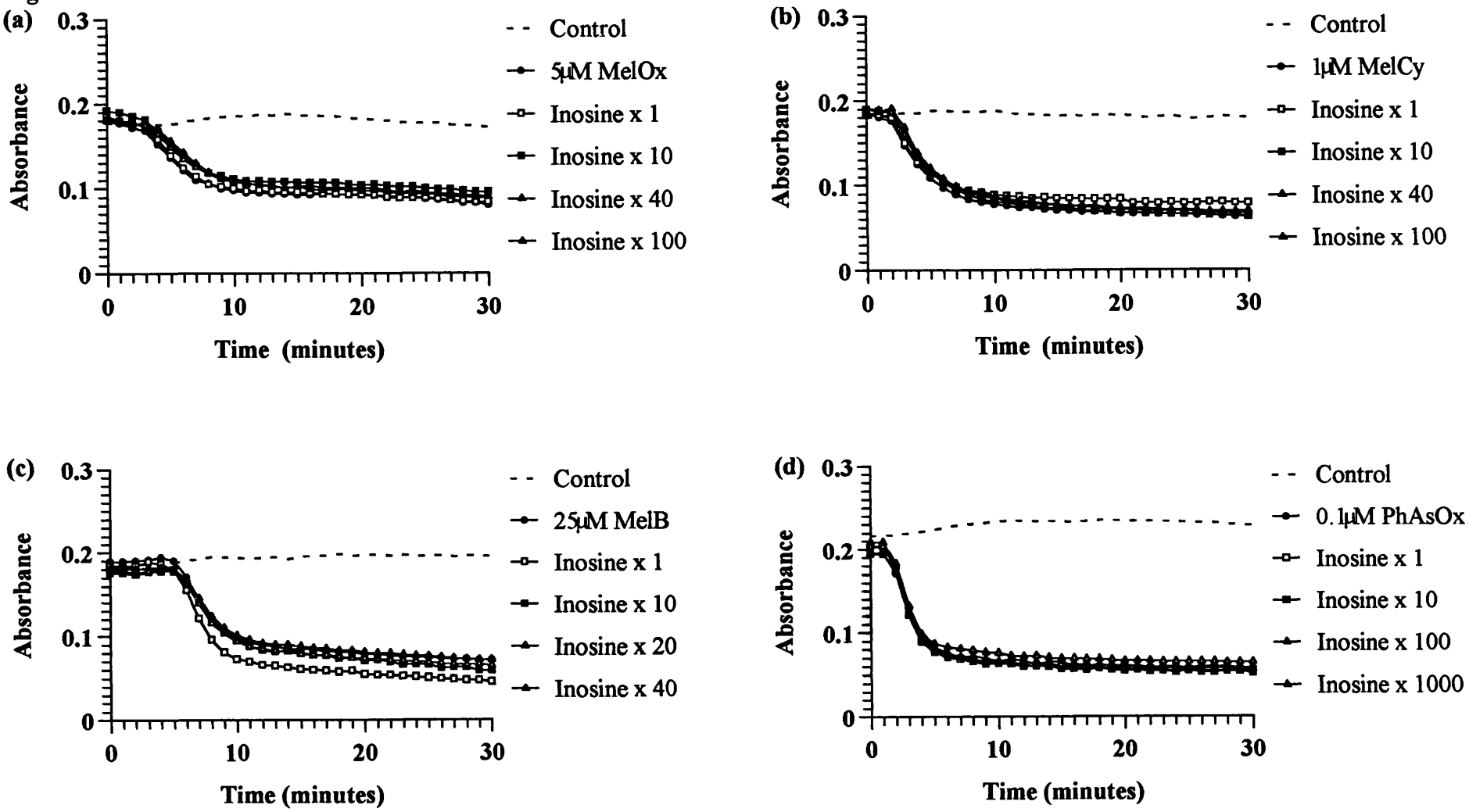
(a)
Absorbance
Time (minutes)
Control
5μM MelOx
Inosine x 1
Inosine x 10
Inosine x 40
Inosine x 100
(b)
Absorbance
Time (minutes)
Control
1μM MelCy
Inosine x 1
Inosine x 10
Inosine x 40
Inosine x 100
(c)
Absorbance
Time (minutes)
Control
25μM MelB
Inosine x 1
Inosine x 10
Inosine x 20
Inosine x 40
(d)
Absorbance
Time (minutes)
Control
0.1μM PhAsOx
Inosine x 1
Inosine x 10
Inosine x 100
Inosine x 1000

Figure 6.4
The Effect of Inosine on the Arsenical-Induced Lysis of 247
(a) with 5μM MelOx
(b) with 1μM MelCy
(c) with 25μM MelB
(d) with 0.1μM Phenyl Arsenoxide

The effects of increasing concentrations of inosine on the arsenical-induced lysis of 247 are shown. See legend for Figure 6.2 for explanation.

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Figure 6.5

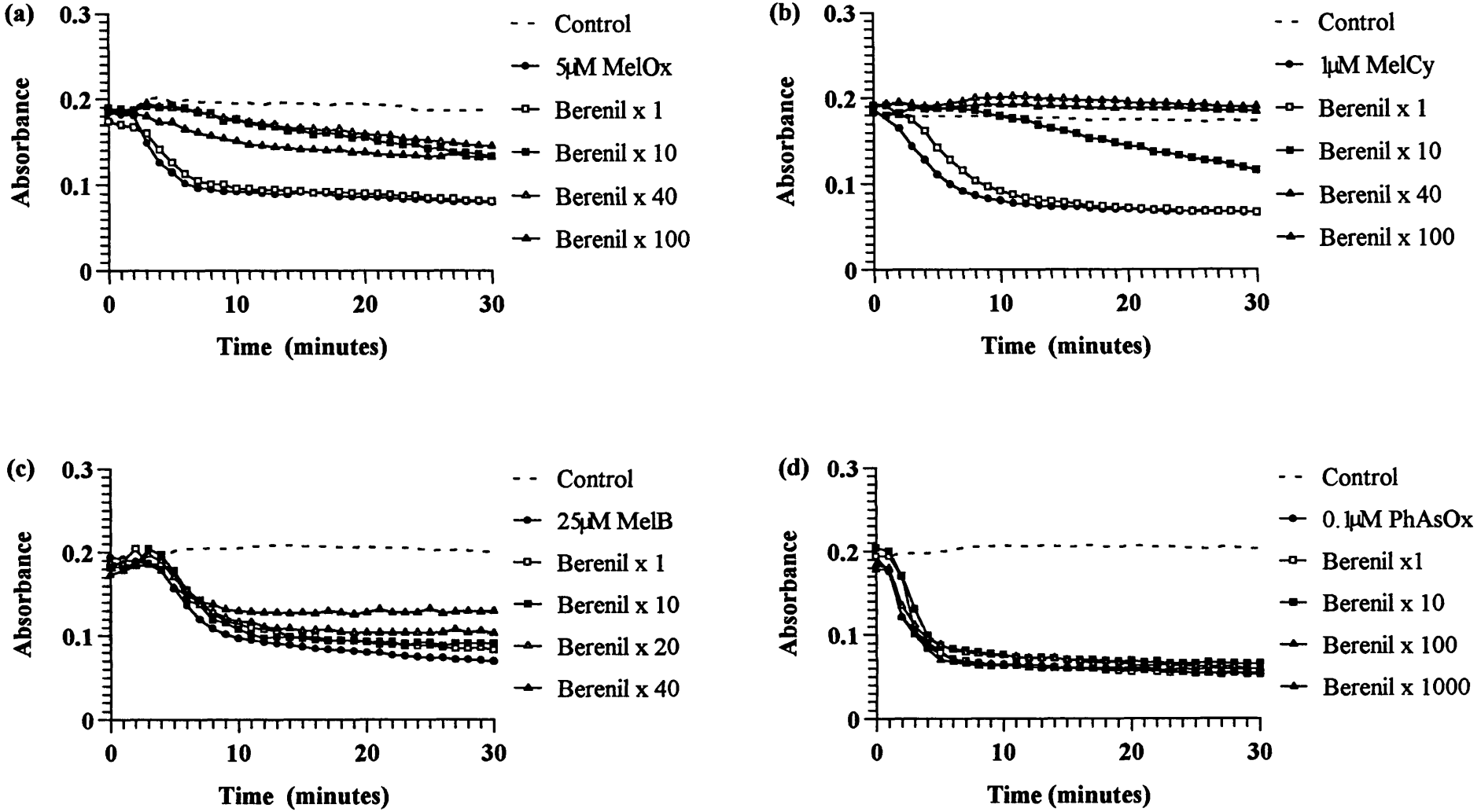


Figure 6.5
The Effect of Berenil on the Arsenical-Induced Lysis of 247
(a) with 5μM MelOx
(b) with 1μM MelCy
(c) with 25μM MelB
(d) with 0.1μM Phenyl Arsenoxide

The effects of increasing concentrations of berenil on the arsenical-induced lysis of 247 are shown. See legend for Figure 6.2 for explanation.

Figure 6.6

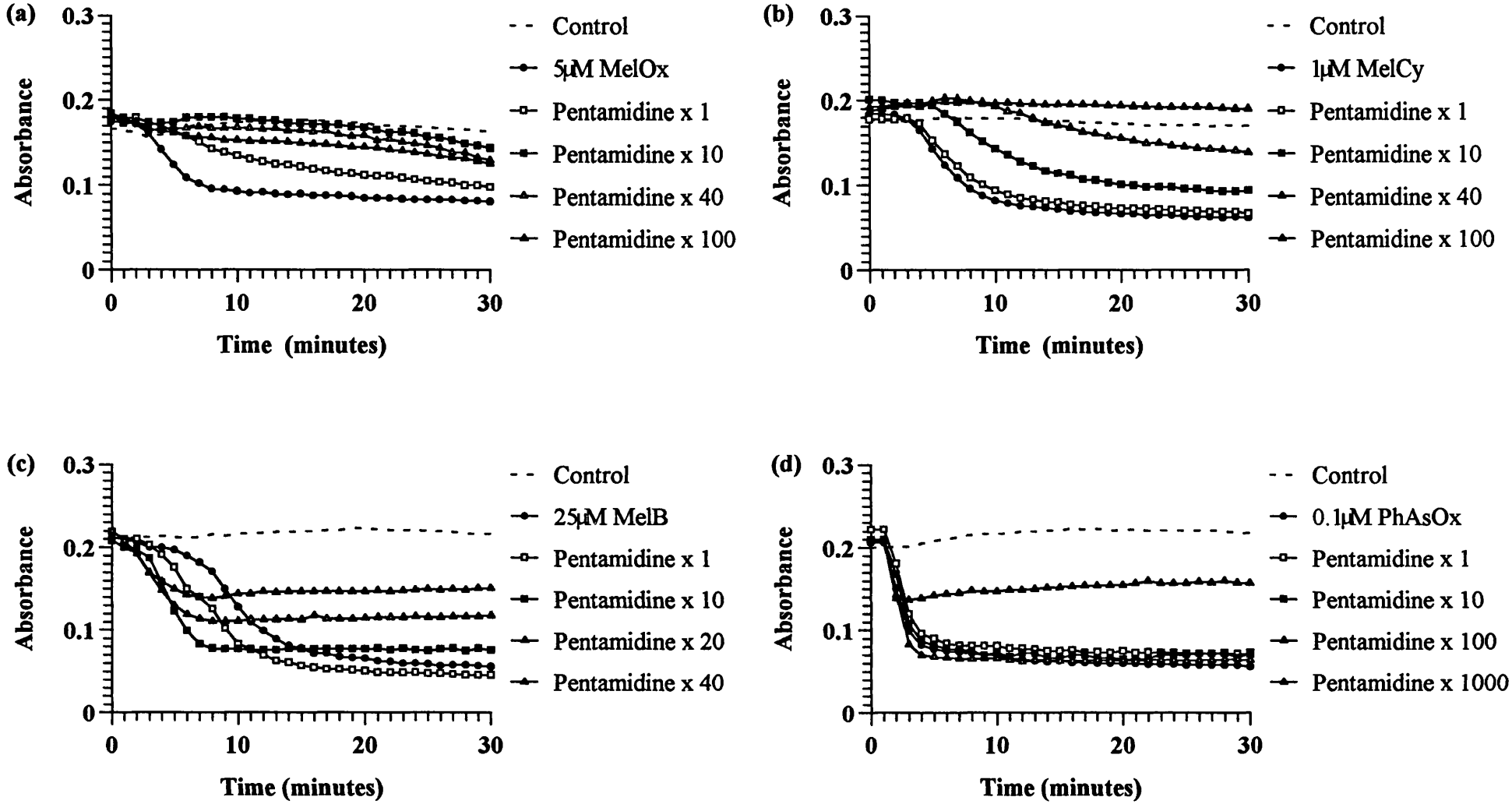

(a)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
5μM MelOx
Pentamidine x 1
Pentamidine x 10
Pentamidine x 40
Pentamidine x 100
(b)
Absorbance
Time (minutes)
Control
1μM MelCy
Pentamidine x 1
Pentamidine x 10
Pentamidine x 40
Pentamidine x 100
(c)
Absorbance
Time (minutes)
Control
25μM MelB
Pentamidine x 1
Pentamidine x 10
Pentamidine x 20
Pentamidine x 40
(d)
Absorbance
Time (minutes)
Control
0.1μM PhAsOx
Pentamidine x 1
Pentamidine x 10
Pentamidine x 100
Pentamidine x 1000

Figure 6.6
The Effect of Pentamidine on the Arsenical-Induced Lysis of 247
(a) with 5μM MelOx
(b) with 1μM MelCy
(c) with 25μM MelB
(d) with 0.1μM Phenyl Arsenoxide

The effects of increasing concentrations of pentamidine on the arsenical-induced lysis of 247 are shown. See legend for Figure 6.2 for explanation.

Figure 6.7

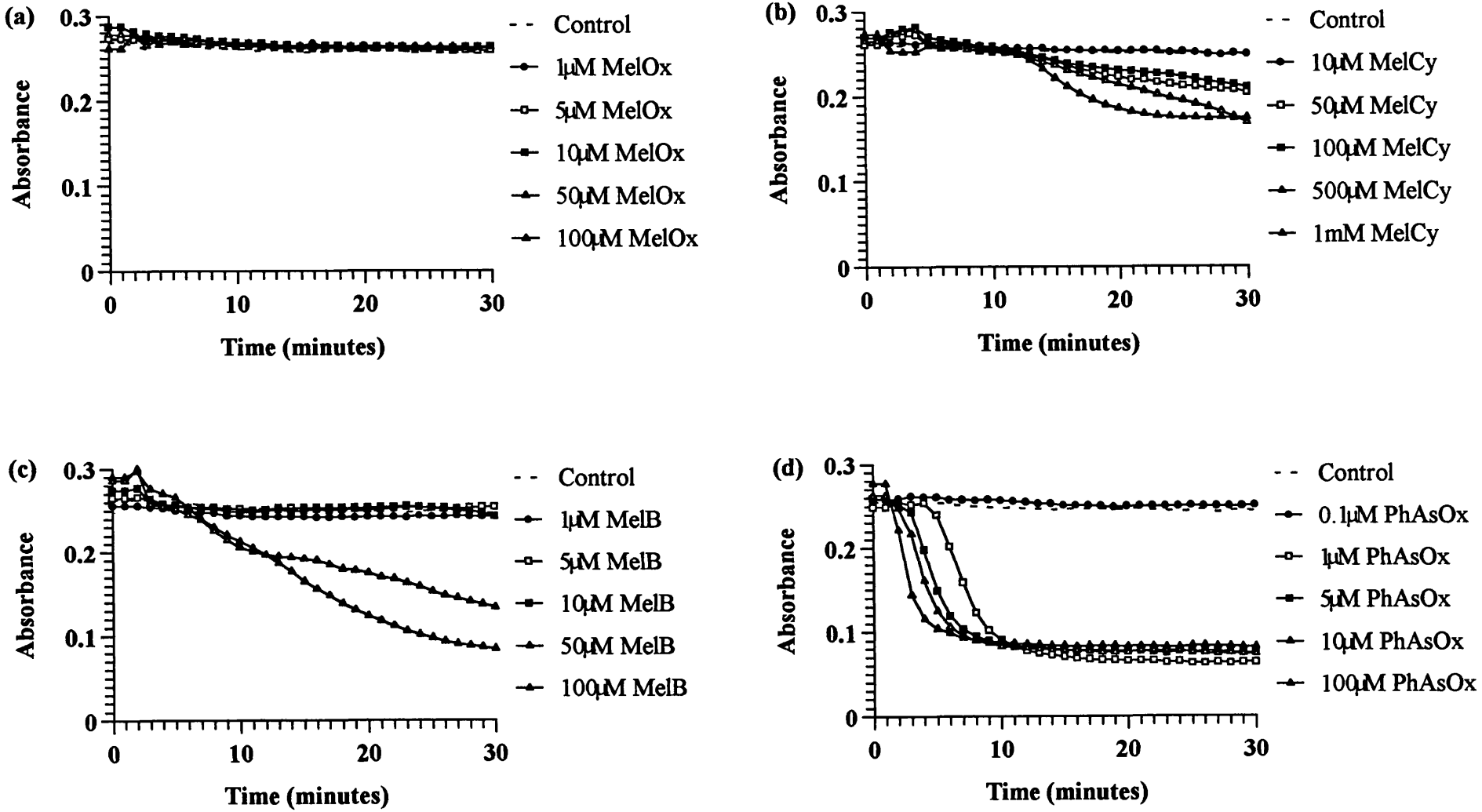
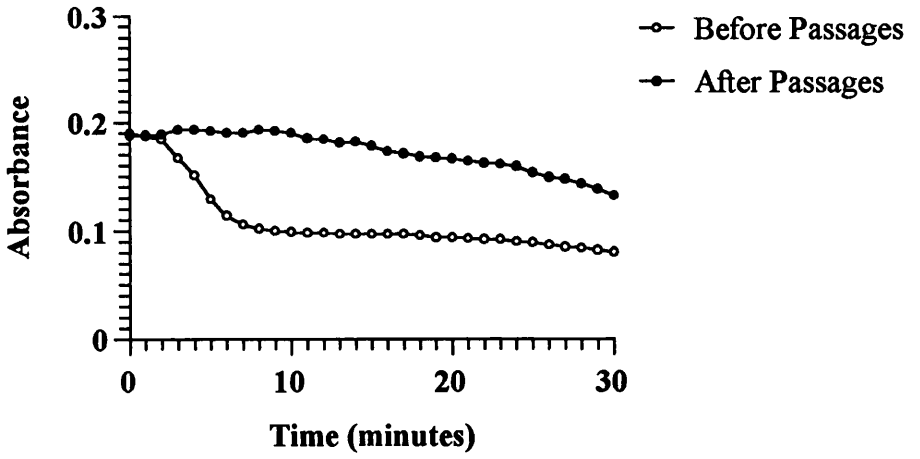

(a)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
1μM MelOx
5μM MelOx
10μM MelOx
50μM MelOx
100μM MelOx
(b)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
10μM MelCy
50μM MelCy
100μM MelCy
500μM MelCy
1mM MelCy
(c)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
1μM MelB
5μM MelB
10μM MelB
50μM MelB
100μM MelB
(d)
Absorbance
0.3
0.2
0.1
0
0
10
20
30
Time (minutes)
Control
0.1μM PhAsOx
1μM PhAsOx
5μM PhAsOx
10μM PhAsOx
100μM PhAsOx

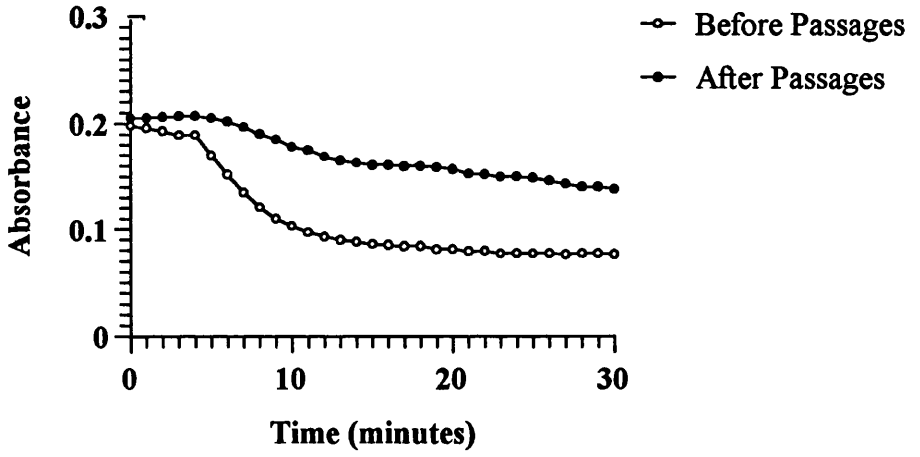
Figure 6.7
Arsenical-Induced Lysis in 247MelCy^R
(a) with MelOx
(b) with MelCy
(c) with MelB
(d) with Phenyl Arsenoxide

The effects of increasing concentrations of arsenical on the lysis of 247MelCy^R are shown. The highest concentrations of each arsenical used were close to the limit in terms of their solubility in the buffer used (CBSS).

Figure 6.8
Arsenical-Induced Lysis of 247 before and after Passages
(a) with 5μM MelOx



(b) with 1μM MelCy



The effect of syringe-passaging on the susceptibility of 247 to arsenical-induced lysis is shown with (a) 5μM melOx and (b) 1μM melCy. The line after passages was syringe passaged for at least 2 months, involving 15 or more passages through rats. The passaged line was clearly less susceptible than the unpassaged line to arsenical-induced lysis.

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Figure 6.9

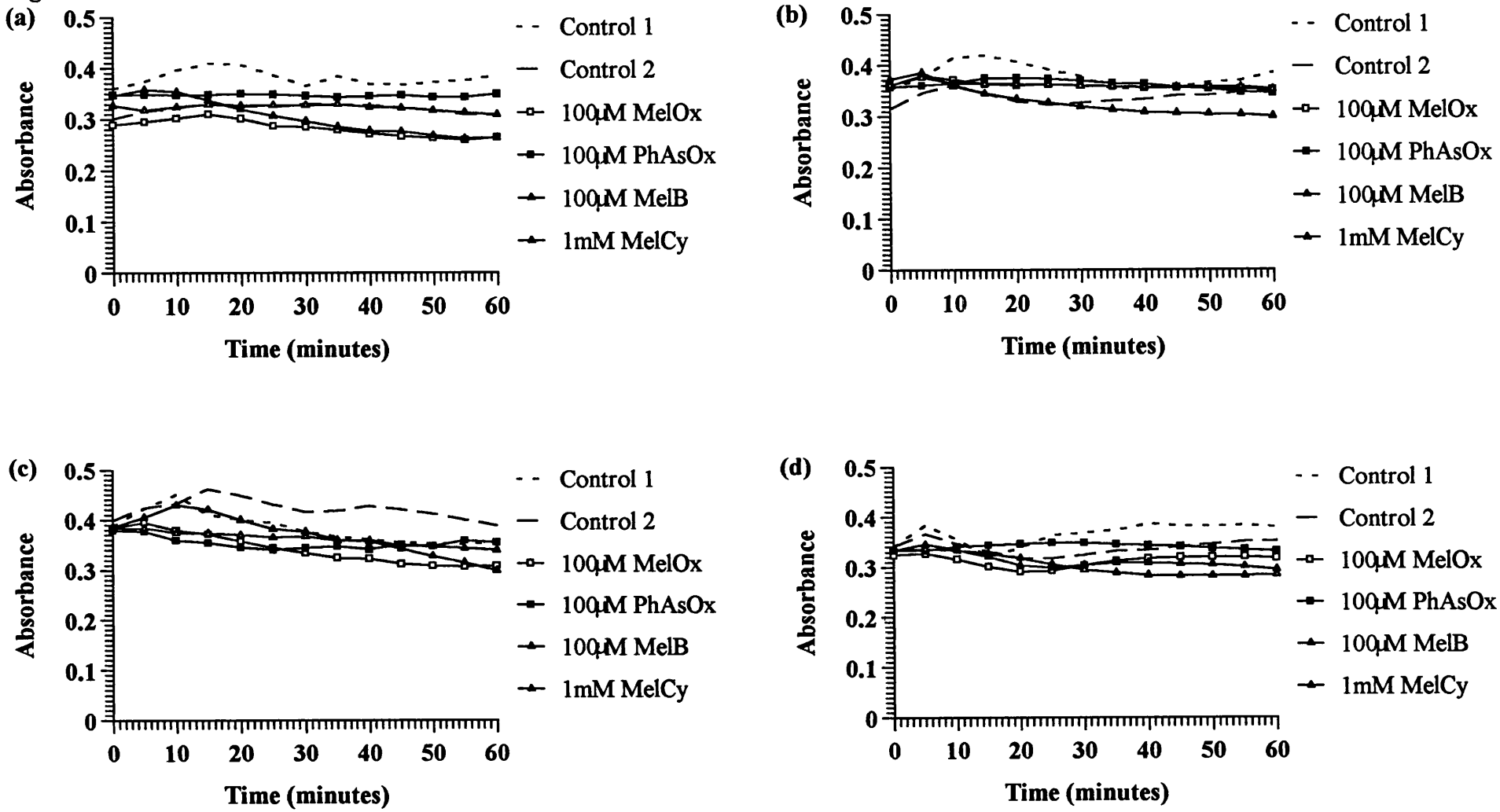
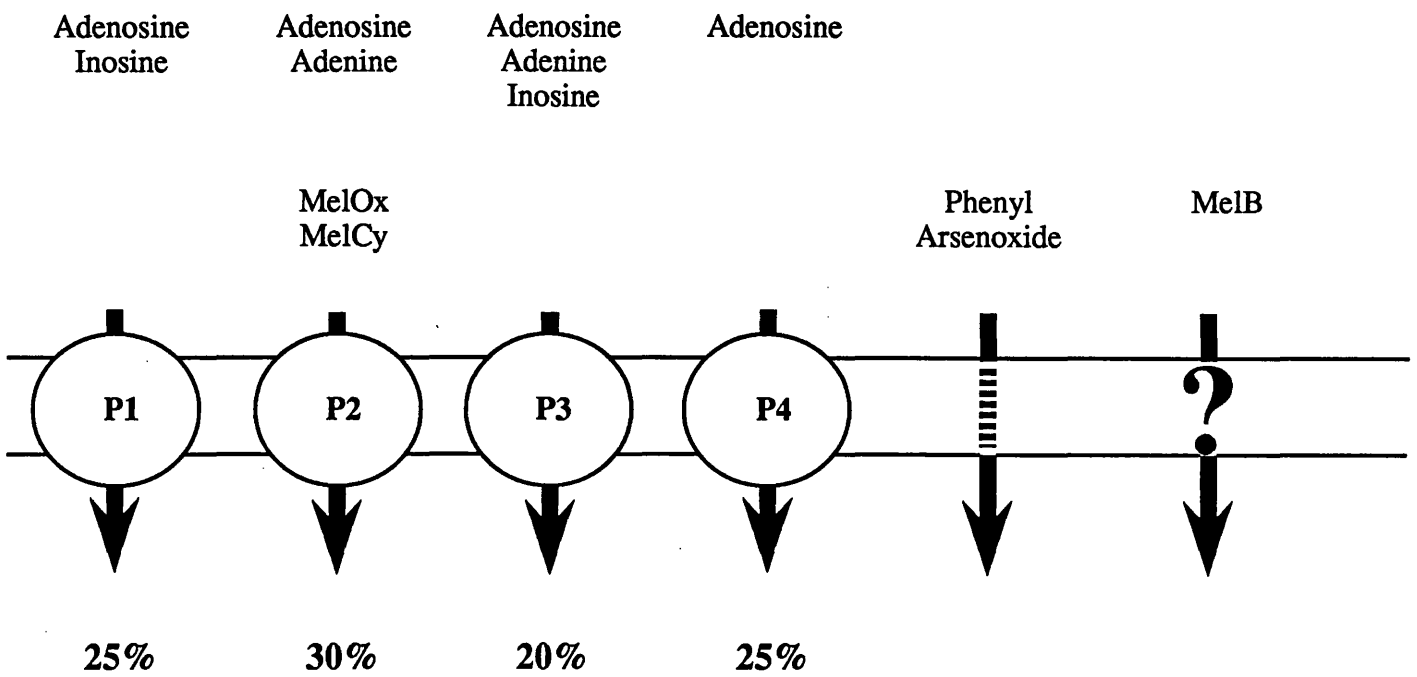


Figure 6.9
The Effect of Arsenicals on Procyclic Culture Forms of 247
(a) in CBSS at 26^0C
(b) in CBSS at 37^0C
(c) in SDM-79 w/o Adenosine at 26^0C
(d) in SDM-79 w/o Adenosine at 37^0C

The effects of arsenicals on procyclic culture forms of 247 are shown. Lysis experiments were conducted in either CBSS or SDM-79 (without adenosine) at either26^0C or 37^0C. The highest possible concentrations of arsenical were used and the absorbance at 750nm was measured for 1 hour.

Figure 6.10
Proposed Uptake of Melaminophenyl Arsenicals in Relation to Adenosine Transport



One possible explanation for the results of the lysis experiments and uptake studies: melOx and melCy, are recognised by the P2 adenosine transporter, which is also responsible for adenine uptake. Phenyl arsenoxide rapidly diffuses into the cell, in agreement with the previous study (Carter and Fairlamb 1993). However, melB appears to be taken up via a non-adenosine transporter (or diffusion).

Table 6.1
Cross-Resistance between MelCy and the Diamidines (Berenil and Pentamidine)

Drug	Minimum Curative Dose (mg/Kg)		Resistance Factor
	247	247MelCy^R	
Berenil	2[a]	32[b]	16
Pentamidine	1.6[c]	>100[d]	>63

The results of the *in vivo* assay for berenil and pentamidine are expressed in terms of the minimum curative dose required to cure all six mice in a group. The resistance factor is a ratio of the minimum curative dose for 247MelCy^R to the minimum curative dose for 247.

[a] Doses tested were 0.5,1,2,4 and 8mg/Kg
[b] Doses tested were 2,4,8,16 and 32mg/Kg
[c] Doses tested were 0.2,0.4,0.8,1.6,3.2 and 6.4mg/Kg
[d] Dose tested was 100mg/Kg

Table 6.2
Rate of Adenosine Uptake in 247 and 247MelCy^R

Trypanosome Line	Rate of Adenosine Uptake (pmol/10^8cells/sec)
247	48
247MelCy^R	5.0

The rate of adenosine uptake between 2 and 7 seconds was measured using [^{3}H]-adenosine. The labelled adenosine was mixed with unlabelled adenosine to give a final adenosine solution with a specific activity of 1.12μCi/ml, and a total concentration of adenosine of 2μM.

Table 6.3
Concentration-Dependant Inhibition of Adenosine Uptake by Adenine with or without 500μM Inosine in 247

[Adenine] (μM)	Inhibition of Adenosine Uptake (%)					
	No Inosine			With Inosine (500μM)		
	Expt1	Expt 2	Mean	Expt 1	Expt 2	Mean
0.1	11.2	11.7	11.5	13.5	72.9	43.2
1.0	33.3	ND	33.3	78.7	42.6	60.7
10.0	60.2	43.2	51.7	76.4	73.8	75.1
50.0	ND	57.3	57.3	62.2	66.9	64.6
250.0	40.8	51.6	46.2	71.4	65.9	68.7

Percentage inhibition of adenosine uptake without inosine is shown in the first three columns, with increasing concentrations of adenine. The putative P1 adenosine transporter is blocked by 500μM inosine, last three columns. The percentage inhibition data from duplicate experiments is shown together with the mean values. Percentage inhibition was calculated by comparison to control samples containing neither adenine nor inosine.

Table 6.4
Concentration-Dependant Inhibition of Adenosine Uptake by Inosine with or without 250μM Adenine in 247

[Inosine] (μM)	Inhibition of Adenosine Uptake (%)					
	No Adenine			With Adenine (250μM)		
	Expt1	Expt 2	Mean	Expt 1	Expt 2	Mean
0.1	40.8	16.0	28.4	ND	51.0	51.0
1.0	8.2	17.8	13.0	34.4	30.6	32.5
10.0	58.5	30.3	44.4	60.1	58.8	59.5
100.0	39.7	45.5	42.6	80.5	69.1	74.8
500.0	52.9	36.4	44.7	72.8	76.3	74.6

Percentage inhibition of adenosine uptake without adenine is shown in the first three columns, with increasing concentrations of inosine. The putative P2 adenosine transporter is blocked by 250μM adenine, last three columns. See legend for Table 6.3.

Table 6.5
Concentration-Dependant Inhibition of Adenosine Uptake by Adenine with or without 500μM Inosine in 247MelCy^R

[Adenine] (μM)	Inhibition of Adenosine Uptake (%)	
	No Inosine	With Inosine (500μM)
0.1	1.7	22.2
1.0	29.0	ND
10.0	28.9	77.8
50.0	35.5	75.1
250.0	34.5	37.7

Percentage inhibition of adenosine uptake without inosine and with 500μM inosine is shown with increasing concentrations of adenine. Duplicate experiments were not conducted with 247MelCy^R. See legend for Table 6.3.

CHAPTER 7
GENERAL DISCUSSION

The ultimate aims of this study were to investigate the genetic and biochemical mechanisms controlling drug-resistance in *T. brucei*. This involved the generation and characterisation of stable cloned lines of trypanosomes *in vivo*, that expressed resistance to melCy and suramin. A suitable *in vitro* assay was developed in order to rapidly measure the levels of drug-resistance in a number of clones generated from a mixed transmission of melCy-resistant and suramin-resistant lines. Biochemical investigations were also conducted into the role of adenosine transporters in the uptake of the melaminophenyl arsenicals, indicating differences between melCy uptake and melB uptake.

This study has demonstrated the use of stable drug-resistant lines of *Trypanosoma brucei* in investigations into the biochemical and genetic basis of drug-resistance and their use as selectable markers in genetic exchange experiments. The successful use of selectable markers in genetic exchange studies has been demonstrated with antibiotic-resistance in two cloned lines of *T. brucei* (Gibson and Whittington 1993). However, the use of genuine trypanocide-resistance rather than antibiotic-resistance has the added advantage of allowing the simultaneous investigation into the inheritance of drug-resistance, and thereby providing information on the number of genes and the nature of the alleles controlling drug-resistance. It should also be possible to use this approach to study the genetic basis of other trypanocides, such as DFMO. However, this process of generating drug-resistant lines of trypanosomes *in vivo*, not only selected for the desired drug-resistance phenotype, but also selected for a monomorphic phenotype (Turner 1990). Consequently, the relatively low proportion of short-stumpy forms in bloodstream infections meant that fly-transmission of the drug-resistant lines was more difficult compared to the

unselected stocks. It was found that a greater number of mature salivary gland infections were produced with the use of an infective feed containing bloodstream forms that were predominantly short-stumpy in morphology, rather than using bloodstream forms from mice with a rising parasitaemia. Another important point to be made concerns the screening of metacyclic populations from infected flies. Screening the salivary gland infections from flies after approximately three weeks post infection provided a more complete view the course of infection in the tsetse flies. However, the process of infecting mice directly from the tsetse flies and screening the salivary gland infections of each fly from day 18 post infection, two or three times each week was relatively expensive and time-consuming. Since the ultimate aim is to generate a whole series of cloned progeny resulting from genetic exchange, it may be more efficient to screen infected flies for mature infections only after approximately 40 days post infection. Although the data in the present study does not support this, the probability of encountering products of genetic exchange is likely to be greatest during the latter part of infection when a mixed salivary gland infection has been established. In this way, the relatively time-wasting and expensive procedure of screening flies during the early stages of infection can be by-passed. Furthermore, since double drug-resistant trypanosomes appeared to bring about only transient, low levels of parasitaemia in double drug-treated mice, it may also prove fruitful to generate metacyclic clones from flies producing mixed infections, on the assumption that flies producing mixed infections after six weeks are likely to also contain the products of genetic exchange that may not be identified in the mouse screen. Uncloned metacyclic populations from flies infected in co-transmission experiments were screened in mice with drug doses of 5mg/Kg. However, the possibility that such levels of drug might generate new mutants rather than select for the pre-existing mutants cannot be overlooked. The dose of 5mg/Kg for melCy and suramin was initially chosen for convenience as it was at least double the amount of drug required to clear drug-sensitive

infections, but was also expected to permit the selection of partially drug-resistant trypanosomes. In hindsight, the doses of drug used should have been calculated in relation to the minimum doses required to clear infections of the drug-resistant lines for the drugs to which these lines were sensitive. Furthermore, a 5-fold greater dose of drug compared to these minimum curative doses (**chapter 2**) would have been more beneficial in the screening of metacyclic populations in fly infected mice. Table 7.1 shows the selection doses that would have been used in further crosses if time had permitted. These changes should remove any remaining doubt from the results of the mouse screen, and mean that only double drug-resistant trypanosomes arising from genetic exchange would give rise to patent infections in double drug treated mice. Furthermore, the doses recommended in Table 7.1 remain sufficiently low to allow double drug-resistant trypanosomes that express low levels of drug-resistance still to survive drug treatment.

The short-term bloodstream culture described in chapter 3 provides a suitable system for the rapid screening of a number of cloned progeny. It was rapid both in terms of the time taken to set up a bloodstream culture, and in terms of the speed with which a result was obtained (24 hours). However, although the repeatability of the assays appeared to be satisfactory for both melCy and suramin, the fact that it was an *in vitro* assessment of drug-resistance remained a drawback. Table 7.2 compares the three different assay systems described in chapters 2 and 3. The most important drawbacks of using the *in vitro* assays are that growth inhibition may not be a true reflection of the trypanocidal nature of either melCy or suramin, and also that a 24 hour incubation in the presence of drug in no way reflects the complex pharmacokinetics that are involved when the drugs are used *in vivo*. These factors are likely to influence the final result of the *in vitro* assays. For this reason, future studies should continue to employ the *in vitro* assay to rapidly

assess the levels of drug-resistance in a series of cloned progeny, but also that clones, that have been identified as hybrids or selfers from other known markers of genetic exchange, should subsequently be analysed *in vivo*. Although, the *in vivo* assay is time consuming and expensive, it would not be necessary to conduct such assays on the scale of those described in chapter 2 for each cloned progeny. Rather, a relatively accurate measurement could be made using only six mice, each receiving 10^5 trypanosomes, followed by a range of drug treatments at, for example, 1.25, 2.5, 5, 10, 20 and 40mg/Kg for melCy, and at 2.5, 5, 10, 20, 40 and 70mg/Kg for suramin. This use of an *in vivo* assay may be particularly important for the measurement of suramin-resistance, as the four clones (466/61M^RS^R bcl1-4) selected at 24mg/Kg in mice expressed relatively low levels *in vitro* (**chapter 5**).

The cloned trypanosome lines expressing stable resistance to melCy and suramin offer a number of alternatives for future co-transmission experiments. Further attempts at generating double drug-resistant cloned progeny could be attempted with 247Sur^R x 386$MelCy^R$. Alternatively, the relative success of co-transmitting the two 247-derived lines together with a different third line could be further improved by the three-way co-transmission of 247$MelCy^R$ x 247Sur^R x 927/4, since the latter line transmits through flies more successfully than 386 (Turner pers. comm.). Another drug-resistant line that is available for co-transmission experiments is 247$Phleo^R$ (resistant to the antibiotic, phleomycin (Jeffries unpublished results)). This line has been generated in procyclic culture and successfully transmitted through tsetse flies, after which, the resulting bloodstream infections can be distinguished from unselected lines by treating infected mice with daily injections (i.p.) of phleomycin at a dose of 3mg/Kg (results not shown). This third drug-resistant 247-derived line could be used as well, or instead of, 247$MelCy^R$ and 247Sur^R. An ambitious co-transmission could be envisaged with 247$MelCy^R$ x 247Sur^R x 247$Phleo^R$ x 927/4, in which

the three possible different double drug-resistant phenotypes ($MelCy^RSur^R$, $MelCy^RPhleo^R$ and Sur^RPhleo^R) resulting from selfing could all easily be screened for directly in mice. These possible co-transmission experiments described could be conducted with the material already available. On the other hand, if a new drug-resistant line were to be generated in mice, it would be beneficial to select a 927/4 line for resistance to melB. This would allow the possibility for further crosses, such as $247Sur^R$ x $927/4MelB^R$ and $247Phleo^R$ x $247Sur^R$ x $927/4MelB^R$, with the latter three-way co-transmission being particularly appealing.

A $927/4MelB^R$ line could also be used to investigate further the role of adenosine transport in the uptake of melaminophenyl arsenicals. Previous studies on the mechanism of melB uptake have employed melOx, showing that melOx uptake was reduced in arsenical-resistant lines (Yarlett *et al.* 1991; Carter and Fairlamb 1993). However, the widely held assumption that melOx and melB have the same mode of action and the same mechanism of uptake has been questioned in the present study. The mode of action and uptake of melB, and the mechanism of melB-resistance must be investigated using melB and not melOx or melCy, and the use of a well characterised melB-resistant line is essential for future studies on arsenical-resistance, allowing a number of anomalies to be resolved. The use of a melCy-resistant line in the present study, has shown that conclusions drawn from studies that have used melOx-resistant lines previously (Yarlett *et al.* 1991; Carter and Fairlamb 1993) cannot necessarily be applied to melB. In terms of actual experimental details, the inability of adenosine and adenine to protect trypanosomes from melB-induced lysis should be investigated. The conjecture that melB is not taken up by an adenosine transporter, and has a different route of uptake from melCy and melOx, leads to the testable prediction that unselected 927/4 should be rapidly lysed by melB, but that this lysis should not be inhibited by adenosine or adenine. If it were found that adenosine and

adenine did not protect 927/4 from melB-induced lysis, then the role of an altered adenosine transporter in melB-resistance would certainly appear unlikely. The work of Carter and Fairlamb (1993) was initiated by screening numerous compounds (such as amino acids, sugars and nucleotides) for their effect on melOx-induced lysis of S427. A similar approach could be used to investigate melB-induced lysis and it may be far more profitable to search for compounds that block melB uptake rather than spend time characterising the kinetics of an adenosine transport system that may have no relevance to melB uptake. Furthermore, a melB-resistant line, such as 927/4MelB^R, should show a large decrease in susceptibility to melB-induced lysis, unlike 247MelCy^R, and also, most significantly, a reduction in the rate of adenosine uptake should not be observed in the melB-resistant line.

In conclusion, the foundations have been laid for future studies into the genetics and biochemistry of drug-resistance, with particular emphasis on the inheritance of arsenical-resistance and the mechanism of uptake of the melaminophenyl arsenicals. The question marks over the route of melB uptake and the possible differences between melB and melCy transport demand further investigations. Although further studies may show that melCy, melOx and melB do indeed share a common adenosine transport mechanism and that the results presented in this study cannot be applied to other lines, investigations are warranted with a view to the possibility that melCy might be of use in the treatment of melB-resistant cases of African sleeping sickness.

Table 7.1
Recommended Selection Doses for Co-Transmission Experiments

Genetic Cross	Selection Doses	
	MelCy (mg/Kg)	Suramin (mg/Kg)
247MelCy^R x 247Sur^R	1.5	7.5
247MelCy^R x 386Sur^R	15	7.5
386MelCy^R x 247Sur^R	1.5	12.5
386MelCy^R x 386Sur^R	15	12.5

The drug doses presented in this table are based on a 5-fold greater dose of drug compared to the minimum curative doses for the different drug-resistant lines **(chapter 2)**.

Table 7.2
Comparison of Assay Systems to Measure Drug-Resistance in Cloned Progeny

Assay System	Advantages	Disadvantages
***In Vivo* Assay** **In Mice**	Pharmacology of drugs similar to clinical cases MelCy-resistance expressed Suramin-resistance expressed Trypanocidal activity of drug measured with cell death	Limited to maximum tolerated doses Expensive Final result takes up to 2 weeks
***In Vitro* Assay** **In Procyclic Culture**	Established culture easy to maintain MelCy-resistance expressed	Transformation takes up to 1 week Growth inhibition is not a true reflection of trypanocidal activity Metabolic and biological differences between procyclic and bloodstream forms Suramin-resistance not expressed Drugs not acting in true environment
***In Vitro* Assay** **In Bloodstream Culture**	Drugs act on bloodstream forms in clinical cases MelCy-resistance expressed Suramin-resistance expressed	Growth inhibition is not a true reflection of trypanocidal activity Drugs are not acting in true environment Simplified medium cannot reflect body fluids

This table critically compares the three different assay systems that were used to screen for drug-resistance. The *in vivo* assay was described in chapter 2, and the two *in vitro* assays were described in chapter 3.

REFERENCES

Allolio, B., Reincke, M., Arlt, W., Deuss, U. & Winkelmann, W. (1989) Suramin for the treatment of adrenocortical carcinoma *Lancet* **2**, 277

Apted, F.I.C. (1970) Treatment of human trypanosomiasis In: *The African Trypanosomiases* Mulligan, H.W. (Editor) London: George Allen & Unwin, 684-710

Bacchi, C.J., Nathan, H.C., Hutner, S.H., McCann, P.P. & Sjoerdsma, A. (1980) Polyamine metabolism: a potential therapeutic target in trypanosomes *Science* **210**, 332-334

Bacchi, C.J., Garofalo, J., Mockenhaupt, D., McCann, P.P., Diekema, K.A., Pegg, A.E., Nathan, H.C., Mullaney, E.A., Cunosoff, L., Sjoerdsma, A. & Hutner, S.H., (1983) *In vivo* effects of α-DL-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei Molecular and Biochemical Parasitology* **7**, 209-225

Bacchi, C.J. (1993) Resistance to clinical drugs in African trypanosomes *Parasitology Today* **9**, 190-193

Baer, H.P., Serignese, V., Ogbunude, P.O.J. & Dzimiri, M. (1992) Nucleoside transporters in *Leishmania major* - Diversity in adenosine transporter expression or function in different strains *American Journal of Tropical Medicine and Hygiene* **47**, 87-91

Baltz, T., Baltz, D., Giroud, Ch. & Crockett, J. (1985) Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense The EMBO Journal* **4**, 1273-1277

Barry, J.D. & Emery, D.L. (1984) Parasite development and host responses during the establishment of *Trypanosoma brucei* infection transmitted by tsetse fly *Parasitology* **88**, 67.84

Bellofatto, V., Fairlamb, A.H., Henderson, G.B. & Cross, G.A.M. (1987) Biochemical changes associated with α-difluoromethylornithine uptake and resistance in *Trypanosoma brucei* *Molecular and Biochemical Parasitology* **25**, 227-238

Berger, B.J, Carter, N.S. & Fairlamb, A.H. (1993) Polyamine and pentamidine metabolism in African trypanosomes *Acta Tropica* **54**, 215-224

Berger, B.J. & Fairlamb, A.H. (1994) Properties of melarsamine hydrochloride (cymelarsan) in aqueous solution *Antimicrobial Agents and Chemotherapy* **38**, 1298-1302

Bitonti, A.J., Dumont, J.A. & McCann, P.P. (1986a) Characterisation of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by berenil, pentamidine and metylglyoxal bis(guanylhydrazone) *Biochemical Journal* **237**, 685-689

Bitonti, A.J., McCann, P.P., Sjoerdsma, A. (1986b) Necessity of antibody response in the treatment of African trypanosomes with α-difluoromethylornithine *Biochemical Pharmacology* **35(2)**, 331-334

Boid, R., Jones, T.W. & Payne, R.C. (1989) Malic enzyme type VII isoenzyme as an indicator of suramin resistance in *Trypanosoma evansi* *Experimental Parasitology* **69**, 317-323

Boreham, P.F.L. (1985) Autocoids: their release and possible role in the pathogenesis of African trypanosomiasis In: *Immunology and Pathogenesis of Trypanosomiasis* Tizard, I. (Editor) Boca Raton: CRC Press, 45-66

Bowman, I.B.R. & Flynn, I.W. (1976) Oxidative metabolism of trypanosomes In: *Biology of the Kinetoplastida* Vol I Lumsden, W.H.R. & Evans, D.A. (Editor) London: Academic Press, 435-476

Brun, R. & Schonenberger, M. (1979) Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium *Acta Tropica* **36**, 289-292

Brun, R., Baeriswyl, S. & Kunz, C. (1989) *In vitro* drug sensitivity of *Trypanosoma gambiense* isolates *Acta Tropica* **46**, 369-376

Brun, R. & Rab, S. (1991) *In vitro* drug sensitivity of *T. congolense* isolates *Parasitology Research* **77**, 341-345

Burri, C., Baltz, T., Giroud, C., Doua, F., Welker, H.A., & Brun, R. (1993) Pharmacokinetic properties of the trypanocidal drug melarsoprol *Chemotherapy* **39**, 225-234

Carter, N.S. & Fairlamb, A.H. (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter *Nature* **361**, 173-175

Cibulskis, R.E. (1988) Origins and organisation of genetic diversity in natural populations of *Trypanosoma brucei Parasitology* **96**, 303-322

Cibulskis, R.E. (1992) Genetic variation in *Trypanosoma brucei* and the epidemiology of sleeping sickness in the Lambwe Valley, Kenya *Parasitology* **104**, 99-109

Clarkson, A.B. & Amole, B.O. (1982) The role of calcium in trypanocidal drug action *Science* **216**, 1321-1323

Clarkson, A.B., Bienen, E.J., Bacchi, C.J., McCann, P.P., Hutner, S.H. & Sjoerdsma, A. (1984) New drug combination for experimental late stage African trypanosomiasis: DL-α-difluoromethylornithine with suramin *American Journal of Tropical Medicine and Hygiene* **33**, 1073-1077

Collins, J.M., Klecker, R.W., Yarchoan, R., Lane, H.C., Fauci, A.S., Redfield, R.R., Broder, S. & Myers, C.E. (1986) Clinical pharmacokinetics of suramin in patients with HTLV-III/LAV infection *Journal of Clinical Pharmacology* **26**, 22-26

Coons, T., Hanson, S., Bitonti, A.J., McCann, P.P. & Ullman, B. (1990) Alpha-difluoromethylornithine uptake in *Leishmania donovani* is associated with increased ornithine decarboxylase activity *Molecular and Biochemical Parasitology* **39**, 77-90

Cross, G.A.M. (1990) Cellular and genetic aspects of antigenic variation in trypanosomes *Annual Review of Immunology* **8**, 83-110

Damper, D. & Patton, C. (1976) Pentamidine transport and sensitivity in *brucei*-group trypanosomes *Journal of Protozoology* **23(2)**, 349-356

De Jongh, R.T. & Friedheim, E.A.H. (1959) Le pentylthiarsphényl-mélamine (melW) dans le traitement de trypanosomiase à *T. gambiense* *Bulletin de la Societé Pathologie exotique* **52**, 769

Demarchi, J. (1958) Rapport sur la chimioprophylaxie de la trypanosomiase à *T. gambiense* In: *International Scientific Committee for Trypanosomiasis Research, Seventh Meeting, Brussels* CCTA, 101-138

Dukes, P. (1984) Arsenic and old taxa: subspeciation and drug sensitivity in *Trypanosoma brucei* *Royal Society of Tropical Medicine and Hygiene* **78,** 711-725

Elrayah, I.E. & Kaminsky, R. (1991) The effect of diminazene aceturate and isometamidium chloride on cultured procyclic forms of susceptible and drug-resistant *Trypanosoma congolense* *Acta Tropica* **49**, 201-213

Evans, D.A. & Ellis, D.S. (1983) Recent observations on the behaviour of certain trypanosomes within their insect hosts *Advances in Parasitology* **22**, 1-42

Fairlamb, A.H. & Bowman, I.B.R. (1980a) Uptake of the trypanocidal drug suramin by bloodstream forms of *Trypanosoma brucei* and its effect on respiration and growth rate *in vivo* *Molecular and Biochemical Parasitology* **1**, 315-333

Fairlamb, A.H. & Bowman, I.B.R. (1980b) *Trypanosoma brucei*: maintenance of concentrated suspensions of bloodstream trypomastigotes *in vitro* using continuous dialysis for measurement of endocytosis *Experimental Parasitology* **49**, 366-380

Fairlamb, A.H., Henderson, G.B. & Cerami, A. (1989) Trypanothione is the primary target for arsenical drugs against African trypanosomes *Procedings of the National Academy of Sciences* **86**, 2607-2611

Fairlamb, A.H., Smith, K. & Hunter, K.J. (1992a) The interaction of arsenical drugs with dihydrolipoamide and dihydrolipoamide dehydrogenase from arsenical resistant and sensitive strains of *Trypanosoma brucei brucei Molecular and Biochemical Parasitology* **53**, 223-232

Fairlamb, A.H., Carter, N.S., Cunningham, M. & Smith, K. (1992b) Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism *Molecular and Biochemical Parasitology* **53**, 213-222

Flynn, I.W. & Bowman, I.B.R. (1974) The action of trypanocidal arsenical drugs on *Trypanosoma brucei* and *Trypanosoma rhodesiense Comparative Biochemistry and Physiology* **48**, 261-273

Friedheim, E.A.H. (1948) Melarsen oxide in the treatment of human trypanosomiasis *Annals of Tropical Medicine, Liverpool*, 357-363

Friedheim, E.A.H. (1949) MelB in the treatment of human trypanosomiasis *American Journal of Tropical Medicine* **29**, 173-180

Frommel, T.O. & Balber, A.E. (1987) Flow cytofluorimetric analysis of drug accumulation by multi drug-resistant *Trypanosoma brucei brucei* and *T. b. rhodesiense Molecular and Biochemical Parasitology* **26**, 183-191

Frommel, T.O. (1988) *Trypanosoma brucei rhodesiense*: effect of immunosuppression on the efficacy of melarsoprol treatment of infected mice *Experimental Parasitology* **67**, 364-366

Fulton, J.D. & Yorke, W. (1941) Studies in chemotherapy XXVII - Further observations on the stability of drug-resistance in trypanosomes *Annals of Tropical Medicine and Parasitology* **35**, 221-227

Galvao-Castro, B., Hochmann, A, & Lambert, P.H. (1978) The role of the host immune response in the development of tissue lesions associated with African trypanosomiasis in mice *Clinical and Experimental Immunology* **33**, 12-24

Gibson, W.C., Marshall, T.F. De C. & Godfrey, D.G. (1980) Numerical analysis of enzyme polymorphism. A new approach to the epidemiology and taxonomy of trypanosomes of the subgenus *Trypanozoon* *Advances in Parasitology* **18**, 175-245

Gibson, W.C., & Wellde, B.T. (1985) Characterisation of *Trypanozoon* stocks from the South Nyanza sleeping sickness focus in Western Kenya *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 671-676

Gibson, W.C. (1989) Analysis of a genetic cross between *Trypanosoma brucei rhodesiense* and *T. b. brucei* *Parasitology* **99**, 391-402

Gibson, W., & Garside, L. (1991) Genetic exchange in *Trypanosoma brucei brucei*: variable chromosomal location of housekeeping genes in different trypanosome stocks *Molecular and Biochemical Parasitology* **45**, 77-90

Gibson, W., Garside, L. & Bailey, M. (1992) Trisomy and chromosome size changes in hybrid trypanosomes from a genetic cross between *Trypanosoma brucei rhodesiense* and *T. b. brucei* *Molecular and Biochemical Parasitology* **52**, 189-200

Gibson, W., & Whittington, H. (1993) Genetic exchange in *Trypanosoma brucei*: Selection of hybrid trypanosomes by introduction of genes conferring drug-resistance *Molecular and Biochemical Parasitology* **60**, 19-26

Gill, B.S. (1971) Resistance of *Trypanosoma evansi* to quinapyramine, suramin, stilbamidine and tryparsamide and analysis of cross-resistance *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**, 352-357

Giordano, C., Clerc, M. & Doutriaux C. (1977) Le diagnostique neurologique au cours des différentes phases de la trypanosomiase humaine africaine *Annales de la Societé Médicine Tropicale* **57**, 213-225

Goodwin, L.G. (1970) The pathology of African trypanosomiasis *Transactions of the Royal Society of Tropical Medicine and Hygiene* **64**, 797-817

Gottesdiener, K., Garcia-Anoveros, J., Lee, M.G. & Van der Ploeg, L.H.T. (1990) Chromosome organisation of the protozoan *T. brucei* *Molecular and Cellular Biology* **10**, 6079-6083

Gottesman, M.M. & Pastan, I. (1988) Resistance to multiple chemotherapeutic agents in human cancer cells *Trends in Pharmacological Science* **9**, 54-58

Grant, P.T. & Sargent, J.R. (1960) Properties of L-α-glycerophosphate oxidase and its role in the respiration of *Trypanosoma rhodesiense* *Biochemical Journal* **76**, 229-237

Gutteridge, W.E. (1969) Some effects of pentamidine on *Crithidia fasciculata* *Journal of Protozoology* **16**, 306-311

Gutteridge, W.E. (1985) Existing chemotherapy and its limitations *British Medical Bulletin* **41**, 162-168

Hajduk, S.L., Cameron, C.R., Barry, J.D. & Vickerman, K. (1981) Antigenic variation in cyclically transmitted *Trypanosoma brucei.* Variable antigen type composition of metacyclic trypanosome populations from the salivary glands of *Glossina morsitans* *Parasitology* **83**, 597-607

Hart, D.T., Misset, O., Edwards, S.W. & Opperdoes, F.R. (1984) A comparison of the glycosomes (microbodies) isolated from *Trypanosoma brucei* bloodstream form and cultured procyclic trypomastigates *Molecular and Biochemical Parasitology* **12**, 25-35

Hawking, F. (1963a) History of chemotherapy In: *Experimental Chemotherapy* Schnitzer, J.R. & Hawking, F. (Editors) NewYork: Academic Press **1**, 1-25

Hawking, F. (1963b) Chemotherapy in trypanosomiasis In: *Experimental Chemotherapy* Schnitzer, J.R. & Hawking, F. (Editors) NewYork: Academic Press **1**, 129-156

Hawking, F. (1978) Suramin: with special reference to onchocerciasis *Advances in Pharmacology and Chemotherapy* **15**, 289-322

Henderson, G.B. & Fairlamb, A.H. (1987) Trypanothione metabolism: a chemotherapeutic target in trypanosomatids P*arasitology Today* **3(10)**, 312-315

Herbert, W.J. & Lumsden, W.H.R. (1976) *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitaemia *Experimental Parasitology* **40**, 427-431

Hide, G., Cattand, P., Le Ray, D., Barry, J.D. & Tait, A. (1990) The identification of *Trypanosoma brucei* subspecies using repetitive DNA sequences *Molecular and Biochemical Parasitology* **39**, 213-226

Hide, G., Welburn, S.C., Tait, A. &Maudlin, I. (1994) Epidemiological relationships of *Trypanosoma brucei* stocks from South East Uganda: evidence for different population structures in human infective and non-human infective isolates *Parasitology* **109**, 95-111

Himmelweit, F. (1960) The collected papers of Paul Ehrlich *Chemotherapy* **3**, *es* Mulligan, H.W. (Editor) London: George Allen & Unwin, 125-221

Hirumi, H., Doyle, J.J. & Hirumi, K. (1977) African trypanosomes: cultivation of animal-infective *Trypanosoma brucei in vitro Science* **196**, 992-994

Jenkins, G.C. & Facer, C.A. (1985) Haematology of African trypanosomiasis In: *Immunology and Pathogenesis of Trypanosomiasis* Tizard, I. (Editor) Boca Raton: CRC Press, 13-43

Jenni, L., Marti, S., Schweizer, J., Betschart, B., Le Page, R.W.F., Wells, J.M., Tait, A., Paindavoine, P., Pays, E. & Steinert, M. (1986) Hybrid formation between African trypanosomes during cyclical transmission *Nature* **322**, 173-175

Jennings, F.W., Urquhart, G.M., Murray, P.K. & Miller, B.M. (1983) Treatment with suramin and 2-substituted 5-nitroimidazoles of chronic murine *Trypanosoma brucei* infections with central nervous system involvement *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 693-698

Jennings, F.W. (1988) Chemotherapy of trypanosomiasis: the potentiation of melarsoprol by concurrent difluoromethylornithine (DFMO) treatment *Transactions of the Royal Society of Tropical Medicine and Hygiene* **82**, 572-573

Jordan, A.M. (1986a) Animal trypanosomiasis In: *Trypanosomiasis Control and African Rural Development* New York: Longman, 44-60

Jordan, A.M. (1986b) Human trypanosomiasis In: *Trypanosomiasis Control and African Rural Development* New York: Longman, 30-43

Jordan, A.M. (1986c) Non-chemical methods of tsetse control In: *Trypanosomiasis Control and African Rural Development* New York: Longman, 109-138

Jordan, A.M. (1986d) Trypanocidal drugs In: *Trypanosomiasis Control and African Rural Development* New York: Longman, 69-84

Kaminsky, R. & Zweygarth, E. (1989) Feeder layer-free *in vitro* assay for screening antitrypanosomal compounds against *Trypanosoma brucei brucei* and *T. b. evansi Antimicrobial Agents and Chemotherpay* **33(6)**, 881-885

Kaminsky, R., Chuma, F. & Zweygarth, E. (1989) *Trypanosoma brucei brucei*: Expression of drug-resistance *in vitro Experimental Parasitology* **69**, 281-289

Kaminsky, R. & Zweygarth, E. (1991) The effect of verapamil alone and in combination with trypanocides on multidrug-resistant *Trypanosoma brucei brucei Acta Tropica* **49**, 215-225

Kaminsky, R. & Brun, R. (1993) *In vitro* assays to determine drug sensitivities of African trypanosomes: a review *Acta Tropica* **54**, 279-289

Kazyumba, G.L., Ruppol, J.F., Tshefu, A.K. & Nkanga, N. (1988) Arsenical resistance and difluoromethylornithine in the treatment of human African trypanosomiasis *Bulletin de la Societé Pathologie exotique Filiales* **81**, 591-594

Kooy, R.F., Hirumi, H., Moloo, S.K., Nantulya, V.M., Dukes, P., Van der Linden, P.M., Duijndam, W.A.L., Janse, C.J. & Overdulve, J.P. (1989) Evidence for diploidy in metacyclic forms of African trypanosomes *Procedings of the National Academy of Sciences* **86**, 5469-5472

Kuzoe, F.A.S. (1993) Current situation of African trypanosomiasis *Acta Tropica* **54**, 153-162

Lambert, P.H., Berney, M. & Kazyumba G. (1981) Immune complexes in serum and in cerebrospinal fluid in African trypanosomiasis *Journal of Clinical Investigations* **67**, 77-85

Lanham, S.W. & Godfrey, D.G. (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose *Experimental Parasitology* **38**, 521-534

Livingstone, D. (1858) Arsenic as a remedy for the tsetse bite *British Medical Journal* 360-361

Lun, Z.R., Min, Z.P., Huang, D., Liang, J.X., Yang, X.F. & Huang, Y.T. (1991) Cymelarsan in the treatment of buffaloes naturally infected with *Trypanosoma evansi* in South China *Acta Tropica* **49**, 233-236

McCann, P.P., Bacchi, C.J., Clarkson, Jr., A.B., Seed, J.R., Nathan, H.C., Amole, B.O., Hutner, S.H. & Sjoerdsma, A. (1981a) Further studies on difluoromethylornithine in African trypanosomes *Medical Biology* **59**, 434-440

McCann, P.P., Bacchi, C.J., Hanson, W.L., Cain, G.D., Nathan, H.C., Hutner, S.H. & Sjoerdsma, A. (1981b) Effect on parasitic protozoa of α-difluoromethylornithine - an inhibitor of ornithine decarboxylase In: *Advances in Polyamine Research* Caldarera, C.M., Zappia, V. & Bachrach, U. (Editors) New York: Raven Press **3**, 97-110

McLintock, L.M.L. (1990) The regulation of pleomorphic *Trypanosoma brucei* infections in immunocompetent hosts *PhD Thesis*, Unpublished, University of Glasgow

Martin, S.K., Oduola, A.M.J. & Milhous, W.K. (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil *Science* **235**, 1238-1285

Maudlin, I. & Dukes, P. (1985) Extrachromosomal inheritance of susceptibility of trypanosome infection in tsetse flies. 1. Selection of susceptible and refractory lines of *Glossina morsitans morsitans* *Annals of Tropical Medicine and Parasitology* **79**, 317-324

Maynard Smith, J., Smith, N.H., O'Rourke, M. & Spratt, B.G. (1993) How clonal are bacteria *Proceedings of the National Acadamy of Sciences* **90**, 4384-4388

Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, P. & Vevert, J.P. (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues *Journal of American Chemical Society* **100**, 2551-2553

Mihok, S., Otieno, L.H. & Darji, N. (1990) Population genetics of *Trypanosoma brucei* and the epidemiology of human sleeping sickness in the Lambwe Valley, Kenya *Parasitology* **100**, 219-233

Misset, O. & Opperdoes, F.R. (1987) The phosphoglycerate kinases from *Trypanosoma brucei*. A comparison of the glycosomal and cytosolic isoenzymes and their selectivity towards suramin *European Journal of. Biochemistry* **162**, 493-500

Mitsuya, H., Popovic, M., Yarchoan, R., Matsushita, S., Gallo, R.C. & Broder, S. (1984) Suramin protection of T-cells *in vitro* against infectivity and cytopathic effect of HTLV-III *Science* **226**, 172-174

Morrison, W.I., Murray, M., Whitelaw, D.D. & Sayer, P.D. (1983) Pathology of infection with *Trypanosoma brucei*: disease syndromes in dogs and cattle resulting in severe tissue damage *Contributions to Microbiology and Immunology* 7, 103-119

Murray, M., Morrison, W.I., Murray, P.K. & Clifford, D.J. (1979) Trypanotolerance - a review *Wild Animal Review* **31**, 2-12

Neal, R.A., Van Bueren, J., McCoy, N.G. & Iwobi, M. (1989) Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovani* by verapamil *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83**, 197-198

Njogu, R.M., Whittaker, C.J. & Hill, G.C. (1980) Evidence for a branched electron transport chain in *Trypanosoma brucei* *Molecular and Biochemical Parasitology* **1**, 13-29

Onyeyili, P.A. & Anika, S.M. (1989) Chemotherapy of *T. b. brucei* infection: Use of DFMO, diminazene aceturate, alone or in combination *Journal of Small Animal Practice* **30**, 505-510

Opperdoes, F.R. & Borst, P. (1977) Localisation of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome *FEBS Letters* **80**, 360-364

Opperdoes, F.R. (1983) Glycolysis as a target for the development of new trypanocidal drugs In: *Mechanism of Drug Action* Singer, T.P., Mansour, T.E. & Undarza, R.N. (Editors) New York: Academic Press, 121-131

Opperdoes, F.R. (1985) Biochemical peculiarities of trypanosomes, African and South American *British Medical Bulletin* **41(2)**, 130-136

Osman, A.S., Jennings, F.W. & Holmes, P.H. (1992) The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice *Acta Tropica* **50**, 249-257

Pepin, J., Milord, F., Mpia, B., Meurice, F., Ethier, L., DeGroof, D. & Bruneel, H. (1989) An open clinical trial of nifurtimox for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness in central Zaire *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83**, 514-517

Phillips, M.A. & Wang, C.C. (1987) A *Trypanosoma brucei* mutant resistant to α-difluoromethylornithine *Molecular and Biochemical Parasitology* **22**, 9-17

Poltera, A.A., Mbuya, B.K. & Nkonge, C (1984) *Trypanosoma brucei brucei*: Immunosuppressive modulation von zirkulierenden Immunkomplexen nach einmaliger Verabreichung von Melarsoprol bei 6-wöchiger experimenteller Infektion In: *Tropenmedizin, Parasitologie* Boch, J. (Editor) Frankfurt: Peter Lang, 7986

Poltera, A.A. (1985) Pathology of human African trypanosomiasis with reference to experimental African trypanosomiasis and infections of the central nervous system *British Medical Bulletin* **41(2)**, 169-174

Pospichal, H., Brun, R., Kaminsky, R. & Jenni, L. (1994) Induction of resistance to melarsenoxide cysteamine (MelCy) in *Trypanosoma brucei brucei Acta Tropica* **58**, 187-197

Raseroka, B.H. & Ormerod, W.E. (1985) Suramin/metronidazole combination for African sleeping sickness *Lancet* **2**, 784-785

Raseroka, B.H. & Ormerod, W.E. (1986) The trypanocidal effects of drugs in different parts of the brain *Transactions of the Royal society of Tropical Medicine and Hygiene* **80**, 634-641

Rickman, L.R. & Robson, J. (1970) The blood incubation infectivity test: a simple test which may serve to distinguish *Trypanosoma brucei* from *T. rhodesiense Bulletin of the World Health Organisation* **42**, 650-651

Robertson, D.H.H. (1963) The treatment of sleeping sickness (mainly due to *Trypanosoma rhodesiense*) with melarsoprol II - An assessment of its curative value *Transactions of the Royal Society of Tropical Medicine and Hygiene* **57**, 176-183

Ross, C.A. & Taylor, A.M. (1990) *Trypanosoma congolense*: an *in vitro* assay to distinguish between drug-resistant from drug-sensitive populations *Parasitology Research* **76**, 326-331

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) Analysis and cloning of eukaryotic genomic DNA In: *Molecular cloning: a laboratory manual* (2nd Edition), 9.34-9.35

Schweizer, J. & Jenni, L. (1991) Hybrid formation in the life cycle of *Trypanosoma (T.) brucei*: Detection of hybrid trypanosomes in a midgut-derived isolate *Acta Tropica* **48**, 319-321

Schweizer, J., Pospichal, H. & Jenni, L. (1991) Hybrid formation betwen African trypanosomes *in vitro* *Acta Tropica* **48**, 237-240

Shapiro, S.Z., Naessens, J., Liesengang, B., Molo, S.K. & Maqondu (1984) Analysis by flow cytometry of DNA synthesis during the life-cycle of African trypanosomes *Acta Tropica* **41**, 313-323

Sjoerdsma, A., Golden, J.A., Schechter, P.J., Barlow, J.L.R. & Santi, D.V. (1984) Succesful treatment of lethal protozoal infections with the ornithine decarboxylase inhibitor, α-difluoromethylornithine *Transactions of the Association of American Physicians* **97**, 70-79

Stein, C.A., La Rocca, R.V., Thomas, R., McAtee, N. & Myers, C.E. (1989) Suramin: an anticancer drug with a unique mechanism of action *Journal of Clinical Oncology* **7**, 499-508

Sternberg, J., Tait, A., Haley, S., Wells, J.M., Le Page, R.W.F., Schweizer, J. & Jenni, L. (1988) Gene exchange in African trypanosomes: characterisation of a new hybrid genotype *Molecular and Biochemical Parasitology* **27**, 191-200

Sternberg, J. & Tait, A. (1989) Genetic exchange in African trypanosomes *Trends in Genetics* **6**, 317-322

Sternberg, J., Turner, C.M.R., Wells, J.M., Ranford-Cartwright, L.C., Le Page, R.W.F. & Tait, A. (1989) Gene exchange in African trypanosomes: frequency and allelic segregation *Molecular and Biochemical Parasitology* **34**, 269-280

Stevens, J.R. & Welburn, S.C. (1993) Genetic processes within an epidemic of sleeping sickness in Uganda *Parasitology Research* **79**, 421-427

Strickland, G.T., Voller, A., Pettit, L.E. & Fleck, D.J. (1972) Immunodepression associated with concomitant toxoplasma and malarial infections in mice *Journal of Infectious Diseases* **126**, 54-60

Sutherland, I., Peregrine, A.S., Lonsdale-Eccles, J.D. & Holmes, P.H. (1991) Reduced accumulation of isometamidium by drug-resistant *Trypanosoma congolense Parasitology* **103**, 245-251

Sutherland, I., Mounsey, A. & Holmes, P.H. (1992) Transport of isometamidium (samorin) by drug-resistant and drug-sensitive *Trypanosoma congolense Parasitology* **104**, 461-467

Tait, A. (1980) Evidence for diploidy and mating in trypanosomes *Nature* **287**, 536-538

Tait, A., Babiker, E.A. & Le Ray, D. (1984) Enzyme variation in *T. brucei* ssp. I Evidence for the sub-speciation of *Trypanosoma brucei gambiense Parasitology* **89**, 311-326

Tait, A., Barry, J.D., Wink, R., Sanderson, A. & Crowe, J.S. (1985) Enzyme variation in *T. brucei* ssp. II Evidence for *T. b. rhodesiense* being a set of variants of *T. b. brucei Parasitology* **90**, 89-100

Tait, A., Turner, C.M.R., Le Page, R.W.F. & Wells, J.M. (1989) Genetic evidence that metacyclic forms of *Trypanosoma brucei* are diploid *Molecular and Biochemical Parasitology* **73**, 247-256

Tait, A. & Turner, C.M.R. (1990) Genetic exchange in *T. brucei Parasitology Today* **6**, 70-75

Tait, A., Buchanan, N., Hide, G. & Turner, M. (1993) Genetic recombination and karyotype inheritance in *Trypanosoma brucei* species In: *Genome Analysis of Protozoan Parasites* Marzana, S.P. (Editor) Nairobi, ILRAD, 93-107

Tait, A., Buchanan, N., Hide, G. & Turner, C.M.R. (1994) Chromosomal organisation, segregation and recombination in genetic crosses of *Trypanosoma brucei*, in preparation

Tetley, L. & Vickerman, K. (1985) Differentiation in *Trypanosoma brucei*: host-parasite cell junctions and their persistence during aquisition of their variable antigen coat *Journal of Cell Science* **74**, 1-19

Tibayrenc, M., Kjellberg, F. & Ayala, F.J. (1990) A clonal theory of parasitic protozoa: The population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas* and *Trypanosoma* and their medical and toxonomical consequences *Procedings of the National Academy of Sciences* **87**, 2414-2418

Tibayrenc, & Ayala, F.J. (1991) Towards a population genetics of micro-organisms: The clonal theory of parasitic protozoa *Parasitology Today* **7**, 228-232

Tréfouel, J. (1962) Le rôle de Maurice Nicolle en chimiothérapie anti-trypanosomes *Bulletin de la Societé Pathologie exotique* **55**, 200

Tsuhako, M.H., Alves, M.J.M., Colli, W., Filardi, L.S., Brener, Z. & Augusto, O. (1991) Comparative studies of nifurtimox uptake and metabolism by drug-resistant and susceptible strains of *Trypanosoma cruzi* *Comparative Biochemistry and Physiology* **99**, 317-321

Turner, C.M.R. & Barry, J.D. (1989) High frequency of antigenic variation in *Trypanosoma brucei rhodesiense* infections *Parasitology* **99**, 67-75

Turner, C.M.R., Sternberg, J., Buchanan, N., Smith, E., Hide, G. & Tait, A. (1990) Evidence that the mechanism of gene exchange in *Trypanosma brucei* involves meiosis and syngamy *Parasitology* **101**, 377-386

Turner, M. (1990) The use of experimental artefacts in African trypanosome research *Parasitology Today* **6**, 14-17

Van der Ploeg, L.H.T, Smith, C.L., Polvere, R.I. & Gottesdiener, K.M. (1989) Improved separation of chromosome-sized DNA from *T. brucei*, stock 427-60 *Nucleic Acids Research* **17**, 3217-3227

Van Hoof, L. (1947) Observation on trypanosomiasis in the Belgian Congo *Transactions of the Royal Society of Tropical Medicine and Hygiene* **40**, 728-754

Van Meirvenne, N., Janssens, P.G. & Magnus, E. (1975) Antigenic variation in syringe passaged populations of *Trypanosoma* (*Trypanozoon*) *brucei* I Rationalistaion of the experimental approach *Annales de la Societé belge de Médecine Tropicale* **55**, 1-23

Van Nieuwenhove, S., Schechter, P.J., Declercq, Bone, G., Burke, J. & Sjoerdsma, A. (1985) Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-α-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 692-698

Van Nieuwenhove, S. (1992) Advances in sleeping sickness therapy *Annales de la Societé belge de Médecine Tropicale* **72 (suppl. 1)**, 39-51

Van Schaftingen, E., Opperdoes, F.R. & Hers, H.-G. (1987) Effects of various metabolic conditions and of the trivalent arsenical melarsen oxide on the intracellular levels of fructose 2,6-bisphosphate and of glycolytic intermediates in *Trypanosoma brucei European Journal of Biochemistry* **166**, 653-661

Vansterkenberg E.L.M., Coppens, I., Wilting, J., Bos, O.J.M., Fischer, M.J.E., Janssen, L.H.M. & Opperdoes, F.R. (1993) The uptake of the trypanocidal drug suramin in combination with low-density lipoproteins by *Trypanosoma brucei* and its possible mode of action *Acta Tropica* **54**, 237-250

Vickerman, K. (1965) Polymorphism and mitochondrial activity in sleeping sickness trypanosomes *Nature* **208**, 762-766

Vickerman, K. (1969) Localisation of variable antigens in the surface coat of *Trypanosoma brucei* using ferritin-conjugated antibody *Nature* **224**, 1125-1126

Vickerman, K. (1985) Developmental cycles and biology of pathogenic trypanosomes *British Medical Bulletin* **41(2)**, 105-114

Vickerman, K., Myler, P.J. & Stuart, K.D. (1991) African Trypanosomiasis In: *Immunology and Molecular Biology of Parasitic Infections* 3rd Edition Warren, K. S. (Editor) Oxford: Blackwell Scientific Publications, 170-212

Voegtlin, C., Dyer, H.A. & Leonard, C.S. (1923) On the mechanism of action of arsenic on protoplasm *Public Health Report* **38**, 1882-1912

Von Jancso, N. & Von Jancso, H. (1934) The role of the natural defence forces in the evolution of the drug-resistance of trypanosomes I - A method for the exclusion of the natural defence mechanisms from chemotherapeutic processes *Annals of Tropical Medicine and Parasitology* **28**, 419-438

Von Jancso, N. & Von Jancso, H. (1935) The role of the natural defence forces in the evolution of the drug-resistance of trypanosomes I - The rapid production of Germanin-fast *T. brucei* in animals with paralysed defence *Annals of Tropical Medicine and Parasitology* **29**, 95-109

Voogd, T.E., Vansterkenberg, E.G.L., Wilting, J. & Janssen, L.H.M. (1993) Recent research on the biological activity of suramin *Pharmacological Reviews* **45**, 177-203

Wells , J.M., Prospero, T.D., Jenni, L. and Le Page, R.W.F. (1987) DNA contents and molecular karyotypes of hybrid *T. brucei* *Molecular and Biochemical Parasitology* **24**, 103-116

Williamson, J. & Rollo, I.M. (1959) Drug-resistance in trypanosomes: cross-resistance analyses *British Journal of Pharmacology* **14**, 423-430

Williamson, J. (1970) Review of chemotherapeutic and chemoprophylactic agents In: *The African Trypanosomiases* Mulligan, H.W. (Editor) London: George Allen & Unwin, 125-221

Willson, M., Callens, M., Kuntz, D., Perié, J & Opperdoes, F.R. (1993) Synthesis and activity of inhibitors of highly specific for the glycolytic enzymes from *Trypanosoma brucei Molecular and Biochemical Parasitology* **59,** 201-210

World Health Organisation (1986a) Epidemiology and control of African trypanosomiasis *Report of a WHO Expert Committee, Technical Report Series* **739**, 1-125

World Health Organisation (1986b) Epidemiology and control of African trypanosomiasis *Report of a WHO Expert Committee, Technical Report Series* **739**, 118-121

Yarlett, N., Goldberg, B., Nathan, H.C., Garofalo, J. & Bacchi, C.J. (1991) Differential sensitivity of *Trypanosoma brucei rhodesiense* isolates to *in vitro* lysis by arsenicals *Experimental Parasitology* **72**, 205-215

Yorke, W., Murgatroyd, F. & Hawking, F. (1931) Studies in chemotherapy V - Preliminary contribution on the nature of drug-resistance *Annals of Tropical Medicine and Parasitology* **25**, 351-358

Zampetti-Bosseler, F., Schweizer, J., Pays, E., Jenni, L. & Steinert, M. (1986) Evidence for haploidy in metacyclic forms of *Trypanosoma brucei Procedings of the National Academy of Sciences* **83**, 6063-6064

Zelleke, D., Kassa, B. & Abebe, S. (1989) Efficacy of RM110, a novel trypanocide, in the treatment of *Trypanosoma evansi* infections in camels *Tropical Animal Health and Production* **21**, 223-226

Zhang, Z.Q., Giroud, C. & Baltz, T. (1992) *In vivo* and *in vitro* sensitivity of *Trypanosoma evansi* and *T. equiperdum* to diminazene, suramin, melCy, quinapyramine and isometamidium *Acta Tropica* **50**, 101-110

Zweygarth, E. & Kaminsky, R. (1990) Evaluation of an arsenical compound (RM 110, melCy, cymelarsan) against susceptible and drug-resistant *Trypanosoma brucei brucei* and *T. b. evansi Tropical Medicine and Parasitology* **41**, 208-212

Zweygarth, E. & Kaminsky, R. (1991) Evaluation of DL-α-difluoromethylornithine against susceptible and drug-resistant *Trypanosoma brucei brucei Acta Tropica* **48**, 223-232

Zweygarth, E., Kaminsky, R. & Webster, P. (1991) *Trypanosoma brucei evansi*: Dyskinetoplasia and loss of infectivity after long-term *in vitro* cultivation *Acta Tropica* **48**, 95-99

Zweygarth, E., Moloo, S.K. & Kaminsky, R. (1993) *Trypanosoma simiae*: *In vitro* studies on drug susceptibility *Acta Tropica* **54**, 301-308