Development and Application of ELISAs to Improve the Chemoprophylactic Control of African Bovine Trypanosomiasis

by

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Abstract

The control of bovine trypanosomiasis in Africa continues to rely heavily on the chemoprophylactic drug isometamidium chloride (Samorin®). However, despite many years of use, no methods are available which are sufficiently sensitive to measure drug levels in treated cattle. Two new enzyme-linked immunosorbent assay (ELISA) procedures for the detection and quantification of isometamidium in bovine serum were developed and validated. The first of these, an indirect competition ELISA (ICE) was capable of detecting isometamidium to approximately 0.1 $ngml^{-1}$. Following the treatment of cattle with isometamidium chloride by intramuscular injection at the recommended prophylactic dose rate of 1.0 $mgkg^{-1}$, the drug could be detected in serum samples for up to four months.

In a controlled laboratory experiment, Boran cattle injected intramuscularly with isometamidium chloride (dose 1.0 $mgkg^{-1}$ body weight) were challenged at monthly intervals with *Glossina morsitans centralis* infected with one of three populations of *T. congolense* (IL 3893, IL 3889 or IL 1180) until all animals became infected. Untreated control cattle confirmed the infectivity of challenge. All cattle challenged with IL 3893 or IL 3889 developed infection following the first challenge, at which time the mean serum drug concentration was 6 $ngml^{-1}$. Cattle challenged with IL 1180 became infected following 6 to 8 challenges. The mean serum drug concentration in these cattle at the time of their third challenge with IL 1180 was 0.75 $ngml^{-1}$. Trypanosome populations IL 3893 and IL 3889 were considered to be highly resistant to isometamidium, while IL 1180, relatively sensitive. Hence, *T. congolense* persisting at serum isometamidium concentrations greater than 0.75 $ngml^{-1}$ can be considered moderately resistant, and at concentrations greater than 6 $ngml^{-1}$ markedly resistant.

In a second, similar experiment isometamidium treated (1.0 $mgkg^{-1}$) Boran cattle were with challenged monthly with *T. congolense* IL 1180 or a moderately isometamidium resistant *T. congolense* clone (IL 3343). Ten treated cattle challenged with *IL* 3343, were all refractory to infection one month after drug administration, but seven cattle succumbed at the second monthly challenge and three cattle at the third. All five treated cattle challenged with IL 1180 resisted four monthly challenges. The mean isometamidium concentrations were 5.6 ± 2.8 $ngml^{-1}$ and 2.0 ± 0.86 $ngml^{-1}$ at the time of the first and second challenges respectively. The mean concentration in cattle refractory to challenge with IL 1180 at the time of the fourth challenge was 0.4 ± 0.18 $ngml^{-1}$.

T. congolense occurring in cattle under chemoprophylaxis may thus be considered moderately drug resistant if the serum isometamidium concentration is 2.0 $ngml^{-1}$; between 0.4 and 2.0 $ngml^{-1}$ a low level of drug resistance may be inferred; below 0.4 $ngml^{-1}$, no inference regarding resistance should be made.

An attempt was made to relate isometamidium concentrations measured by the ICE in serum from Jersey cattle under a failing chemoprophylactic regimen in coastal Kenya, to the incidence of trypanosome infection. The distribution of trypanosome challenge over a number of three month prophylactic periods was uneven, and almost all challenge (based on the infection rate in untreated control cattle) occurred at the end of such periods. At the time of challenge, concentrations in the treated group were at their lowest and no inferences could be made about drug resistance in trypanosome infections, all of which were caused by *T. vivax*.

A simpler, competitive enzyme immunoassay (CEIA) developed for isometamidium in bovine serum has several advantages over the ICE; fewer incubation steps; frozen storage of microtitre plates in batches; overnight competition incubation. The response variance of 57 untreated cattle was small (CV approximately 10%); partitioning showed 77% of this variance to be intrinsic to the samples, and 23% due to procedure (CV of duplicates wells approximately 5%). The CEIA could detect isometamidium in serum of treated cattle for up to ten weeks following treatment, with a high level of reproducibility. The limit of detection is approximately 0.5 $ngml^{-1}$.

The CEIA was used to investigate the pharmacokinetics of isometamidium in Friesian cattle (*Bos taurus*) treated intravenously and intramuscularly (dose rate 1.0 $mgkg^{-1}$ body weight). The major pharmacokinetic parameters were calculated using standard pharmacokinetic equations. The large V_{SS} (mean 24 lkg^{-1}), and the prolonged MRT (83 h) and terminal phase half-life (136 h) following intravenous injection were consistent with extensive uptake of the drug into tissues such as liver, kidney and spleen. The relatively low bioavailability (60%), and prolonged MAT (7.8 days) following intramuscular injection was consistent with a primary depot at the site of injection.

Isometamidium could be detected using the CEIA in the sera of all but one of 24 cattle treated intramuscularly (1.0 $mgkg^{-1}$ body weight) in an area of natural tsetse challenge; for up to 14 weeks in five cattle; for 12 weeks in 12 cattle; for 10 weeks in 20 cattle; and for eight weeks in 22 cattle. The geometric mean apparent half-life of isometamidium in 22 cattle was 23.2 days. Treated

cattle were protected against trypanosome infections for at least 18 weeks; thereafter three trypanosome infections were detected, between 20 and 22 weeks following treatment. In contrast, in 18 untreated control cattle, nine trypanosome infections were detected over the first 18 weeks. While there was no evidence of drug-resistant trypanosomes, the CEIA was capable of quantifying drug-levels in 20 out of 23 cattle for at least 70 days.

Finally, the CEIA was used to investigate the role of isometamidium in an experimentally induced wasting syndrome in Maasai Zebu cattle under a regimen of frequently repeated isometamidium treatments. Significant weight loss and mortality occurred in poorly nourished cattle after three or four isometamidium treatments and additional treatments with diminazene. Weight losses and glutamate dehydrogenase levels were correlated with isometamidium concentrations which showed small but significant elevations with successive treatments, but no marked increases. Such frequently repeated treatment regimens must be considered inadvisable.

In conclusion, the two ELISAs are capable of determining isometamidium concentrations in sera from cattle in the field, and promise to provide a practical means of rationalising chemoprophylactic drug regimens, particularly where they can assist in identifying the development of trypanocidal drug resistance.

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Declaration

I hereby declare that the work presented in this thesis is original and was conducted solely by the author except where the contribution of others has been acknowledged.

I also hereby certify that no part of this thesis has been submitted previously in any form to any university. It has in part been published in the following scientific publications:

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Laboratory records and computer data files related to the work described in this thesis are deposited in the Department of Veterinary Physiology, the University of Glasgow.

Table of Contents

List of Tables

List of Figures

List of Plates

Chapter 1:

African Bovine Trypanosomiasis

Introduction

Geographic distribution

The trypanosomiases of domestic animals occur over a vast area occupying the greater part of the tropics and subtropics of the Americas, Africa and Asia. Tsetse-transmitted trypanosomiasis occurs in the sub-Saharan region of Africa where the distribution of the disease approximates to that of the tsetse fly vector species, over an area of 11 million km² (Jordan 1986). In the north the limit of distribution depends on rainfall, being close to the 500mm isohyet, and extends from 14° N in Senegal in West Africa, across the southern boundaries of the Sahara desert, to 4° N in East Africa at the southern edge of the Somali desert. The southern limits of distribution vary in the west between 10 and 20° S, following the northern limits of the Kalahari and Namib deserts, while further to the east, the southern limit, which depends on minimum temperature, is approximately 20° S although on the East African coast it extends as far as 29° S. Between the northern and southern limits of the tsetse belts the precise distribution of the tsetse fly is not ubiquitous, but depend on factors influencing its survival, namely rainfall, vegetation and temperature. Altitude has a significant influence on all three of these factors, such that few tsetse survive above approximately 1800m in the equatorial region, and correspondingly lower altitudes closer to the southern limits of the fly belts.

Economic importance

In the majority of the 37 sub-Saharan countries affected by tsetse-transmitted trypanosomiasis the problem is classified as severe, and figures among the first three priority veterinary diseases (Anon, 1992). Tsetse flies pose a threat of trypanosomiasis over almost 40% of the continent of Africa (FAO/WHO/OIE, 1982). Seven million km² of this area would probably be suitable for livestock and agricultural development if trypanosomiasis were controlled (Finelle, 1974; MacLennan, 1980), and an extra 120 million cattle and an equivalent number of small ruminants might be supported (FAO, 1987; FAO/WHO/OIE, 1982). However, heavily infested fly belts are virtually devoid of cattle: for example, in Tanzania the distribution of cattle is almost exactly the converse of that of the tsetse (Jordan, 1986). Of a total African population of approximately 173 million cattle, only 25% is located in tsetse-infested zone (IBAR, 1989).

Where cattle are kept in areas of tsetse challenge, it is generally on the fringes of the tsetse belts; livestock owners have to balance the need for better nutrition for their animals, which is usually available within the fly-infested areas, with the associated threat of disease (Jordan, 1986). In these areas, losses in livestock production and performance include poor growth, weight loss, lowered milk yield, reduced animal traction output, infertility, high abortion rates, and mortality (FAO/WHO/OIE, 1963; McDowell, 1977). Losses in food production from mixed agriculture due to lack of manure, draught power, and cash income are incalculable (Stewart, 1986). For example, in Africa 80% of traction is non-mechanical; it has been estimated that a draught ox can increase the agricultural output of a family six-fold (McDowell, 1977). Moreover, the manure provided by livestock is essential for the production of food and cash crops.

Trypanosomiasis is thus the most economically important disease of Africa (Jawara, 1990), and the most significant factor in Africa's deteriorating food production situation (Trail, Sones, Jibbo, Durkin, Light and Murray, 1985). It has been estimated that controlling trypanosomiasis in tsetse-infested areas could generate an additional US$ 750M annually (Finelle, 1980).

Aetiology

Tsetse transmitted trypanosomiasis of domestic livestock in Africa is caused by various species of the genus *Trypanosoma*, which are parasitic protozoa found in the blood and tissues of their hosts. Within this genus a large number of species occur, of which only a few are recognised as disease causing parasites of man and his domestic animals. Other species infect non-domestic animals and / or are considered to be harmless. These parasites are generally transmitted by an arthropod vector, although exceptions to this rule occur. Of greatest importance as vectors of disease-causing *Trypanosoma* sp. in sub-Saharan Africa are blood-sucking flies of the genus *Glossina*, known collectively as the tsetse flies. The eminent British medical scientist David Bruce (1895) working in Southern Africa around the turn of the century described a trypanosome (*Trypanosoma brucei*) as being the aetiological agent of both the disease of cattle known as "Nagana" and tsetse fly disease. The importance of the genus *Trypanosoma* as disease causing parasites of domestic animals was, however, discovered fifteen years earlier by a British Army veterinary surgeon Griffith Evans (1880) posted to the Royal Artillery in the Punjab Frontier Force, who recognised a parasite (today known as *Trypanosoma evansi*) in the blood

of horses suffering from a disease known locally as "Surra" (lit. rotten), and shortly afterwards in the blood of diseased camels.

The trypanosomes of the genus *Trypanosoma* are, in the trypomastigote form in which they occur in the mammalian host, unicellular mononuclear flagellate protozoa approximately 10 - 35 μm in length. These organisms are elongate and motile by virtue of the flagellum which originates from a basal body (usually in association with a kinetoplast) at the posterior end of the trypanosome and extends the length of the organism to which is loosely attached by a fold of the pellicle, known as the undulating membrane. There may be a variable length of free flagellum extending beyond the anterior end of the trypanosome, depending on the species of trypanosome. These morphological features, visible under the light microscope, particularly in Romanowski stained slide preparations have been the traditional basis of the sub-classification of the genus, although more recently, newer molecular techniques have been used to investigate the phylogenetic relationships of various trypanosome isolates (Hide and Tait, 1991).

The genus *Trypanosoma* can be divided into two sections on the basis of the mode of transmission, the Salivaria and the Stercoraria (Hoare, 1964). The pathogenic trypanosomes of Africa and Asia are generally transmitted by the bites of the vector arthropod, and are therefore classified in the section Salivaria, while the important human parasite of South America *Trypanosoma cruzi* (Chagas, 1909) is transmitted in the faeces of the arthropod vectors, triatomid bugs of the family Reduviidae, and is thus an example of the Stercoraria. Non-pathogenic stercorarian trypanosomes are widely distributed an example being the common cattle parasite *Trypanosoma theileri* (Laveran, 1902) important as a potential source of confusion in the diagnosis of bovine trypanosomiasis.

All the tsetse-transmitted trypanosomes of mammals are members of the Salivaria, which has been further divided into four sub-genera on the basis of the morphological and epidemiological characteristics of its members, these being *Duttonella*, *Nannomonas*, *Trypanozoon* and *Pycnomonas*. The first of the sub-genera, *Duttonella* contains the important parasite of cattle and other ungulates *Trypanosoma vivax* (Zeimann, 1905) and the less important *Trypanosoma uniforme* (Bruce, Hamerton, Bateman, Mackie and Bruce, 1911). The second sub-genus, *Nannomonas*, contains another important parasite of domestic ruminants, *Trypanosoma congolense* (Broden, 1904), and a second

species *Trypanosoma simiae* (Bruce, Harvey, Hamerton, Davey and Bruce, 1912) important as a parasite of domestic and wild pigs. The sub-genus *Trypanozoon* contains three morphologically indistinguishable forms, all classified as sub-species of a single species, *T. brucei*, which are distinct in terms of their epidemiology, particularly in relation to their role in human African trypanosomiasis or sleeping sickness. *Trypanosoma brucei brucei* (Plimmer and Bradford, 1899) is distinguished by its inability to infect man, while it causes acute disease in dogs and horse, and chronic disease in pigs and cattle. *Trypanosoma brucei rhodesiense* (Stephens and Fantham, 1910) appears to be very similar to *T. b. brucei* except that it has acquired the ability infect man, classically causing sporadic outbreaks of "Rhodesian" sleeping sickness, a relatively acute disease in Eastern and Southern Africa. *Trypanosoma brucei gambiense* (Dutton, 1902), on the other hand, is a rather different organism which is not considered to be of significance in causing disease in domestic animals, although their role in its epidemiology is far from clear. In man it causes the more chronic "Gambian" form of sleeping sickness of central and west Africa. Also included in this subgenus are *T. evansi* (Steel, 1885) and *T. equiperdum* (Doflein, 1901), important parasites of domestic animals, but not transmitted by tsetse flies. Finally, the fourth sub-genus of *Trypanosoma*, *Pycnomonas*, contains but a single species, *Trypanosoma suis* (Ochmann, 1905), of limited distribution and pathogenic only to young domestic pigs.

The cycle development of trypanosomes in the invertebrate host differs between the three main sub-genera of tsetse-transmitted trypanosomes. Trypanosomes are ingested by the tsetse fly in a blood meal from a mammalian host as trypomastigote forms which are found exclusively in the fly gut. The parasites then undergo a transformation in the mouthparts or salivary glands of the fly into the epimastigote form, a form similar to the trypomastigote, but characterised by the close apposition of the basal body and kinetoplast to the nucleus (Hoare and Wallace 1966). Finally the parasites transform into metacyclic forms, which are infective for the mammalian host, the entire cycle taking between 5 and 35 days depending upon the species of trypanosome, species of tsetse fly, and the temperature (Jordan 1986; Vickerman, Tetley, Hendry and Turner, 1988). The importance of the species of trypanosome in the length of the development cycle in the fly is related to differences in the site of development within the fly of the various sub-genera of trypanosome. While *Duttonella* complete the cycle entirely within the fly's proboscis, *Nannomonas* develop in the midgut and proboscis, and *Trypanozoon* in the midgut and

salivary glands. The site of development within the fly can be used together with morphological criteria to identify the infecting trypanosomes at least to the sub-genus level.

Transmission

Trypanosomes are generally transmitted by haematophageous arthropods, either mechanically as in the case of *T. evansi*, as suggested by Evans (1880), or cyclically, with replication in the arthropod host as discovered by Kleine (1909) in tsetse flies (*Glossina* sp.). A notable exception to this rule in Africa and elsewhere is the salivarian trypanosome parasite of the horse and donkey, *T. equiperdum*, which is transmitted by coitus and thus no longer requires an intermediate host for its propagation.

The tsetse flies comprise 22 species of *Glossina*, divided between three well-marked species groups (Newstead, 1911), and are the principal vectors of trypanosomiasis in sub-Saharan Africa. These three groups, distinguishable on the basis of differences between the male and female genital armature, are the *fusca* group, the *palpalis* group, and the *morsitans* group, containing 14, 9 and 7 individual species and subspecies respectively (Jordan, 1986).

The *fusca* group generally occur in lowland rainforest habitats, and are the least important in terms of transmission of bovine trypanosomiasis. The *palpalis* group species also occur in lowland rainforest habitat but also extend into humid savannah, and along watercourses into drier savannah, and includes three species of importance in the transmission of bovine trypanosomiasis: *G. palpalis*, *G. fuscipes*, and *G. tachynoides*.

Species of the *morsitans* group are restricted to savannah woodlands and vegetation along watercourses. These species are therefore the ones most likely to come into contact with grazing herds of cattle. The three subspecies of *G. morsitans*, *Glossina m. morsitans*, *Glossina m. submorsitans* and *Glossina m. centralis* (see Plate 4), together with *G. pallidipes*, occupy a very large area and are major vectors of animal trypanosomiasis. Three other species in this group, *G. swynnertoni*, *G. longipalpis* and *G. austeni* are also important vectors of bovine disease in particular areas, but are more restricted in their distributions.

The life-cycle of *Glossina* is remarkable in that the female gives birth to a fully formed larva of approximately its own size, which immediately burrows into the ground and forms a puparium, from which a single fly emerges after

approximately 30 days (Jordan 1986). Once deposited the larvae do not feed, and thus the species is entirely dependent for nutrition on the blood meals of the adults.

Glossina species differ in their ability to transmit trypanosomes: all species can be infected with some species of trypanosomes, but not all can be infected with all trypanosomes (Jordan 1986). For example, *palpalis* group flies are rarely if ever infected with *T. congolense.* The ability of tsetse to support trypanosomes is hereditary (Maudlin, 1982), refractoriness to infection being associated with a midgut lectin, while susceptibility appears to be associated with the presence of rickettsia-like-organisms (RLO) in fly ovaries and midguts (Maudlin, 1993). RLO have been shown to posses endochitinase activity which results in the excess production of n-acetyl glucosamine which inactivates the lectin.

Finally, the occurrence of *T. vivax* well outside Africa, notably Central and South America, and the West Indies, is evidence of transmission of this species in the absence of tsetse, transmission being thought to be due to the non-cyclical, "mechanical" transfer of bloodstream-form trypanosomes directly from one host to another on the mouthparts of biting flies (Stephen 1986). *T. vivax* has also been found in areas of Africa far from the known distribution of tsetse, suggesting mechanical transmission of this species may also be important within Africa, although the interpretation of such findings remains controversial because of difficulties in detecting tsetse at very low densities.

Pathogenesis, immunology and pathology

Pathogenesis

The pathogenesis of animal trypanosomiasis has been the subject of excellent reviews by Fiennes (1970), Murray, Morrison, Emery, Akol, Masake, and Moloo (1980), Stephen (1986), and Losos (1986).

The course and severity of the disease depends on a number of factors, of which the species and origin of infecting trypanosomes, the breed of cattle involved and the history of prior exposure to infection, and treatment with trypanocidal drugs are the most readily defined (Stephen, 1986). There is a spectrum of disease states ranging from the peracute haemorrhagic condition associated with some particularly virulent *T. vivax* infections which may result in death within two weeks of the infective tsetse fly bites, through the more usual chronic condition characterised by anaemia and progressive wasting common in *T. congolense* infections, to almost inapparent chronic infections which may occur with *T. brucei*, particularly in indigenous cattle. Spontaneous recovery from infection may also occur. It would however be a gross oversimplification to state that these clinico-pathological syndromes typify infections with each of the three species in the field. Different isolates of the same trypanosome species differ markedly in their virulence, so that in East Africa *T. congolense* has traditionally been considered the major parasite of cattle, whereas in West Africa *T. vivax* holds the same status. Moreover, in the field, as opposed to experimental situations, mixed infections with various combinations of the three species are common and the clinico-pathological picture is thus further confused. Finally, the effects of trypanosomiasis are most pronounced in pure-bred, "exotic" breeds of imported cattle, less so in indigenous local breeds, and often mild or inapparent in the so called "trypanotolerant" taurine breeds of West Africa such as the N'dama, the Baoulé and the West African Shorthorn.

Fiennes (1970) described bovine trypanosomiasis as a relapsing condition, with successive waves of parasitaemia, accompanied by pyrexia, being followed by parasite destruction and an afebrile period. He divided the disease caused by *T. congolense* and *T. vivax* into two distinct stages, an acute stage with pre-critical and critical phases, and a chronic stage which is post critical. Although a "crisis", or sudden resolution of fever, is not universally recognised as a feature of bovine trypanosomiasis, most workers describe the acute stage of disease as being characterised by febrile periods accompanying

parasitaemic peaks, which become less frequent during the chronic stages in which parasites may be difficult to detect by conventional parasitological techniques.

Studies on the pathogenesis of bovine trypanosomiasis have centred on the development of anaemia, which is the single most significant lesion in this condition. The anaemia in bovine trypanosomiasis is essentially an extravascular haemolytic anaemia (Mamo and Holmes, 1975; Dargie, Murray, Murray, Grimshaw and McIntyre, 1979), with evidence of erythrophagocytosis in many tissues, and, initially, a vigorous secondary haemopoietic response characterised by erythroid hyperplasia and extramedullary haematopoiesis. The mechanism of development of anaemia is complex and only partially understood, and has been variously attributed to pyrexia, production of toxins by trypanosomes (Tizard, Nielsen, Seed, and Hall, 1978), the adsorption of trypanosome antigens or immune complexes on the erythrocyte surface, with complement fixation and erythrophagocytosis or complement-mediated lysis (Kobayashi and Tizard, 1976), disseminated intravascular coagulation, and overactivity of the mononuclear phagocytic system.

The second most obvious clinico-pathological feature of bovine trypanosomiasis is that it is a wasting condition, with progressive emaciation and cachexia which becomes increasingly apparent in cases progressing into the chronic stages of disease. Tracey (1992) reviewed the role of the cytokine tumour necrosis factor (TNF-α) in the pathogenesis of septic shock and cachexia. Through the action of a number of secondary mediators this cytokine can induce a range of biological responses including the clinico-pathological manifestations of septic shock syndrome (not unlike peracute haemorrhagic *T. vivax* infection) and also chronic cachexic states (not unlike chronic bovine trypanosomiasis). TNF-α together with other cytokines such as interleukin -2 and interferon-γ, has recently received increasing attention as an important immunological mediator in trypanosomiasis (de Baetselier, 1993). These cytokines have now been shown to have a potential role not only in the pathogenesis of the disease, but also as an important part of the mechanism by which trypanosomes regulate their own proliferation and suppress the host immune system.

Immunology

Generalised increases in immunoglobulin (Ig) G and particularly IgM have been shown to occur in trypanosome infections (Luckins 1972; Luckins, 1976;

Luckins and Mehlitz, 1978). This hyperglobulinaemia occurs in spite of a generalised immunodepression which results in reduction in the responses of infected cattle to a range of bacterial and viral vaccines (Holmes, Mamo, Thompson, Knight, Lucken, Murray, Murray, Jennings and Urquhart, 1974; Scott, Pegram, Holmes, Pay, Knight, Jennings and Urquhart, 1977).The phenomenon of antigenic variation in trypanosomes is the principal means by which the trypanosome evades the host's immune response and was reviewed by Gray and Luckins (1976), and Vickerman (1978). Trypanosomes comprising a particular parasitaemic peak are usually a mixture of variable antigenic types (VATs) in various proportions (van Miervenne, Janssens, Magnus, Lumsden and Herbert, 1975). The host immune response may eliminate the predominant VATs by the action of specific immunoglobulin, while parasites possessing less well represented VATs are able to proliferate and generate the subsequent parasitaemic peak. The site of antigenic variation has been shown to be the variable surface glycoprotein (VSG) coating the plasma membrane or pellicle of trypanosomes. Individual trypanosomes may switch between a wide repertoire of VATs, by altering the VSG amino acid sequence, a process which is under genetic control (Borst, Arnberg, Bernards, Cross, Frasch, Hoeijmakers and van der Ploeg, 1980; Borst, Frasch, Bernards, Hoeijmakers, van der Ploeg, and Cross 1980; Borst, Fase-Fowler and Gibson, 1981). The destruction of trypanosomes associated with the production of specific immunoglobulin directed against their surface glycoprotein coat has been demonstrated in bovine infections with *T. brucei* (Nantulya, Musoke, Barbet and Roelants, 1979; Musoke, Nantulya, Barbet, Kironde and McGuire, 1981) and *T. congolense* (Masake, Musoke and Nantulya, 1983).

Gross pathology

Gross pathological lesions in trypanosomiasis are generally non-specific and most are secondary to the anaemia. They include emaciation, generalised pallor, atrophy of body fat, splenomegaly, fatty change in the liver, enlargement and flabbiness of the heart, and straw-coloured effusions in the body cavities. The lymph nodes may be enlarged, oedematous or haemorrhagic. More acute cases may show evidence of generalised petechial and ecchymotic haemorrhages (Stephen, 1986).

Histopathology

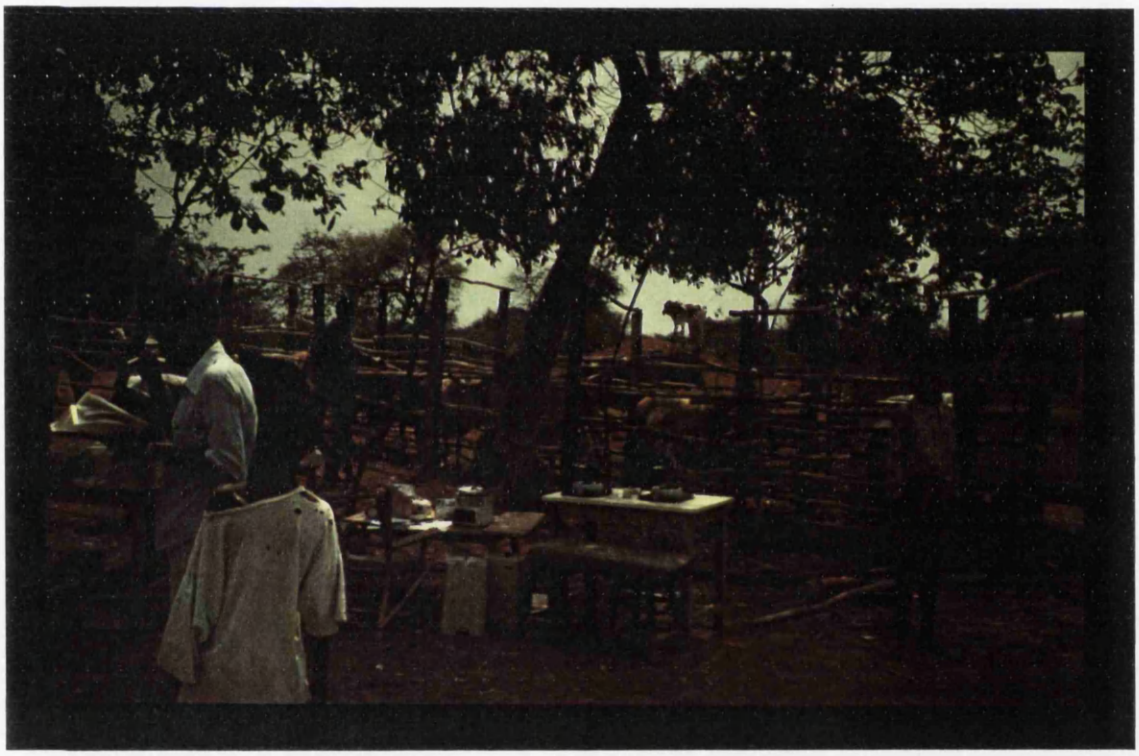
The principal histopathological lesions of bovine trypanosomiasis are hyperplasia of the mononuclear phagocyte system, hyperplasia or hypoplasia of

the lymphoid system, disseminated intravascular coagulation, pulmonary alveolar septal thickening, and testicular degeneration (Anosa and Isoun, 1983). The principal changes affecting the haemopoietic system may be evidence of erythrophagocytosis and haemosiderosis in the lymph nodes, spleen, haemolymph nodes, liver and lungs, erythroid hyperplasia in the bone marrow, and extramedullary haematopoiesis (Stephen 1986).

Akol and Murray (1983) described the morphology and histopathology of local skin reactions in susceptible cattle, frequently termed "chancres" by analogy to similar lesions in primary human syphilis, which developed following the bites of *T. congolense* infected *G. morsitans centralis*. These reactions of delayed onset and variable duration are the first clinical indications of trypanosome infection, although they are rarely observed outside experimental settings. The chancres appeared as small nodules of a few millimetres at the site of challenge after as little as five days, reached maximum size after 10 to 13 days, and had disappeared by 20 to 30 days. At their height, they were raised, indurated, hot, painful swellings up to 100 mm in diameter. An intense inflammatory reaction was observed histologically, with an increase in total cellularity up to 10-fold. This was caused initially by a polymorphonuclear leucocyte infiltration, which was later replaced by a mononuclear infiltrate of mainly small to medium lymphocytes. These changes preceded parasitaemia and other clinical signs by several days, and it was concluded that the skin was acting both a focus of infection and a site for local proliferation of parasites.

Plate 1. Trypanosome-infected Zebu cow in Somalia, showing severe emaciation. Swellings due to the intramuscular injection of isometamidium chloride are visible on the neck. Photograph courtesy of Professor P.H. Holmes.

Plate 2. A well-organised field arrangement for the diagnosis of bovine trypanosomiasis using the HCT-BCT in Busia District, Western Province, Kenya. Adequate facilities for restraining cattle, field microscopes and a microhaematocrit centrifuge, powered by a portable generator, are visible.



Diagnosis

The diagnosis of trypanosomiasis in domestic animals, including tsetse-transmitted bovine trypanosomiasis has been the subject of a number of extensive reviews (Molyneux, 1975; Stephen, 1986; Nantulya, 1990; Luckins 1992; Luckins 1993).

Clinical diagnosis

Clinical signs of acute bovine trypanosomiasis include anaemia, pyrexia, weight loss, abortion, and in the absence of treatment, death (Stephen, 1986). Cases progressing to a more chronic disease state may be characterised by anaemia, cachexia, poor productivity and infertility (see Plate 1). The clinical picture depends to some extent on the species of infecting trypanosomes, and the geographic location. Hyperacute disease associated with *T. vivax* may resemble an acute septicaemia or result in a haemorrhagic syndrome, cases of which may often be found dead.

Classically in West Africa, acute *T. vivax* has been considered more important than *T. congolense*, whereas in Central or East Africa *T. congolense* was considered the more important parasite (Stephen, 1986). Exceptions to this slightly simplistic view occur, such as outbreaks of acute haemorrhagic disease in Coastal Kenya caused by *T. vivax* (Maloo, 1993).

T. congolense infections tend to be less acute and less dramatic, and in that sense are less pathogenic than *T. vivax* infections, although the end result is almost as lethal (Stephen, 1986). *T. brucei* infections are generally regarded as being of low pathogenicity for African cattle which may be infected without showing overt clinical signs, although exotic breeds and imported cattle may be more susceptible (Jordan, 1986).

Under certain circumstances a presumptive diagnosis may be made on the basis of clinical signs alone, particularly anaemia (Luckins, 1992), and the administration of trypanocidal drugs may be warranted on this basis, with a positive response to treatment being interpreted as confirmation of the diagnosis. However, these signs are all non-specific and a number of other disease states which occur in the endemic area may result in the same clinical picture. Hence diagnosis is usually confirmed by the use of more specific diagnostic tests.

Parasitological diagnosis

Blood films

The examination of wet blood films, and Giemsa-stained thick and thin fixed blood films with the aid of the light microscope have been used as diagnostic methods ever since they were first used to identify the aetiological agents of trypanosomiasis. The use of these methods has been reviewed by Killick-Kendrick (1968).

Concentration techniques

Major advances in the diagnosis of trypanosomiasis came with the haematocrit centrifugation technique (HCT) of Woo (1970) in which trypanosomes are visualised in the buffy coat layer of a microhaematocrit centrifuge tube, and its subsequent modification, the phase contrast / buffy coat technique (HCT-BCT), in which the buffy coat is expressed from the microhaematocrit tube onto a glass slide and examined microscopically using phase-contrast or dark ground illumination (Murray, Murray and McIntyre, 1977). A method using miniature anion-exchange columns for the separation of trypanosomes from erythrocytes prior to concentration by centrifugation (Lumsden, Kimber, Evans and Doig, 1979) has not seen widespread application under field conditions. Paris, Murray and McOdimba (1982), compared the effectiveness of some of these newer diagnostic methods with the traditional blood films, and found the HCT-BCT to be the most sensitive technique, followed in order of decreasing sensitivity by the HCT, stained thick film, stained thin film and wet blood film. The sensitivity of the HCT-BCT depended on the species of trypanosome, with the smallest numbers detectable per ml of blood being 2.5×10^2, 5×10^2 and 5×10^3, for *T. congolense*, *T. vivax* and *T. brucei* respectively. A well-organised field diagnostic set-up is shown in Plate 2

Subinoculation methods

Subinoculation methods are those by which trypanosomes are demonstrated by transferring infection from the suspected case to another vertebrate host, an invertebrate host or to an in-vitro culture system. These methods have the additional advantage that stabilates of the isolated parasites may be prepared for further investigation.

Animal subinoculation

Subinoculation of blood from suspected cases into another species, especially laboratory rodents has been widely used, although not all

trypanosomes are infectious for these species. Immunosupression of laboratory rodents either by irradiation or using chemical immunosuppressants such as cyclophosphamide may increase the proportion of trypanosomes resulting in infection (F.W. Jennings, personal communication). Inoculation of susceptible rodents may be more effective for some trypanosomes species, particularly *Trypanozoon* sp. (Godfrey and Killick-Kendrick, 1961), than others. Robson and Ashkar (1972), found far more cases of *T. brucei* infection in Kenya using mouse inoculation than using blood examination, although for *T. congolense* infections mouse inoculation revealed only half as many positive animals as were identified using blood examination. Mouse inoculation failed to pick up any *T. vivax* infections. For this reason subinoculation of domestic ruminants (usually sheep or goats on grounds of expense) rather than rodents may be recommended. *T. vivax* may vary in its ability to give rise to parasitaemias in goats, depending on the geographical origins of the parasites (Peregrine, Moloo and Whitelaw, 1991), and it may be necessary to subinoculate calves to maximise the chance of detecting this species of trypanosome.

Xenodiagnosis

Xenodiagnosis is the feeding of a clean susceptible vector species on a suspected case of trypanosomiasis, after which it is either dissected and examined for the presence of infection, or allowed to feed on a clean animal which is itself examined for the development of infection. It may be the only parasitological means of demonstrating chronic-phase human infection with *T. cruzi (*van Miervenne and le Ray, 1985). Because of the scarcity of laboratory-reared *Glossina* sp. in Africa, this method of diagnosis is rarely attempted for tsetse-transmitted bovine trypanosomiasis. However it may be warranted in particular circumstances if the presence of a trypanosome infection in a particular animal or cattle population is suspected on the basis of surrogate tests (i.e. tests which do not conclusively demonstrate the presence of the parasite, such as antigen-ELISA, see below), but cannot be conclusively demonstrated by other means. The differential susceptibility of different species of *Glossina* should be taken into account if this technique is used, for example, *G. palpalis* is unlikely to become infected with *T. congolense* (Stephen, 1986).

In Vitro Culture methods

The first *in vitro* cultures of bloodstream form trypanosomes were initiated from an intermediate rodent host in which parasites were isolated. Zweygarth and Kaminsky (1990) described a method for isolating *T. brucei brucei* and *T.*

evansi directly in culture from host animals with low parasitaemias which were in some cases not detectable on wet blood film or HCT. A kit for the *in vitro* isolation of trypanosomes (KIVI) has been developed as a means of isolating *T. gambiense* from man (Aerts, Truc, Penchenier, Claes and Le Ray, 1992) and has also been shown to be useful in diagnosis as it may demonstrate the presence of this organism at very low levels in patients' circulation (Truc and Formenty, 1993). The method has also been assessed for use in domestic animals, and shown to detect more *T. brucei* infections than conventional parasitological techniques (McNamara, 1993; Truc and Formenty, 1993).

Immunological diagnosis

Demonstration of trypanosomes, either directly in the blood, or indirectly by subinoculation of animals, culture media or xenodiagnosis all have their limitations in terms of sensitivity and practicability. This, together with a recognition of the invaluable contribution of serological methods to the control of diseases of viral and bacterial aetiologies, has led to considerable research into the development of immunological methods in the diagnosis of trypanosomiasis.

Detection of anti-trypanosomal antibodies

The complement fixation test (CFT) was used extensively and exclusively in the successful eradication campaign in North America (Watson, 1920) of equine *T. equiperdum* infection (dourine), in which trypanosomes are rarely demonstrable in blood or body fluids. Although it has been compared favourably with the indirect fluorescent antibody test in the diagnosis of *T. congolense* infections in cattle (Lotzsch and Deindl, 1974), and has been used in the diagnosis of surra (*T. evansi* infection) in buffalo (Randall and Schwartz, 1936), this test suffers problems in reagent preparation, standardisation and anticomplimentary activity in sera (Luckins, 1993), and has not found a useful role in the diagnosis of bovine trypanosomiasis. The indirect haemagglutination (IHA) test has also found some use in then diagnosis of *T. evansi* infections (Shen, 1974), but was found to be unreliable for use for *T. vivax* infection (Clarkson, Cottrell and Enayat, 1971).

The development of the indirect fluorescent antibody test (IFAT), a primary binding assay, and thus one in which the antibody-antigen reaction is measured directly, was a significant step forward in the detection of anti-trypanosomal antibodies. In this test, blood films from infected animals with high

parasitaemias are fixed, and used as a source of trypanosomal antigens to which anti-trypanosomal antibodies in test sera may bind specifically. Bound antibodies are visualised using anti-species immunoglobulin (e.g. anti-bovine immunoglobulin) conjugated to a fluorescent dye which may be observed using an ultraviolet microscope. The IFAT has been shown to be both sensitive and specific in the detection of bovine anti-trypanosomal antibodies (Wilson, 1969; Luckins and Mehlitz, 1978). Although a degree of cross-reactivity between *T. brucei*, *T. congolense* and *T. vivax* (Ashkar and Ochilo, 1972) means that the IFAT is not reliably species specific, cross-reactivity is not complete and thus all three antigens must be used for maximum efficiency (Luckins, 1993). Other disadvantages of IFAT are the requirement for an expensive ultraviolet microscope, and the subjectivity of the results.

The enzyme-linked immunosorbent assay (ELISA), another primary binding assay, was developed by Engvall and Perlmann (1971) and, in various forms, has since become one of the most widely used and useful techniques in biomedical science. In the ELISA, in its original form, antigens are immobilised by passive adsorption onto a solid-phase, frequently a 96-well polystyrene microtitre plate, and detected using specific antibody labelled with an enzyme which catalyses the conversion of a colourless substrate to a visible coloured product. A more useful variant, the indirect ELISA, is analogous to the IFAT, in that specific antibody in a test sample, if present, binds to the immobilised antigen, and is itself detected by means of enzyme-labelled anti-species (e.g. anti-bovine) conjugate and conversion of substrate to coloured product. In practical terms the attraction of the ELISA is twofold. Firstly the test may be performed without specialised equipment, so that it may be adapted to inexpensive versions for use in the field or under-resourced laboratories. Secondly, with suitable equipment various degrees of automation are possible, allowing a high throughput of test samples in suitably equipped laboratories (Luckins 1992).

The indirect ELISA was adapted for a number of protozoan diseases, including trypanosomiasis (Voller, Bidwell and Bartlett, 1975; Voller, Bidwell, Bartlett and Edwards, 1977), it was shown to be capable of detecting specific antibodies in trypanosome infected cattle (Luckins, 1977) and it was shown to detect more serologically positive cattle than the IFAT (Luckins and Mehlitz, 1978). As with the IFAT, cross reactivity between the three major tsetse-transmitted trypanosome species occurs in the indirect ELISA using crude

antigen preparations, and sera must be screened against all three antigens for optimum sensitivity (Luckins and Mehlitz, 1978; Luckins 1992). The fractionation of trypanosomal antigens by column chromatography has improved the species specificity of antibody detection by ELISA (Ijagbone, Staak and Reinhard, 1989), although this approach has yet to see widespread application.

Another simple antibody detection method which is particularly suitable for field use in Africa is the card agglutination test (CATT), which has recently found widespread application in the diagnosis of *T. gambiense* sleeping sickness (Magnus, Vervoort, and van Miervenne, 1978). The use antigens from particular variable antigenic types (VATs) of *T. gambiense* which are highly conserved across the range of parasites, and thus the majority of infected individuals develop antibodies which cause visible agglutination when whole blood or serum is mixed with the antigen on a card. Although this test has been adapted for use for diagnosis of *T. evansi* in animals (Bajyana-Songa, Hamers-Casterman, Hamers, Pholpark, Pholpark, Leidl, Tangchaitrong, Chaichanopoonpol, Vitoorakool and Theirapataskum, 1987), it is less likely to be applicable to *T. congolense* or *T. vivax* than the *Trypanozoon* species because of the difficulty in identifying suitable VATs in these species (Luckins 1992).

Detection of trypanosomal antigens

While significant advances have been made in the detection of anti-trypanosomal antibodies, all these techniques suffer the drawback that they are unable to distinguish current active infection from infections which have resolved spontaneously, or which have been treated successfully, as antibodies may persist (Luckins, Boid, Rae, Mahmoud, el Malik and Gray, 1979; Luckins 1992). Demonstration of the parasite itself rather than antibodies is necessary for the confirmation of active infection, although it is recognised that the conventional parasitological techniques are relatively insensitive. Modifications of the ELISA have enabled the technique to be used for the detection of antigens, which may be a better indicator of active infection than antibodies. The double sandwich ELISA method for the detection of antigens (Voller, Bidwell and Bartlett, 1976), was shown by Rae and Luckins (1984) to detect antigens in animals within 10 to 14 days of infection with *T. congolense* and *T. evansi*, and these antigens were shown to disappear within 21 days of trypanocidal drug treatment. In this ELISA, polyclonal antibodies raised against

crude trypanosomal antigen preparations were used to coat microtitre plates, and antigen present in test sera bound to the antibody. The bound antigen was then detected using the same antibody conjugated with enzyme and a suitable substrate.

Antigen-detection ELISAs (Ag-ELISA) for tsetse-transmitted trypanosomiasis were developed at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya, using the sandwich ELISA methodology, but incorporated trypanosome species-specific monoclonal antibodies (Köhler, and Milstein, 1975) which reacted with determinants of *T. brucei*, *T. congolense* or *T. vivax* (Nantulya, Musoke, Rurangirwa, Saigar and Minja, 1987). The aim of the development of these tests was to increase the sensitivity of diagnosis, in both the analytical sense (i.e. a smaller quantity of the analyte [trypanosome] can be detected), and in the epidemiological sense (i.e. a greater proportion of infected animals react in the test). In addition it was hoped to maximise the specificity of the tests (i.e. to minimise the number of false positive results). In practice this was equated to lack of cross-reactivity to other protozoon pathogens (Nantulya *et al.*, 1987), and lack of cross reactivity of each species specific test to the other two trypanosome species (Nantulya and Lindqvist, 1989).

Development of Monoclonal Antibodies

The monoclonal antibodies (Mabs) initially described for use in the Ag-ELISA (Nantulya *et al.*, 1987) were raised in Balb/c mice immunised using procyclic forms of *T. brucei brucei* LUMP 427 (Lugala, Uganda), *T. brucei rhodesiense* B704 (Lugala, Uganda), *T. congolense* STIB 212 (Serengeti, Tanzania) and *T. vivax* IL 1392 (Zaria, Nigeria) propagated in-vitro. The hybridomas obtained after fusion of spleen cells of immunised mice with NS1 myeloma cells were screened by indirect immunofluorescence (IFAT), and doubly cloned. Ascitic fluids produced in Balb/c mice were purified using protein-A sepharose (IgG Mabs) or Sepharose 6b filtration (IgM Mabs).

Subgroup specificity

Purified Mabs were tested by IFAT against formaldehyde-fixed whole procyclic trypanosomes. Interestingly, all of seven randomly selected Mabs all gave 100% positivity against homologous (with respect to subgenus) procyclics, and 0% positivity against heterologous procyclics (Nantulya *et al.*, 1987). Within the *T. brucei* subgroup, three IgM Mabs, of which two (TB7/8.1.48 and TB7/8/13.12) were raised against *T. b. brucei*, and one (TR7/47.34.16) against

T. b. rhodesiense, all bound in IFAT to all procyclics of six stocks of *T. b. brucei*, three stocks of *T. b. rhodesiense* and two stocks of *T. b. gambiense*, but not to any procyclics of five stocks of *T. congolense*, five stocks of *T. vivax*, or one stock of *T. simiae*. Tested by IFAT against the same panel of procyclics, an IgG_1 Mab (TC3/17.1.13) and an IgG_3 Mab (TC6/25.25.4) raised against *T. congolense*, bound to all *Nannomonas* procyclics, but not to any *Duttonella* or *Trypanozoon* procyclics; an IgG_3 Mab (TV8/8.33.42) and an IgM Mab (TV8/8.5.38) raised against *T. vivax*, bound to all *Duttonella* procyclics, but not to any *Nannomonas* or *Trypanozoon* procyclics.

Later monoclonal antibodies

Another IgM Mab (TC40/31.15.45), was raised against procyclic trypomastigotes of *T. congolense* KILIFI/83/IL/97 which had been treated with periodate-lysine-paraformaldehyde fixative (periodate digestion was to destroy surface carbohydrates to enhance production of Mabs to protein epitopes), and was tested by ELISA using experimentally infected bovine sera (Nantulya and Lindqvist, 1989).

Later, two further Mabs raised against bloodstream forms were described. The IgM Mab TC39/30.38.16 was used in an attempt to assess test sensitivity by Masake and Nantulya (1991), who state it was raised against *T. congolense* (stock unspecified) as reported by Nantulya *et al.* (1987). However when Mab TC39/30.38.16, and yet another Mab (class unspecified), TV27/9.45.15, were used by Nantulya, Lindqvist, Stevenson, and Mwangi (1992) in Ag-ELISA for field diagnosis, they were described as having being raised against total lysates of bloodstream forms of *T. congolense* and *T. vivax* respectively (stocks unspecified).

Monoclonal antibody specificity

There is now a confusing number of apparently species specific Mabs against salivarian trypanosomes (Table 1). Of these some have been tested in IFAT (Nantulya *et al.*, 1987), some in Ag-ELISA using procyclic lysates (Nantulya *et al.*, 1987), some in Ag-ELISA on samples derived from experimental infections of cattle and or goats, with (Nantulya and Lindqvist 1989) and without treatment (Masake and Nantulya, 1991; Masake, Moloo, Nantulya, Minja, Makau and Njuguna, submitted), some in Ag-ELISA on bovine field samples (Nantulya *et al.*, 1992) and one on field samples from camels (Waitumbi and Nantulya, 1993). In other, numerous publications, Ag-ELISA results from various species, including cattle, pigs, monkeys and man are

presented (e.g. IAEA, 1993; OAU/STRC, 1993), without the precise identity of the Mabs used being identified. Clearly, it is difficult to assess the performance of an immunoassay technique in which there appears to be little consistency in the primary immunological reagents.

Antigen recognition

The first group of anti-trypanosomal Mabs to be described for use in Ag-ELISA (Nantulya *et al.*, 1987), were shown by IFAT to react with molecules on the plasma membrane of live and formaldehyde-fixed homologous procyclic forms, but not bloodstream forms. In the same work, ELISA reactions were obtained with supernatants of procyclic lysates, procyclic culture supernatants and, significantly, lysates of bloodstream forms prepared from stocks isolated in various countries. This suggested that the antigens detected were water soluble, present in more than one stage of the life cycle, and relatively conserved over the parasites' geographic ranges. However, with regard to the bloodstream forms, neither the details of the species, number and origins of the trypanosome isolates tested, nor which Mabs they reacted with, were described.

FAO/IAEA Ag-ELISA kit

The antigen-detection ELISA developed at ILRAD has been introduced in the form of a standardised kit to a number of African institutes involved in tsetse and trypanosomiasis control during the period 1987 - 1992 under a Co-ordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (Ooijen, 1993).

The FAO/IAEA trypanosomiasis Ag-ELISA kit uses three species-specific Mabs, directed against invariant surface antigens of *T. brucei*, *T. congolense* and *T. vivax*, these being of the IgM, IgM and IgG_1 classes respectively. It is understood that these are **TR7/47.34.25**, **TC39/30.38.11** and **TV27/9.45.15** respectively (D. Rebeski, personal communication), of which the first two are assumed to be equivalent to TR7/47.34.16 and TC39/30.38.16 respectively.

Table 1. Monoclonal antibodies to trypanosome invariant antigens.

Mab I.D.	Immunogen	Mab class	Subgroup specificity
TB7/8.1.48[1]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TB7/8/13.12[0]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TR7/47.34.16[0,2,4,5,6]	*T. b. rhodesiense* B704 pt	IgM	*Trypanozoon*
TC3/17.1.13[0]	*T. congolense* STIB 212 pt	IgG_1	*Nannomonas*
TC6/25.25.4[1]	*T. congolense* STIB 212 pt	IgG_3	*Nannomonas*
TC40/31.15.45[2]	*T. congolense* KILIFI/83/IL/97pt	IgM	*Nannomonas*
TC39/30.38.16[3,5]	*T. congolense* bf	IgM	*Nannomonas*
TV8/8.33.42[1,2]	*T. vivax* IL 1392 pt	IgG_3	*Duttonella*
TV8/8.5.38[0]	*T. vivax* IL 1392 pt	IgM	*Duttonella*
TV27/9.45.15[5]	*T. vivax* bf	IgG_1?	*Duttonella*

[0]Reactivity tested in IFAT but not Ag-ELISA against procyclic lysates (Nantulya *et al.*, 1987).

[1]Reactivity tested in IFAT and Ag-ELISA against procyclic lysates (Nantulya *et al.*, 1987).

[2]Reactivity tested in Ag-ELISA against infected cattle sera (Nantulya and Lindqvist, 1989).

[3]Used in Ag-ELISA for *T. congolense* in goats and cattle (Masake and Nantulya, 1991).

[4]Used in Ag-ELISA for *T. evansi* in dromedary camel (Nantulya *et al.*, 1989; Waitumbi and Nantulya, 1993).

[5]Used in Ag-ELISA for field diagnosis in cattle at Nguruman (Nantulya *et al.*, 1992).

[6]Used in Ag-ELISA for *T. brucei* infections in cattle (Masake *et al.*, in preparation).

bf = bloodstream forms

pt = procyclic trypomastigotes

Molecular biological diagnosis

The molecular diagnosis of parasites was reviewed by Nantulya (1991). This approach to diagnosis is based on detection of specific evidence of the presence of the parasite genome, i.e. trypanosome nucleic acids, in samples obtained from the host.

DNA probes

DNA probes utilise nucleotide sequences of no known function, known as "satellites", which are repeated many thousands of times in the parasite genome and which show species-specific differences. The sequences in the probe, which is conveniently labelled with a tracer, usually a radioisotope a non-nuclear label, are hybridised with complementary sequences in the DNA of the test sample which has previously been treated with denaturing agents and split into single strands.

DNA probes have been used to distinguish *Trypanozoon* species from other trypanosome species (Massamba and Williams, 1984), to identify *T. brucei*, *T. vivax* and two types of *T. congolense* in experimentally infected flies (Kukla, Majiwa, Young, Moloo and ole-MoiYoi, 1987), to distinguish the two species of *Nannomonas* and subspecific groups of *T. congolense* (Gibson, Dukes and Gashumba, 1988), and to differentiate *T. congolense* and *T. simiae* in wild caught tsetse flies (McNamara, Dukes, Snow and Gibson, 1989). They have also been used to detect DNA of *T cruzi* in mammalian blood samples (Nantulya, 1991).

Polymerase chain reaction (PCR)

DNA probes may suffer lack of sensitivity if there is an inadequate number of repeats of the diagnostic sequence, or there is insufficient nucleic acid in the test sample. The polymerase chain reaction (PCR) technique provides a means of massive amplification of the diagnostic signal. Segments of DNA flanking the diagnostic sequence, known as primers, are incubated with the test material, together with a DNA polymerase. If the diagnostic sequence is present the complementary strands will be synthesised by DNA chain extension in each direction from the opposing primers. The resulting double stranded DNA can again be split and the cycle repeated several times with an exponential increase in the number of copies of the diagnostic sequence until sufficient material is present to be detected using a hybridization probe. This technique has been applied to the diagnosis of bovine trypanosomiasis using plasma buffy coat

samples from cattle which are aparasitaemic but antigenaemic on the basis of Ag-ELISA (Majiwa Maloo, Thatti and Moloo, 1993).

The main drawback of the PCR technique is its remarkable sensitivity. In theory, a single copy of the diagnostic sequence could be identified, and yet the presence of a minute quantity of trypanosome DNA is insufficient evidence for a viable active infection. Such DNA could have resulted from an infection which failed to become established, or an infection already cleared by self-cure or chemotherapy, or even laboratory contamination. More recent work had attempted to overcome this problem by detecting trypanosome-specific messenger RNA sequences which will only be present within metabolically active viable trypanosomes (Dr. J. Burke, personal communication).

Control methods

Vector control

In spite of attempts to control tsetse flies for more than 60 years, the area of tsetse infestation is actually expanding in many areas of the African continent (MacLennan, 1980).

Bush Clearance

Selective bush clearance removes breeding habitats for tsetse flies, which are highly dependant on correct vegetation types for shelter. For example, clearance of medium sized bushes and trees from riverine vegetation belts may remove the dry season "concentration area" of savannah tsetse, although such techniques are rarely practised today (Jordan, 1986).

Clearance of entire tracts of land using bulldozers and chains, followed by firing of stumps and use of herbicides may be used to create barriers to tsetse re-invasion of cleared areas. However this is extremely costly as a zone as wide as 5 km may be required, with regular re-slashing of regenerating vegetation as.

Elimination of Wildlife

In many areas wildlife may be the main source of blood meals for tsetse. The great rinderpest panzootic of 1895 to 1896 in the Zambezi and Limpopo basins killed nearly all the wild ruminants, and this resulted in the elimination of tsetse and trypanosomiasis from the area (Jordan, 1986). This observation led to the use in the first half of this century of mass destruction of game animals in many countries, a method now considered unacceptable. Selective shooting of particular hosts such as warthog, based on the feeding preferences of particular tsetse species, and the use of fences to separate livestock areas from game sanctuaries may still play important roles in integrated tsetse and trypanosomiasis control programmes.

Insecticidal Spraying

Spraying campaigns have cleared sizeable areas of Nigeria, Zimbabwe, Botswana, and Zambia (Jordan, 1986). However complicated logistics and organisational infrastructure are required and these operations are generally costly. There are also problems related to the ever-present danger of re-invasion of tsetse from adjacent areas and the environmental objections due to non-target effects of insecticides (Douthwaite, 1992).

Residual insecticides

Ground spraying of tsetse habitat usually involves the use of residual insecticides, such as DDT, and dieldrin. Hand spraying of the tree trunks and undersides of low branches is aimed at depositing residual insecticide that will remain active for weeks or even months on tsetse resting sites. Detailed entomological and botanical data are required to achieve high selectivity for particular tsetse species in particular areas, and military-style logistical support is needed. Nevertheless this has been used successfully as the sole control method to clear large tracts of land of tsetse (Jordan, 1986). Some attempts have also been made to use helicopters to replace ground spraying of residual insecticides onto vegetation, but this is difficult and expensive.

Non-residual insecticides

The aim of non-residual insecticide spraying is to produce a fine aerosol mist which will drift through vegetation and kill tsetse directly in flight or on resting sites. Twin engined fixed wing aircraft or occasionally helicopters are used for this technique which is most effective against *G. morsitans*. Passively driven, spinning-cage type sprayers are used to produce droplets of exactly the right size. Ultra low volume applications of the organochlorine endosulphan or the synthetic pyrethroid deltamethrin are used to minimise flight times and thereby costs. Temperature inversion conditions are necessary for effective spraying, and this may require night flying and expensive navigational equipment. Detailed meteorological and entomological data are essential.

Biological control

Biological control is the deliberate use of predators, parasites or pathogens for the control of tsetse. Such techniques are potentially highly specific and environmentally non-contaminating, but have not generally passed beyond the experimental stage.

Sterile Insect Technique

This technique of control of insect pests has been used extremely successfully against the New World screw worm (*Cochliomyia hominivorax*) in the southern United States and Mexico, a serious ectoparasite of cattle in that region, and has been evaluated for use against tsetse in a number of countries (Jordan, 1986). Sterile insect technique (SIT) uses the fact that female tsetse mate only once, so that if the male is sterile, there will be no offspring. By saturating the environment with artificially reared males which have been sterilised by irradiation or chemical means, the tsetse population will no longer

be sustained, and eradication will be the end result. The technique is theoretically attractive, but has a number of practical drawbacks, such as the number of different tsetse species involved, the difficulty in maintaining colonies of tsetse flies in sufficient numbers, and the need for the artificially-reared sterilised male tsetse to be competitive with wild males. Normally other methods, such as use of insecticides are required to reduce tsetse numbers to the level at which SIT is practicable, and pains must be taken to prevent re-invasion from surrounding areas. A current FAO/IAEA project for the elimination of *G. austeni* from the island of Zanzibar is an example of a situation where the technique may likely to succeed, although the operation has been extremely costly.

Traps and Targets

Originally designed for the purpose of monitoring tsetse flies, various types of trap are now also employed for the control of tsetse numbers. Traps may be mono- or bi-conical, or mono- or bi-pyramidal in design, consisting of various combinations of white, blue and black fabric, and targets are usually simple screens of the same materials, impregnated with a residual insecticide with a knock-down effect, such as deltamethrin. These use visual (colour) and olfactory stimuli, such as bovine urine, acetone and octenol to attract flies. Traps and targets have been used successfully to control tsetse flies in a number of areas, such as the Upper Didessa river valley system in south-western Ethiopia (Slingenbergh, 1992). Some recent tsetse control projects have placed emphasis on local community participation in the manufacture and maintenance of targets as an important element of sustainability (Williams, 1993).

Application of Insecticides onto Cattle

In what has effectively been a logical extension of the principle of insecticide-impregnated targets, the application of residual insecticides directly onto cattle has generated great interest in recent years (Wilson, 1993). This technique use cattle themselves a "walking targets" for tsetse flies, and thereby eliminates the requirement for, and costs of research, development and production of the targets themselves, and the chemoattractants for tsetse used with them: cattle themselves produce the necessary olfactory stimuli. Synthetic pyrethroid insecticides, such as deltamethrin (Okello-Onen, Heinonen, Ssekitto, Mwayi, Kakaire and Kabarema, 1993) and cypermethrin (Leak, Woudyalew Mulatu, Rowlands and d'Ieteren, 1993), which have both knock-down and cidal

effects on tsetse, are used. The knock-down effect may last over two months following a single application of insecticide, and while the flies are not killed directly by this effect, in the natural environment they are normally predated by ants within minutes of falling to the ground (Wilson, 1993).

The aim is not to prevent infected tsetse biting the treated cattle, but to reduce and eventually eliminate tsetse populations in the grazing areas. It is therefore essential that a sufficient proportion of the tsetse obtains bloodmeals from cattle rather than untreated wildlife, which could sustain the fly population, and bloodmeal analysis of trapped tsetse is recommended prior to embarking on such a control programme (S.G.A. Leak, personal communication). The insecticides may be applied as pour-on preparations (Leak *et al.*, 1993), which require animals to be reasonably well restrained, or as dips (Okello-Onen *et al.*, 1993) or sprays, in which case the cost of the preparation is lowered because the expensive spreading agent is not required, but dipping and spraying facilities are themselves costly. Where dipping and spraying are in use as methods of control of ticks and tick-borne diseases, the overall cost of changing from a purely acaricidal dip to one which will control both tsetse and ticks may be relatively small, although there may be a conflict of interests in the control of these two types of parasitic diseases. In many areas, development of acaricidal resistance in ticks is considered to be a major threat, and synthetic pyrethroids are being held in reserve to be released for use only when significant resistance to the currently used organophosphorus and organochlorine dips becomes a serious problem. Finally there may be a conflict of interests where overt tick-borne disease, such as babesiosis and anaplasmosis, is held in check by maintaining a situation of enzootic stability within the cattle population. This requires that all stock are exposed to sufficient challenge to these diseases at a young age when they are relatively resistant, so that mild or inapparent infections of little economic consequence are followed by a lifelong state of premunity. Tsetse control programmes utilising applications of insecticides to cattle could potentially perturb the situation of enzootic stability and result in an increase in the incidence of overt tick-borne disease. In spite of these potential problems these techniques are becoming increasingly popular, probably because of the relatively small capital investment required and the fact that there is no need for the extensive organisational infrastructure of spraying or trapping campaigns. In some instances there are early signs of sustainability, as small-scale livestock owners are already seeing economic benefits sufficient

to enable them to bear the costs of the insecticides themselves (Woudyalew Mulatu, personal communication).

Trypanotolerant livestock

The wild animals which are the reservoir of the tsetse-transmitted trypanosome species harbour the parasites without any apparent ill effects, and this is considered to be the result of a long ancestral association over which period the parasites have adapted to cause the minimum harm to their hosts and thereby improve their own chances of survival. Similarly those cattle which have been exposed the longest to tsetse-transmitted trypanosomiasis appear to be the most resistant to the effects of the disease. This trait, termed trypanotolerance is attributed to the taurine breeds (*Bos taurus*) of cattle in West and Central Africa, which can be divided into two groups, the long-horned N'dama breed, and the West African shorthorn breeds such as the Lagune, Baoulé, and Muturu breeds. The N'dama is believed to be descended from the Hamitic longhorn breed which arrived in the Nile Delta from the Near East in about 5000 B.C., while the taurine shorthorn breeds arrived in the same area somewhat later between 2750 and 2500 B.C. (Murray, Trail, Davis and Black, 1984). The more widespread humped cattle (*Bos indicus*), now the most prevalent indigenous cattle in Africa and regarded as highly susceptible to trypanosomiasis did not become numerous in Africa until after the Arab invasion of 699 A.D.

Trypanotolerant cattle have the ability to survive and be productive in tsetse-infected areas without the aid of trypanocidal drugs. Nevertheless these cattle are susceptible to trypanosome infections, although like the wild animal reservoir hosts of trypanosomes they often show relatively few ill effects. The prevalence, level and duration of parasitaemia in trypanotolerant cattle such as N'dama is less than in susceptible Zebu (*Bos indicus*). Moreover, trypanotolerant cattle appear to have the ability to control the anaemia which is a central feature of the disease in susceptible cattle (Murray *et al.*, 1984). This resistance to trypanosomiasis appears to have a genetic basis, rather than being the result of acquired resistance to local strains of trypanosomes, as which have never previously been exposed to trypanosomes manifest the trait on experimental infection, and West African trypanotolerant cattle have been successfully established in distant areas of West and Central Africa where susceptible cattle could not survive.

In spite of these advantages, the distribution of trypanotolerant cattle in Africa is limited, and these breeds only represent about 5% of the total 147 million cattle in the 38 tsetse-infested countries (ILCA, 1979). There are various reasons why trypanotolerant cattle have not become more widespread. The trypanotolerant breeds of cattle are generally smaller than Zebu cattle, and in the past small size has been equated with poor productivity. Recent work, however, has shown productivity to be similar in both types of cattle in conditions of no or low tsetse challenge, and in areas of higher challenge, data could not be obtained because only trypanotolerant cattle survived (ILCA, 1979). The genetic basis of resistance to trypanosomiasis shown by these cattle has been misunderstood, and it has been assumed that trypanotolerance would break down if cattle were exposed to other than local strains of trypanosomes. Finally, cattle are kept for a number of reasons in Africa, including status and economic security, and in this context the size of livestock may have important social and cultural implications; for example, among some cattle herding people, such as the Ankole of Uganda, the size of an animal's horns may be a more important determinant of its perceived worth than any other biological measure of productivity.

The trypanotolerant trait is not absolute, and cattle of trypanotolerant breeds may succumb to the effects of the disease under circumstances of stress, such as poor nutrition, overwork, intercurrent disease or just particularly heavy tsetse challenge. Roelants (1986) analysed data from a number of experimental studies which compared the survival of Zebu and trypanotolerant cattle under different levels of natural tsetse challenge. Under conditions of light tsetse natural challenge, whereas 75% of Zebu cattle died, 98% of N'dama and cattle survived. However, under heavy natural tsetse challenge, overall mortality among Zebu cattle rose to 94%, and 31% of cattle of the trypanotolerant N'dama, Muturu and Baoulé breeds also died. The use of trypanotolerant livestock is thus often supplemented by the use of trypanocidal drugs in areas of heavy tsetse challenge (Roelants, 1986; Otesile, Akpokodje and Ekwuruke, 1991).

Immunological control

The search for an effective vaccine against bovine trypanosomiasis has received considerable input from international research funding bodies in the latter half of the present century, but most workers now accept that one is unlikely to emerge in the foreseeable future. Attempts have been made to

vaccinate cattle using living trypanosomes of low virulence, or attenuated by ionising radiation; using dead parasites exposed to or combined with various chemicals, and drugs; using trypanosome homogenates from organisms disrupted by presses, ultrasonication, lyophilisation, and freezing and thawing; using cell free exoantigens and trypanosomes altered by many other ingenious methods (Stephen, 1986). Although varying degrees of success have been claimed for these methods, a satisfactory vaccine affording protection against natural challenges has eluded all attempts.

The development of a vaccine protective against bovine tsetse-transmitted trypanosomiasis is probably one of the greatest challenges facing the veterinary immunologist. Firstly there are three distinct species of trypanosome involved in the disease complex, *T. brucei*, *T. congolense* and *T. vivax*. Secondly, each of these species is composed of a large but unknown number of antigenically distinct strains. Finally the phenomenon of antigenic variation which the trypanosome uses to evade the natural immune response of the host is as effective against artificially induced immune responses directed against the parasites surface. Immune responses against particular VATs may be induced which are effective against homologous challenge, but protection has not been achieved against heterologous strains or VATs (Akol and Murray, 1983). A vaccine incorporating components directed against the entire repertoire of VATs presented by all trypanosome strains of all species is most unlikely as a single clone of *Trypanozoon* has been shown capable of expressing over 100 distinct VATs (Capbern, Girond, Baltz and Mattern, 1977).

Trypanocidal drugs

Introduction

Because of the difficulties associated with vector control, the absence of an effective vaccine, and the limited numbers and distribution of trypanotolerant cattle, in most tsetse-infected African countries the use of trypanocidal drugs is the mainstay of control of bovine trypanosomiasis (Jordan 1992), and is likely to remain for the foreseeable future. The use of trypanocidal drugs has been the subject of a number of valuable reviews (Davey, 1957; Whiteside, 1962a; Williamson, 1962, 1970, 1976; Hawking, 1966; Leach and Roberts, 1981; Stephen, 1986; and Losos, 1986).

There are two main strategies for the use of trypanocidal drugs in the control of bovine trypanosomiasis. Drugs may be used for the therapy of existing trypanosome infections, in which case they are termed chemotherapeutic, or alternatively drugs with a prolonged period of biological activity may be administered at intervals suitable to uninfected cattle at risk of becoming infected, in which case they are termed chemoprophylactic. Some drugs may be used for either purposes, although dose rates and routes of administration may be adjusted for the particular circumstances of use, while others, particularly those which are eliminated rapidly are limited to therapeutic use. The principal drugs used in the control of African bovine trypanosomiasis are listed in Table 2.

Table 2. The principal drugs used in the control of African bovine trypanosomiasis (Adapted from Sones, 1988).

Chemical Name	Chemical Class	Use	Commercial Names
Diminazene aceturate	Aromatic diamidine	Chemotherapy	Berenil (Hoechst), Veriben Ganaseg
Homidium bromide Homidium chloride	Phenanthridine	Chemotherapy	Ethidium (Laprovet) Novidium (Rhône Mérieux)
Isometamidium chloride	Phenanthridine	Chemotherapy & Chemoprophylaxis	Samorin, Trypamidium (Rhone Mérieux)
Quinapyramine sulphate	Quinoline - pyramidine	Chemotherapy	Trypacide sulphate (Rhone Mérieux)
Quinapyramine sulphate/chloride	Quinoline - pyramidine	Chemotherapy & Chemoprophylaxis	Trypacide Prosalt[1] (ICI) Tribexin Prosalt[2] (Indian Drugs and Pharmaceuticals Ltd).

Historical Perspective

Only a small number of trypanocidal drugs have survived preliminary field trials to become established for use in the control of bovine tsetse-transmitted trypanosomiasis (Williamson, 1970). These are tartar emetic, quinapyramine, the phenathridinium drugs homidium, prothidium and isometamidium, and the diamidine diminazene aceturate, and of these only three, homidium, isometamidium and diminazene remain in widespread use.

Tartar Emetic

The first trypanocidal agents to be used to any significant extent in cattle were potassium antimony tartrate (tartar emetic), and the more soluble, less irritant sodium analogue which were shown by Plimmer and Thompson (1908) to eliminate *T. brucei* and *T. evansi* in laboratory rodents. The drug was subsequently used in cattle, but severe tissue reactions meant the intravenous route of administration was necessary, with the attendant difficulties in range cattle unused to handling. A dose of 1.0 - 1.5 mg per animal as a 5% aqueous solution was given daily or weekly. The efficacy of tartar emetic against *T. congolense* and *T. vivax* in cattle was confirmed by Bevan (1928) and Curson (1928), but not against *T. brucei* infections. Up to 6% mortality occurred, but mortality rates in unprotected cattle exposed to tsetse challenge was considerably worse. In spite of the development of newer, safer drugs, tartar emetic continued to be used up to early 1950s (Leach and Roberts, 1981).

Suramin

Suramin synthesised by Bayer chemists in Germany during WW1, became available 1920, and was shown to be effective against experimental *T. equiperdum* infections and naturally occurring *T. evansi* infections in camels (Knowles, 1925), and other species (Edwards, 1926). Suramin has significant prophylactic activity, conferring protection for up to two months (Findlay, 1930), and has been widely used human, equine, and cameline trypanosomiases, but not in cattle. On its own, suramin has no pronounced activity against *T. congolense* or *T. vivax* infections, and is thus not particularly useful for bovine tsetse-transmitted disease.

The use of suramin complexes, or "suraminates" was discussed at length by Williamson (1970). The salts produced by complexing suramin with other trypanocidal drugs, originally the human trypanocide pentamidine (Guimaraes and Lourie, 1951), are insoluble and may confer long periods of prophylaxis

with little systemic toxicity. However because of severe local reactions, including sloughing of the entire injection site (Stephen, 1986) these complexes have not been widely used in spite of their ability to confer long prophylactic periods in cattle.

Antimosan, Stibophen and the Styrylquinolines

Two other groups of compounds were investigated at about the same time as suramin. Antimosan, a sulphonated pyrocatechol was derived from trivalent antimony following research on tartar emetic, and its sodium salt, stibophen, were found to be effective against *T. congolense* or *T. vivax* , but not as effective against *T. brucei* (Parkin, 1931; 1935). This drug could be administered intramuscularly or subcutaneously, but repeated doses were required at four weekly intervals. The styrylquinolines were found to be active against the subgenus *Trypanozoon*, but suffered problems of toxicity, both systemic, and at the site of injection (Browning, Cohen, Ellingworth and Gulbranson, 1926).

Phenanthridines: dimidium

A significant advance in the development of trypanocides came with the investigation of the phenanthridines. Browning, Morgan, Robb and Walls (1938) reported that phenidium chloride was active against *Trypanozoon*, and this activity was confirmed in field trials in Africa, although the drug was poorly soluble and had a narrow therapeutic index. Another phenathridine, dimidium had better solubility, and eliminated the majority of *T. congolense* infections in the field when administered subcutaneously at a dose rate of 1.0 mg per kg body weight (Carmichael and Bell, 1944). The use of this drug was applied in mass treatment campaigns in East and Central Africa, and heralded a new era in trypanocidal chemotherapy: by 1952 trypanosomes resistance to this drug were considered to be widespread. The narrow therapeutic index of the drug prevented increasing the dose rate as a means of overcoming drug resistance, as photosensitization occurred with doses of just 2.0 $mgkg^{-1}$ (Randall and Beverage, 1946), and sometimes as little as 1.0 $mgkg^{-1}$ (Evans, 1948).

Quinapyramine

The synthesis of quinapyramine by Barret, Curd and Hepworth (1953) followed earlier work on an unsuccessful trypanocide, the 4-aminoquinoline derivative, surfen C (Jensch, 1937). Surfen C had marked activity against *T. congolense*, but cattle treated in field trials in Africa suffered serious local and

systemic toxicity, including death within 15 minutes of injection (Le Roux, 1936; van Rensberg, 1938). Quinapyramine, a bis-quarternary compound synthesised by restructuring the surfen C molecule, was far more successful as it retained the trypanocidal activity, but toxicity was far less of a problem as it is generally well tolerated in cattle. The dimethosulphate was shown experimentally by Curd and Davey (1949, 1950) to be active against most of the African trypanosomes pathogenic to livestock, and this was confirmed in field trials (Davey, 1950). The drug had a marked prophylactic activity, particularly when a mixture of three parts of quinapyramine dimethosulphate to four parts of the relatively insoluble quinapyramine chloride was used (the chloride alone would not attain curative blood levels). This mixture was marketed by ICI as Antrycide Prosalt, and the formulation was later changed in 1958 to three parts dimethosulphate to two parts chloride in the revised formulation (Prosalt RF) which was cheaper to produce and equally effective (Marshall, 1960). Prosalt RF provided three months protection to cattle in areas of low tsetse challenge and two months where challenge was rated medium (Davey, 1958). The prophylactic activity of quinapyramine was however seen partly as a disadvantage, as it was considered to be a possible factor in the development of resistance to this drug. By 1977 resistance was widespread, and Antrycide was withdrawn from the market, although quinapyramine has since been reintroduced by May and Baker as Trypacide®, for the treatment of *T. evansi* infections in camels and horses.

Homidium

Watkins and Woolfe (1952) reported the development of homidium bromide, a new aminophenanthridium compound, by the substitution of an ethyl for a methyl-group on the quaternary nitrogen atom of dimidine. Homidium bromide was as effective against *T. congolense* and *T. vivax* as dimidine bromide, but was significantly less toxic at 1.0 $mgkg^{-1}$ (Ford, Wilmshurst and El Karib, 1953a,b). The new drug had limited prophylactic activity (Leach, El Karib, Ford and Wilmshurst, 1955). Homidium bromide was marketed as Ethidium® by Boots (later by Camco, and more recently by Laprovet), while May and Baker patented the chloride salt, which had the advantage over the bromide of solubility in cold water (Wragg, 1955), as Novidium.

Quinapyramine and homidium were used in mass treatment campaigns of cattle exposed to tsetse challenge. In six years treatments in Northern Nigeria increased over ten-fold to 641 000 in 1957-8 (Wilson, 1960). The availability of

these drugs allowed susceptible cattle to be kept commercially in tsetse-infested areas, such as Mkwaja ranch in north-east Tanzania (Ford and Blazer, 1971). As usual, with the advent of mass treatment, reports of drug resistance soon followed, and by 1966 homidium-resistant strains of trypanosomes were reported to be widespread (Jones-Davies and Folkers, 1966). Antrycide and Ethidium were withdrawn from field use in Northern Nigeria on account of drug resistance in 1963 and 1965 respectively (Williamson, 1970).

Diminazene Aceturate

Another major development in the chemotherapy of bovine trypanosomiasis was the introduction of the aromatic diamidine, diminazene aceturate by chemists at Hoechst in Germany (Bauer 1955a,b; Fussgänger, 1955). This followed earlier work which showed that synthalin, a synthetic hypoglycaemic agent, was active against trypanosomes *in vivo* (von Jansco and von Jansco, 1935) and *in vitro* (Lourie and Yorke, 1937). The finding of trypanocidal activity *in vitro* was of significance, because showed the compound had direct trypanocidal activity, rather than merely an indirect effect on trypanosome metabolism secondary to the lowering of blood glucose levels. This focused synthetic activity on the diamidines, and led to the synthesis of pentamidine and many experimental compounds including, phenamidine, propamidine, stilbamidine, and M & B 2242 (Williamson, 1970).

Like quinapyramine, diminazene aceturate was developed from a systematic dissection of the structure of the surfen C molecule (Jensch, 1958). On the basis of earlier work, the inter-ring bridge grouping of symmetrical "bis guanylphenyl-Typ" molecules derived from structure-activity studies on surfen C was varied, and the maximal trypanocidal activity was found when two amidophenyl moieties were joined by a triazene bridge to produce the diminazene molecule. In extensive field trials, this compound was found to be active not only against trypanosomes, particularly *T. congolense* and *T. vivax* (Fussgänger and Bauer, 1958), but also against *Babesia*, for which it is still used as one of the main therapeutic agents in cattle. The drug was less active against *T. brucei* infections in cattle, which required an increase in dosage from the 3.5 $mgkg^{-1}$ found to be effective for *T. congolense* and *T. vivax* to 5.0 $mgkg^{-1}$. The use of this drug was quickly adopted in many parts of Africa, so that by 1961 an average of 190 000 annual doses were being used in Kenya (Fairclough, 1963b), and in Northern Nigeria it replaced quinapyramine and

homidium (MacLennan, 1968) which were becoming less useful as a result of drug resistance.

Williamson (1970) considered the three reasons for the success of diminazene to be, firstly its higher therapeutic index than any other curative or prophylactic drug, secondly its efficacy against trypanosome infections resistant to other trypanocides, and finally the absence of any serious drug-resistance to the compound even after ten years of widespread use. The apparent inability of the compound to give rise to resistant trypanosomes was attributed to its rapid excretion and consequent lack of appreciable prophylactic activity (Fairclough, 1963b, c). Nevertheless, with continued use resistance even to diminazene became apparent, with isolates of resistant *T. congolense* (MacLennan and Jones-Davies, 1967) and *T. vivax* (Jones-Davies, 1967a).

Pyrithidium

By the substitution of the pyrimidyl moiety of quinapyramine into a phenathridine resembling phenidium, Watkins and Woolfe (1956) synthesised a new trypanocide, pyrithidium bromide (Prothidium, Boots) which was shown to possess therapeutic and prophylactic activity (Watkins, 1958). Doses of 0.2 to 0.4 $mgkg^{-1}$ were curative for *T. congolense* infections in cattle (Whiteside, Fairclough and Bax, 1960), and prophylaxis of cattle in an area of high tsetse challenge was demonstrated (Finelle and Lacotte, 1965). Toxicity problems were however observed, with severe local reactions when 2.5 $mgkg^{-1}$ was administered subcutaneously (Stephen, 1962a), or deaths following intramuscular doses of 5.0 $mgkg^{-1}$ (Leach and El Karib, 1960). The problem of local reactions could be reduced if the drug was incorporated into a mixture of lanolin and olive oil, and in this form doses of up to 6.0 $mgkg^{-1}$ could be injected subcutaneously into the dewlap (Cawdery, 1963). Resistance to pyrithidium occurs readily, with cross-resistance to quinapyramine and homidium because of the close relationship of the chemical structures (Leach and Roberts, 1981) and the product was withdrawn from the market in 1985.

Isometamidium

The most recent trypanocide to be introduced for the chemoprophylaxis and chemotherapy of African bovine trypanosomiasis was isometamidium chloride, marketed by May and Baker (now Rhône-Mérieux, France) in 1961 as Samorin® in Anglophone countries, and later as Trypamidium® (Specia, now Rhône-Mérieux) in Francophone countries. This product resulted from work by Wragg, Washbourne, Brown and Hill (1958), who prepared the p-

amidinophenyldiazoamino derivative of homidium chloride. The linking group was selected on the basis of the similarity of the resulting product to diminazene. The reaction was reported to produce two isomers, a relatively water-insoluble purple isomer (melting point 287 - 289°C), and a red isomer (melting point 236 - 240°C), which could be separated by precipitation from water with sodium chloride, followed by fractional crystallisations from water and methanol. Extensive testing of structure-activity relationships between similar pairs of isomers with various substituents showed the most promising product to be a mixture of the purple and red isomers at a ratio of approximately 55% to 44%, which was designated M & B 4404, and later called metamidum chloride hydrochloride. This product was highly active therapeutically and unlike homidium or diminazene, which had no appreciable prophylactic activity at 1/3 of the LD_{50}, had significant prophylactic activity at 1/9 of its LD_{50}. The structure of the more water soluble and more active red isomer was described by Berg (1960, 1963), who isolated what he considered to be a purer form in sufficient quantities for field trials. The new product, 7-(m-amidinophenyldiazoamino)-2-amino 10-ethyl 9-phenylphenanthridinium chloride hydrochloride, ($C_{28}H_{25}ClN_7$.HCl, molecular weight 531.5) was named isometamidium (M & B 4180A) and marketed as Samorin® or Trypamidium®. The commercial product contains approximately 70% isometamidium chloride (i.e. the red isomer), 20% of the purple isomer, 10% of bis compound (Hutchinson, 1981) small quantities of another positional isomer, and traces of homidium (J. Wilkes, personal communication).

Fairclough (1958) reported the results of trials in Africa on the metamidium isomeric mixture (M & B 4404) in terms of tolerance, therapeutic activity, prophylactic activity and activity against drug-resistant trypanosomes. Sub-cutaneous doses of 5.0 and 10 $mgkg^{-1}$ were poorly tolerated and resulted in signs of local and systemic toxicity, but 1.0 $mgkg^{-1}$ was considered to be "non-toxic". Doses of 0.2 $mgkg^{-1}$ were curative for *T. congolense* infected cattle, while as little as 0.05 $mgkg^{-1}$ was curative for *T. vivax*. In other therapeutic trials doses of 0.5, 1.0 and 2.0 $mgkg^{-1}$ of M & B 4404 resulted in pronounced local reactions, but the drug was considered curatively excellent (Kirkby, 1961a).

In prophylactic trials, 1.0 and 2.0 $mgkg^{-1}$ doses of M & B 4404 gave periods of protection similar to, or slightly longer than those of Antrycide Prosalt (Kirkby 1961c). In trials in West Africa with controlled infection, intramuscular doses of 3.0 $mgkg^{-1}$ were found to give 19 weeks protection (Gray and Stephen, 1962),

although in another trial in Northern Nigeria, Kirkby (1961b) concluded the same dose to be too toxic for use. Smith and Brown (1960) using the drug at 4.0 $mgkg^{-1}$ obtained significantly longer periods of prophylaxis (mean 18 weeks) in an area of heavy tsetse challenge than that obtained using Antrycide Prosalt (mean 10 weeks). Finally, 29 weeks protection against West African *T. vivax* were obtained using 5.0 $mgkg^{-1}$ (Stephen, 1960).

Later attention focused on the newer formulation isometamidium (M & B 4180A), which was recommended in 1961 by the manufacturers to be administered by deep intramuscular injection at dose rates of 0.5 $mgkg^{-1}$ as a curative agent for normal trypanosome strains, at 1.0 - 2.0 $mgkg^{-1}$, as a curative agent for drug-resistant strains, and at 2.0 $mgkg^{-1}$ for prophylaxis.

Future Prospects

New trypanocidal drugs

Since the introduction of isometamidium chloride in 1961 no new trypanocides suitable for the control of African bovine trypanosomiasis have been released onto the market. Melarsenoxide cysteamine (MelCy, Cymelarsan, Rhône Mérieux), an organic arsenical suitable for the treatment of *Trypanozoon* infections, was recently launched particularly for the treatment of *T. evansi* infections in camels (Raynaud, Sones, and Friedheim, 1989). However, this compound is not effective against *T. congolense* or *T. vivax*, and it also lacks significant prophylactic activity.

The scarcity over the last 30 years of new trypanocidal drugs suitable for the control of African bovine trypanosomiasis has been attributed to the high costs of drug development, licensing and marketing, which represents a large investment to pharmaceutical companies. The potential markets for these drugs are almost entirely confined to developing countries with limited foreign exchange and frequently uncertain political and economic stability (Williamson, 1976; Holmes and Torr, 1988; Murray, Stear, Trail, d'Ieteran, Agyemang and Dwinger, 1991). These same countries may nevertheless have stringent drug registration requirements which necessitates a major investment with no guarantee of successful economic return. These factors combine to make this market relatively unattractive to the major multinational pharmaceutical companies, a few of whom somewhat reluctantly continue to manufacture the existing trypanocidal drugs. The lack of new drugs highlights the importance of optimising the use of the existing limited range of trypanocidal compounds,

particularly in view of the ever present threat of the development of drug resistance.

New Formulations of Existing Drugs

New formulations of the existing trypanocidal drugs may provide a means of improving chemotherapy and chemoprophylaxis with as great an investment as the development of completely new drugs. Two aspects of drug delivery show considerable potential for improvement. Firstly many trypanocidal drugs, particularly those used for chemoprophylaxis cause considerable local side effects at the site of injection. Secondly, new administration strategies may increase the duration of action of trypanocidal drugs, thus extending the effective prophylactic period afforded by chemotherapeutic agents, or allowing chemotherapeutic agents to be used for prophylactic purposes.

Sustained release preparations

Hope-Cawdery and Simmons (1964) experimented with formulations of isometamidium in a high viscosity base, but with disappointing results. Other workers have used isometamidium-dextran complexes, with reduction in local toxicity and extended prophylaxis in rodents (James, 1978; Aliu and Sannusi, 1979). Subcutaneous injections of isometamidium-dextran complexes produced small nodules at the site of injection in cattle, but there was no severe local reaction with either subcutaneous or intramuscular injection (Aliu and Sannusi, 1979). There was no improvement in the cure rate of *T. vivax* infections in cattle using the complexes at 0.5 $mgkg^{-1}$, although it was concluded that the complex given at 1.0 or 2.0 $mgkg^{-1}$ could be expected to protect cattle against infection, whilst yielding a good carcass and hide grading. Nevertheless, the use of isometamidium-dextran complexes has not progressed beyond the experimental stage.

Other investigators have experimented with the incorporation of isometamidium into multilamellar phospholipid lysosomes. This system of drug delivery was developed for treatment of leishmaniasis, and was found particularly suitable because the drug-containing lysosomes, once injected, are phagocytosed by the very cells of the mononuclear phagocytic system which are parasitised by the intracellular stages of *Leishmania* (Alving, Steck, Chapman, Waits, Hendricks, Swartz and Hansen). In experiments in goats using lyposomes incorporating tritiated isometamidium, Fluck (1985) found reduced tissue reactions on intramuscular injection, and more sustained blood levels based on plasma radioactivity, when the lysosomal preparation rather

than the standard aqueous isometamidium solution was injected. Again this system of drug delivery has not progressed beyond the experimental stage.

Slow-Release devices

Slow release devices (SRDs) prepared by extrusion of a mixture of a polyester composed of a copolymer of ε-caprolactone and L-lactide (molar ratio 80:20) containing 25% by weight of homidium bromide as cylindrical fibres of 1.7 mm diameter have been developed by Geerts, De Deken, Kageruka, Lootens and Schacht (1993). These SRDs were evaluated in prophylaxis experiments in rabbits challenged with different stocks of *T. congolense*, in which they were compared with conventional intramuscular injections of 1.0 $mgkg^{-1}$. Ninety-five percent of rabbits implanted with SRDs were protected against challenge for three months, whereas only 30.8% of rabbits which received the conventional intramuscular injection of homidium bromide at 1.0 $mgkg^{-1}$ were protected for one month.

Similar devices containing isometamidium chloride are currently being evaluated by the same workers in cattle under field tsetse challenge in Africa. The potential advantages of such devices are a longer, more uniform and more reliable prophylactic period, and a significant reduction in the local tissue reaction at the site of injection / implantation. A potential disadvantage of SRDs might be the prolongation of the period during which the drug is circulating at levels below the effective trypanocidal concentration, and which may therefore be conducive to the development of drug resistance. The economic constraints on the licensing and marketing of new trypanocidal drugs may however apply to some extent to these SRDs, and it is unlikely that their use will become widespread in the near future.

Chemotherapy

Chemotherapy of bovine tsetse-transmitted trypanosomiasis now relies on the use of three compounds, isometamidium, homidium and diminazene, of which isometamidium and possibly homidium also have significant prophylactic activity. Jordan, (1986) considered the ideal circumstances for chemotherapy to be when livestock are under occasional risk of trypanosomiasis, such as during the seasonal occupation of tsetse-infested areas, on the fly-infested margins of naturally fly-free or reclaimed areas, or following sporadic infection caused by flies carried by vehicles, or in limited foci of infestation.

Properties of the ideal chemotherapeutic agent.

Stephen (1986) listed the properties of an ideal drug for the treatment of bovine trypanosomiasis as being that it should:

- cause a minimum of irritation and tissue necrosis at the site of injection
- have a wide therapeutic index, with special reference to *T. congolense* and *T. vivax* infections.
- be transparent at normal concentrations
- be readily soluble in water
- be safe for human consumption in residual quantities
- be cheap
- be effective over a dose range proportional to the weight estimation error in the field.

Chemoprophylaxis

Since the withdrawal of prothidium and quinapyramine, isometamidium chloride is now the only widely used compound for the chemoprophylaxis of African bovine tsetse-transmitted trypanosomiasis. Jordan, (1986) considered the conditions necessary for effective chemoprophylaxis to be even more stringent than those necessary for effective chemotherapy, in particular the strict supervision of drug administration and animal husbandry. The administration of repeat doses before drug levels fall below trypanocidal levels is necessary to avoid conditions liable to induce the development of drug resistance (see below). Trained staff, reliable transport, and access to livestock are therefore important (Leach and Roberts, 1981). Hence, chemoprophylaxis is more appropriate for well organised ranches and probably inappropriate for nomadic or semi-nomadic livestock in pastoral management systems (Jordan, 1986). Prophylactic administration of isometamidium chloride to a small East African Zebu cow by intramuscular injection is shown in Plate 3.

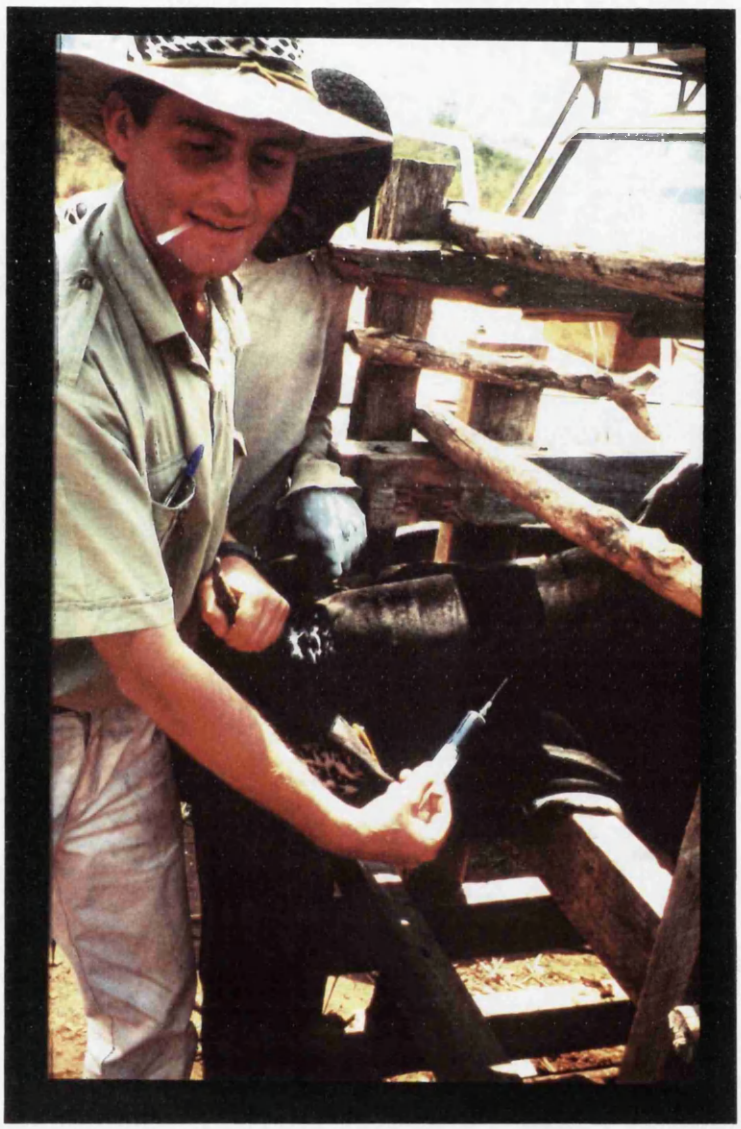
Properties of the ideal chemoprophylactic agent.

Stephen (1986) listed the properties of an ideal chemoprophylactic drug for the prevention of bovine trypanosomiasis as being that it should:

- cause a mild reaction at the site of injection - at least not more severe than that with Antrycide Pro-salt
- be non-toxic at the recommended dose, and produce no harmful effects in the developing embryo
- be easy to make up and administer
- be administered in modest total volumes e.g. 15 to 20 ml

- be suitable for subcutaneous injection, without causing damage or injury to the skin or underlying muscles
- provide prophylaxis against *T. vivax* and *T. congolense* for periods satisfactory to the policy of the administering and control agency, by the expediency of the varying the dosage rate
- not induce resistant strains in 'breakthrough' trypanosomes
- have chemotherapeutic activity at the recommended prophylactic doses
- present a resolved or insignificant deposit by the end of the prophylactic period
- be reasonably cheap
- be effective over a dose range proportional to the weight estimation error in the field.

Plate 3. Prophylactic administration of a dose of Samorin® (isometamidium chloride) by intramuscular injection to a small East African Zebu cow in Busia District, Western Province, Kenya.



Pharmacology of Isometamidium

The pharmacology of isometamidium was reviewed by Kinabo and Bogan (1988d) and Kinabo (1993). The name "Isometamidium" has been used extensively in the literature to refer to the commercial preparation Samorin® (also Trypamidium®), rather than in the pure sense to refer to the red isomer present in the mixture. This usage will also be adopted in this thesis: where the red isomer is being referred to exclusively, this will be stated explicitly. The commercial preparation is presented as a dark reddish-brown powder with a solubility of 6% (w/v) in water at 20°C, with lower solubility in pure organic solvents. It is labile under low and high pH conditions and at high temperatures (Kinabo and Bogan 1988d).

Studies on the pharmacokinetics of isometamidium have been hampered by the lack of an analytical method capable of measuring levels of the drug in animals for more than a few hours following treatment (Kinabo, 1993). Nevertheless, studies have been conducted in goats (Kinabo and McKellar, 1990), pigs (Kinabo, McKellar and Eckersall, 1991), camels (Ali and Hassan, 1984) and cattle (Kinabo and Bogan, 1988b).

Following intramuscular injection of cattle, isometamidium is detectable in plasma almost immediately, and maximal concentrations are observed within an hour, after which they fall rapidly to very low concentrations (Kinabo and Bogan, 1988b). In goats, the mean bioavailability of isometamidium has been calculated to be very low, at around 27% (±13%) (Kinabo and McKellar, 1990). The low bioavailability was considered to be a reflection of binding of the drug to tissue macromolecules and ground matrix components at the injection site. This tissue-drug complex was considered to be analogous to a slow-release drug formulation and to constitute the primary depot from which drug was slowly absorbed at low levels to exert its prophylactic activity (and see Plate 6). The low bioavailability was therefore not necessarily as disadvantageous as would be held conventionally for other drugs.

The apparent volume of distribution of isometamidium in goats was greater than 1 lkg^{-1}, which indicates that the drug is accumulated in tissues at concentrations higher than those of the plasma (Kinabo and McKellar, 1990). High concentrations of the drug have been found at the injection site, in the liver, kidney and spleen of calves up to six weeks following intramuscular injection (Kinabo and Bogan, 1988b).

The studies of Hill and McFadzean (1963) on rats and mice shed light on the relative importance of the primary deposit at the injection site and secondary drug deposits in other organs in maintaining prophylactic activity over a prolonged period. When mice were given subcutaneous injections of isometamidium they were protected against intraperitoneal challenge with *T. congolense* for four weeks as opposed to one week for mice which received the drug intravenously. Rats in which the drug was injected into the tail were protected against challenge for up to 16 weeks, but if the tail was amputated 24 hours after treatment, the period of protection was reduced to one - two weeks. Fluorescence of the drug was observed at the injection site for several weeks following subcutaneous treatment of both rats and mice, but fluorescence in liver and kidney was only observed for up to one week. From these results it was concluded that the depot at the injection site was of primary importance for prophylaxis, while the depots in the liver and kidney were of secondary importance only.

The discovery that phenanthridinium derivatives, including isometamidium have activity against certain types of tumour cells led Philips, Sternberg, Cronin, Sodergren and Vidal (1967) to consider the drug as a potential anti-cancer agent in humans and to conduct extensive experiments to obtain information for use in initiating a clinical study. Using a spectrophotometric method to measure the level of isometamidium in the liver of rats, 41% of a 1.0 $mgkg^{-1}$ intravenous dose was present within 1 minute of injection, and by 10 minutes this figure rose to 62%. In addition, approximately 5% of the administered dose was detected in kidney as early as 1 minute after injection. The drug was only detected in blood in the two earliest samples taken at one and 1.5 minutes following injection; none was detected thereafter. None of the drug could be detected in urine over three days following a 20 $mgkg^{-1}$ subcutaneous injection, which was considered remarkable since the analysis was capable of detecting as little as 0.07% of the dose administered. Amounts equivalent to 0.7 - 2.3% of the dose per day were detected in faeces over the same period. In other experiments isometamidium could not be detected in quite large volumes of bile (up to 4ml) obtained by cannulation bile ducts of treated rats, although it was readily detectable in the livers.

Experiments to investigate the distribution of isometamidium in sub-cellular fractions derived from rat hepatic cells obtained 24 hours following treatment, showed that 60% of the drug had been concentrated in the mitochondrial

fraction, with the remainder distributed between the nuclei and the microsomal fractions, but essentially none was present in the supernatant (Philips *et al.*, 1967). The binding of isometamidium to rat serum was also investigated, by partitioning between hexanol and aqueous phases, and measurement at the absorbance maximum of the drug in solvent saturated with Tris (380nm). At equilibrium, when the total concentration of isometamidium in the aqueous phase (serum) was 16 to 23 μgml^{-1}, between 86% and 97% of the drug was bound (Philips *et al.*, 1967). Similar results were obtained using 2% bovine serum albumin (BSA) as the aqueous phase; at equilibrium when the drug concentration in the aqueous layer was 14 to 18 μgml^{-1}, 86% to 89% of the drug was bound. When the concentration of BSA was reduced to 0.2%, isometamidium concentrations in the aqueous phase fell to 9 to 13 μgml^{-1}, of which 66% to 74% was bound. Binding of isometamidium to dialysed rat serum was also found to alter the absorption spectrum of the drug by increasing the wavelength of absorption maxima and minima; BSA had a similar effect, suggesting that albumin was the major binding component in serum (Philips *et al.*, 1967). Major spectral changes were also obtained using nucleic acids, heparin and hyaluronic acid, which were distinguishable from those of BSA and from each other.

Smith, Mayambo, Dunlop and Holmes (1991) used Western blotting and an enhanced chemiluminescence detection system to demonstrate the binding of isometamidium to bovine serum protein macromolecules. An isometamidium-positive band of approximate molecular weight 140 kD was observed in blots of either bovine serum albumin or normal bovine serum albumin which had been spiked with isometamidium. A maximum of one major 140 kD band and two minor bands were detected in blots of serum from cattle two and 16 days following isometamidium treatment. These results were suggestive of the fact that the major drug-binding serum macromolecule in isometamidium-treated cattle has an approximate molecular weight of 140 kD, and could be a dimer of albumin.

Studies by Kinabo and Bogan (1987) also demonstrated the binding of isometamidium to calf thymus DNA and to acid phospholipid. The binding of isometamidium to intracellular macromolecules may in part explain its rapid uptake into hepatocytes and other cells, which is otherwise difficult to understand for a cationic substance which appears to be firmly bound to serum proteins, although a mechanism, possibly an active transport system (Kinabo

and Bogan, 1988d), capable of transporting isometamidium into cell has also been proposed (Philips *et al.*, 1967). Similar active transport systems have been demonstrated to be responsible for hepatocellular uptake of other quarternary ammonium compounds (Solomon and Schanker, 1963).

The metabolism of isometamidium has not been reported, although studies have not been extensive. No putative metabolites have been detected in plasma or serum following injection of the drug in cattle (Kinabo and Bogan, 1988b) or in rats (Philips *et al.*, 1967). The related phenanthridinium derivative homidium has been shown to be metabolised primarily by N-acetylation in the liver, with subsequent excretion via the bile (MacGregor and Clarkson, 1971; 1972). Although isometamidium could not be detected in bile of treated rats (Philips, 1967), excretion of ^{14}C-labelled isometamidium in faeces, but not urine, of intramuscularly treated cattle suggests the biliary route at the main excretory pathway of the drug or its metabolites (Kinabo, Bogan, McKellar and Murray, 1989; Kratzer, Turkson, Karanja, and Ondiek, 1989).

Mechanism of Action

On the basis of experimental work *in vitro*, a number of mechanisms of action have been proposed for the anti-trypanosomal activity of isometamidium, although none has been formally demonstrated as being of primary importance *in vivo*. Blockade of nucleic acid synthesis through intercalation between DNA base pairs (Wagner, 1971), inhibition of RNA polymerase (Richardson, 1973), inhibition of DNA polymerase (Marcus, Kopelman, Koll, and Bacchi, 1982), and incorporation of nucleic acid precursors into DNA and RNA (Lantz and Van Dyke, 1972) have all been proposed as mechanisms of action of the phenanthridinium drugs. Modulation of glycoprotein biosynthesis (Casero, Porter and Bernacki, 1982), of lipid metabolism (Dixon, Ginger and Williamson, 1971), ATP metabolism (Frank-Henderson, Battel, Zombor and Khoo, 1977), membrane transport (Girgis-Takla and James, 1974) and alteration of kinetoplast DNA minicircles through the selective inhibition of mitochondrial type II topoisomerase (Shapiro and Englund 1990; Shapiro 1993) may also contribute to the effects of these drugs. The selective toxicity of isometamidium on trypanosomes as opposed to mammalian cells may be related to some of the biochemical peculiarities of trypanosomes (Kinabo, 1993), or alternatively to higher intracellular drug concentrations resulting from active drug uptake by trypanosomes (Sutherland, Mounsey and Holmes, 1992; Zilberstein, Wilkes, Hirumi and Peregrine, 1993).

Isometamidium Chemoprophylaxis

The efficacy of isometamidium chloride as a chemoprophylactic agent for the prevention of tsetse-transmitted bovine trypanosomiasis has been recognised since its introduction in the 1960s. However, over most of the thirty odd years of its use in Africa the understanding of its efficacy and the duration of prophylaxis, and hence the selection of the optimal dosage strategy have been based on field observations. Investigations in which the prophylactic activity of isometamidium against trypanosomiasis has been investigated in cattle using naturally and experimentally infected tsetse challenge, and intravenous and intradermal needle challenge are summarised in Table 3.

Table 3. Prophylactic activity of isometamidium against bovine trypanosomiasis (Modified from Kinabo and Bogan, 1988d).

Trypanosome species	Mode of Transmission	Geographic location / origin	Dose* ($mgkg^{-1}$)	Prophylactic period (months)	Reference
T. congolense	TN	Kenya	0.5	3.5	Fairclough, 1963a
T. congolense	TN	Tanzania	0.5	6.0	Wiesenhütter, *et al.*, 1968
T. congolense (IL 1180)	TE / ID	Kenya	0.5	3.0	Peregrine *et al.*, 1988
T. congolense	TN	Nigeria	1.0	3.0	Kirkby, 1964
T. congolense	TN	Tanzania	1.0	7.5	Wiesenhütter, *et al.*, 1968
T. congolense (IL 1180)	TE / ID	Kenya	1.0	5.0	Whitelaw *et al.*, 1986
T. congolense (IL 1180)	TE / ID	Kenya	1.0	4.0	Peregrine *et al.*, 1988
T. congolense (IL Nat 285)	TE / ID	Kenya	1.0	5.0	Peregrine *et al.*, 1988
T. congolense	TN	Nigeria	2.0	4.0	Kirkby, 1964
T. vivax	TN	Kenya	0.5	3.5	Fairclough, 1963a
T. vivax	TN	Tanzania	0.5	6.0	Wiesenhütter *et al.*, 1968
T. vivax (IL 2969)	TE	Kenya	0.5	< 1.0	Peregrine *et al.*, 1987
T. vivax (IL 2982)	TE	Kenya	0.5	< 1.0	Peregrine *et al.*, 1991
T. vivax (IL 2986)	TE	Kenya	0.5	1.0	Peregrine *et al.*, 1991
T. vivax	IV	Senegal	0.5 (IV)	1.5 - 2.0	Touré, 1973
T. vivax (IL 2968)	TE	Nigeria	0.5	2.0	Peregrine *et al.*, 1987
T. vivax	TN	Nigeria	1.0	3.0	Kirkby, 1964
T. vivax	IV	Venezuela	1.0	5.5	Toro *et al.*, 1983
T. brucei	IV	Senegal	0.5 (IV)	1.0	Touré, 1973

TN: tsetse challenge, natural
TE: tsetse challenge, experimental
ID: intradermal challenge using metacyclic trypanosomes
IV: intravenous challenge or drug administration
*Route of drug administration intramuscular unless indicated otherwise

In the field, the use of intramuscular doses of 0.5 to 1.0 $mgkg^{-1}$ isometamidium chloride has been reported to afford from as long as 14 to 36 weeks prophylaxis against natural tsetse challenge (Robson, 1962; Fairclough, 1963a; Kirkby, 1964; Wiesenhütter, Turner and Kristensen, 1968), to as little as 2 to 3 weeks (Dolan, Stevenson, Alusha and Okech, 1992; Münstermann, Mbura, Maloo and Löhr, 1992), although the reasons for this variation have not been adequately addressed. For example, Whiteside (1962a) suggested that the duration of chemoprophylaxis was directly related to the level of trypanosome challenge, whilst others have held the view that variation in the susceptibility of trypanosomes to this trypanocide, i.e. drug-resistance, is more significant.

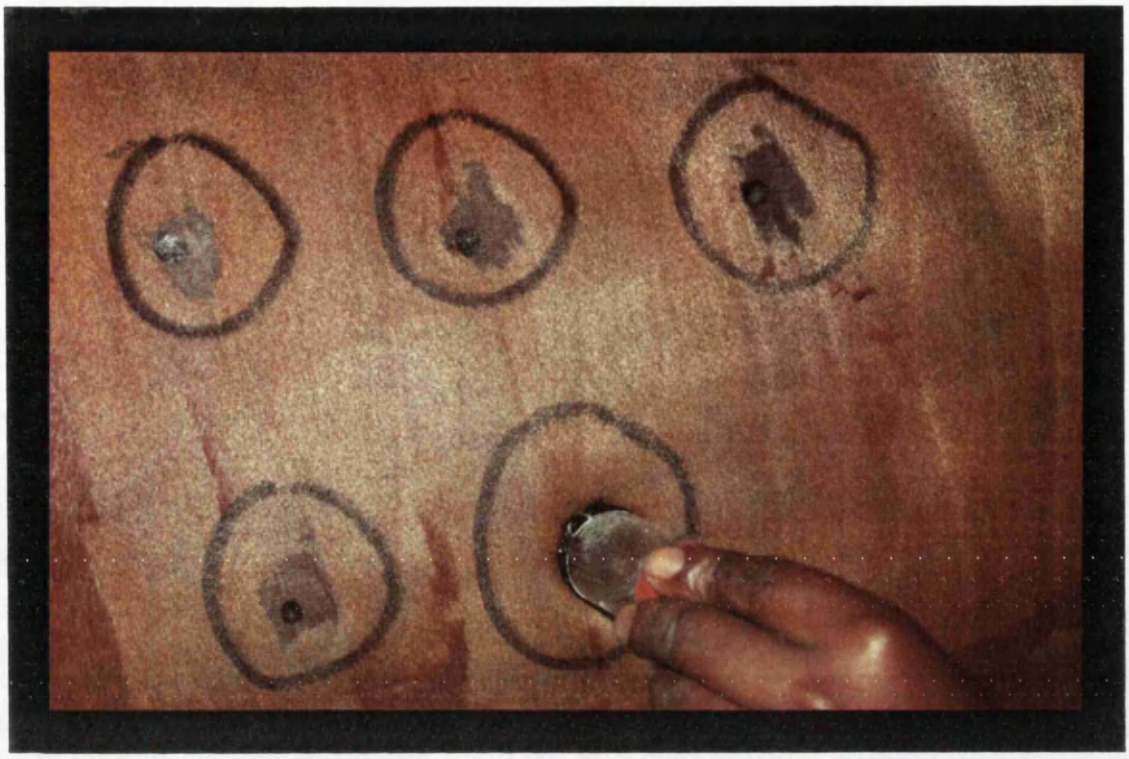
Logan, Goodwin, Tembeley and Craig (1984) demonstrated that Zebu-type cattle could produce beef in an area of West Africa infested with *G. palpalis gambiensis* and *G. morsitans submorsitans.* Maure cattle treated with isometamidium at a dose rate of 0.5 $mgkg^{-1}$ body weight at quarterly intervals were compared with a challenge control group which were treated with diminazene if found to be infected, over a period of 21 months. Whereas only one case of trypanosomiasis, but no losses, occurred in the isometamidium group, there was a total of 159 cases of trypanosomiasis with five fatalities in the diminazene group. Although the cost of drugs was almost identical under the two regimens, average daily weight gains were significantly higher in the isometamidium treated cattle than the diminazene group; overall weight gains were respectively 110 and 73 kg per head.

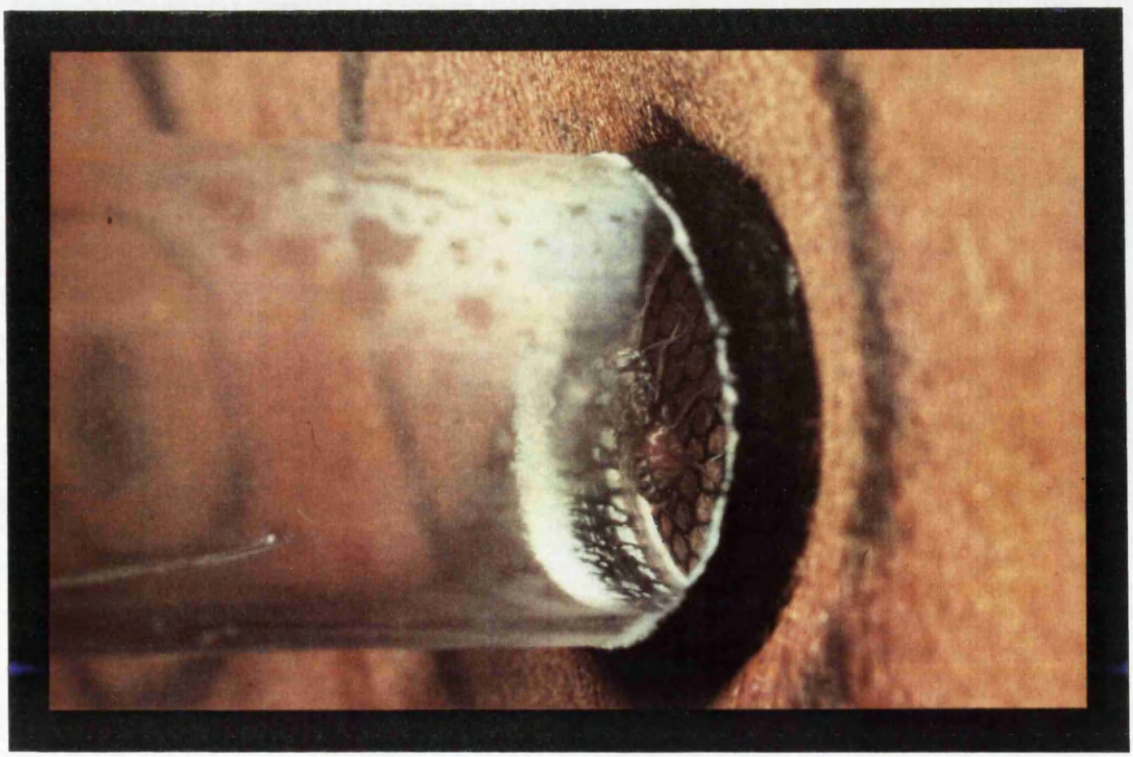
One of the most comprehensive studies of the use of isometamidium prophylaxis was that of Trail, Sones, Jibbo, Durkin, Light and Murray (1985) who examined a unique set of 10 years of matching productivity and health records from Mkwaja Ranch in Pangani District in Coastal Tanzania (Ford and Blaser, 1971), a commercial beef ranch infested with *G. morsitans morsitans*, *G. pallidipes*, *G. brevipalpis* and probably *G. austeni.* The data set equated to 134 000 trait-years of new data, and amounted to approximately twenty times as much information on livestock productivity under chemoprophylaxis as had been available in the whole of Africa over the previous 25 years. Planned experiments demonstrated that cattle were unable to survive without trypanocidal drugs: untreated animals died or succumbed to predators. Isometamidium prophylaxis was shown to be clearly superior to chemotherapy using diminazene, even in pre-weaning cattle, in which isometamidium had not

previously been used in large scale commercial herds. Boran cattle at Mkwaja under isometamidium prophylaxis were 80% as productive as Boran cattle on trypanosomiasis-free ranches in Kenya, and 35% more productive than ranched trypanotolerant N'dama cattle under medium to high trypanosomiasis risk in West Africa. It was therefore concluded that profitable cattle ranching is possible in Africa in areas of high risk of trypanosomiasis through the use of isometamidium prophylaxis.

Another significant study on the use of isometamidium prophylaxis in the field was that of Bourn and Scott (1978), who described the use of the drug to control trypanosomiasis among work-oxen introduced to Angar-Gutin, a lowland area of south western Ethiopia heavily infested with G. morsitans submorsitans. In 1972, as part of a resettlement project, 40 Zebu-type oxen, which, unusually for Africa, are widely used in the tsetse-free highlands of Ethiopia for ploughing, traction and other purposes. Over the next five years, the success of the project, which relied on high standards of management and veterinary supervision, was such that the number of oxen introduced had increased to 450. Initially, diminazene was employed in a strategic curative regime, on average every 28 days, but later isometamidium prophylaxis was used. When isometamidium prophylaxis was relied upon alone, there was evidence of the development of resistance to this drug, and its use was therefore alternated with diminazene (as a "sanative", see below), but only in animals clinically affected by trypanosomiasis. This regimen was used successfully for the final three years of the study, and it appeared that a degree of "non-sterile immunity" (see below) had developed since only 20% of the oxen required treatment at the end of every month, even though 50-69% had positive blood films (and, arguably, a higher percentage might have been found to be infected, had a more sensitive diagnostic technique been used).

Plate 4. Laboratory-reared *Glossina morsitans centralis* being applied to shaved areas of the abdominal flank of Kenyan Boran cattle. The presence of infective metacyclic *Trypanosoma congolense* in the saliva of each tsetse fly was confirmed by microscopic examination.
a) Method of application of individual tsetse flies.
b) Fed fly, showing abdomen engorged with host blood.





Factors Influencing the Success and Duration of Isometamidium Chemoprophylaxis

Only in the last decade have some of the factors influencing the chemoprophylactic activity of isometamidium been investigated under controlled experimental conditions. This work has been performed at the International Laboratory for Research on Animal Diseases (ILRAD), in collaboration with the University of Glasgow veterinary School. Whitelaw , Bell, Holmes, Moloo, Hirumi, Urquhart and Murray, (1986) investigated the period of prophylaxis in Kenyan Boran cattle after intramuscular (i.m.) injection with isometamidium at a dose rate of 1.0 $mgkg^{-1}$ body weight. Laboratory-reared tsetse flies (*G. morsitans centralis*) which had been fed on goats experimentally infected with a doubly-cloned isolate of *T. congolense* (IL 1180 [also referred to as ILNat 3.1]) were used for challenge of cattle (see Plate 4). The presence of infectious metacyclic trypanosomes was confirmed for each fly used for challenge by microscopic examination of salivary probes onto a glass slide.

Twenty cattle were each challenged on a single occasion, using five infected tsetse flies; four cattle at one, two, three, four and five months after the isometamidium treatment had been administered. Another four cattle were challenged repeatedly at the same monthly intervals, using five similarly infected flies at each occasion. Whether challenged singly, or repeatedly, none of the cattle developed infections detectable using the buffy-coat technique, or by sub-inoculation of mice. In contrast to the responses of challenge control cattle, which became infected following every monthly challenge, skin lesions (chancres) and lymph node enlargement were not detectable in these cattle. Moreover, anti-trypanosomal antibody titres, as measured by immune-lysis of trypanosomes, and by neutralisation of trypanosome infectivity for mice, only developed in challenge control cattle but not cattle in which prophylaxis was effective.

In a second phase of the experiment, the same 24 cattle were again challenged at six months after the isometamidium treatment, either by the application of five infected flies, or by the intradermal injection of 5×10^2, 5×10^3, 5×10^4 or 5×10^5 *in vitro* cultured metacyclic forms of the same challenge stock of trypanosomes. By this time, eight of the cattle were susceptible to infection, but the remaining 16 cattle were still apparently protected by the drug. No correlation was observed between susceptibility to infection the previous challenge history in the first phase of the experiment. Nor was there any

correlation with the method of challenge or, in the case of challenge with *in vitro*-derived metacyclic trypanosomes, the number of trypanosomes used. The apparent absence of any relationship between the level of challenge using intradermal inoculation of titrated doses of *in-vitro* derived metacyclic and the efficacy of prophylaxis was unexpected; the duration of chemoprophylaxis had previously been considered to be related to the level of challenge (Whiteside, 1962a). In an attempt to relate the level of challenge with *in vitro*-derived metacyclic trypanosomes to field challenge, the upper dose of 5×10^5 metacyclic trypanosomes was equated to attack by 45,000 tsetse flies per month (Whitelaw *et al.*, 1986).

In this second phase of the experiment, it was again demonstrated that immune responses (measured in terms of skin reactions, local lymph node enlargement, and the development of anti-metacyclic trypanosome antibody activity) were not primed in isometamidium-treated animals which resisted challenge (Whitelaw *et al.*, 1986). In those treated animals in which infections did develop, chancre development and lymph node enlargement were less severe than in untreated challenge controls, and prepatent periods of infection (i.e. the number of days between challenge, and the development of parasitaemia) were significantly longer in the isometamidium-treated cattle. These findings suggested that, in contrast to the situation with therapeutic use of trypanocidal drugs in the field (Bourn and Scott, 1978), non-sterile immunity was unlikely to play a role in the protection afforded by prophylactic isometamidium, provided that prophylaxis was effective in preventing infections from developing to the extent that priming of the immune system could occur.

They also indicated that the prophylactic effect of isometamidium is likely to be exerted at the site of inoculation, i.e. the skin, and for this reason, the authors, in concurrence with Stephen (1986), suggested that experiments on prophylactic drugs should use cyclical challenge; intravenous inoculation of trypanosomes, as used by many experimenters, not only represents an artificial route of infection, but may also bypass an important site of drug activity.

In a similar experiment, Peregrine, Ogunyemi, Whitelaw, Holmes, Moloo, Hirumi, Urquhart and Murray (1988) again examined factors influencing the duration of intramuscular isometamidium prophylaxis, in particular the dose of drug, the level of metacyclic challenge, and the influence of infection with an unrelated serodeme at the time of treatment. Again laboratory-reared *G. morsitans centralis* infected with *T. congolense* IL 1180, and 5×10^3 or 5×10^5

in vitro-cultured metacyclic forms of the same *T. congolense* stock were used for challenge, as well as *G. morsitans centralis* infected with a doubly cloned stock of an unrelated serodeme of *T. congolense* (IL 285). Prophylactic doses of 1.0 $mgkg^{-1}$ afforded complete protection against both serodemes for four months, and seven out of eight cattle remained for uninfected following five monthly challenges. Even after the sixth and seventh monthly challenges two cattle challenged with each serodeme remained uninfected. Doses of isometamidium of 0.5 $mgkg^{-1}$ protected all of 16 cattle challenged with *T. congolense* IL 1180 for at least 3 months and 50% of the cattle for four months. Although the authors concluded there was no relationship between the method of challenge (infected tsetse compared with intradermal challenge with metacyclic forms), or the weight of challenge (5×10^3 compared with 5×10^5 metacyclic forms), the number of cattle in each treatment group (four) would suggest that the experiment would be a rather insensitive method of detecting one should it exist. This experiment also demonstrated that an established *T. congolense* infection (IL 285) at the time of isometamidium treatment did not influence the subsequent duration of prophylaxis against an unrelated serodeme (*T. congolense* IL 1180) of the parasite.

The duration of prophylaxis conferred by isometamidium against *T. vivax* has also been investigated (Peregrine, Moloo and Whitelaw, 1987, 1991). Using experimental tsetse challenge of cattle with stocks of *T. vivax* from East Africa (IL 2982, Galana, Kenya, and IL2969, Kilifi, Kenya) and West Africa (IL 2968, Zaria, Nigeria), protection after intramuscular isometamidium treatment at a dose rate of 0.5 $mgkg^{-1}$ varied between less than one month (*T. vivax* IL 2982, and IL2969) and at least two months (*T. vivax* IL 2968). Three *T. vivax* populations originating from locations in Kenya were shown to be sensitive to the therapeutic activity of isometamidium, but resistant to the prophylactic activity of the drug, as defined by "early" breakthroughs at dosages recommended for field use. The basis for this apparent difference in sensitivity to the prophylactic and therapeutic use of the drug was not established, although the authors suggested different isomers or metabolites of the drug might be responsible for its different activities.

Studies on a tsetse-transmitted drug-resistant clone of *T. congolense* derived from a field isolate (Burkina Faso) without drug selection (Sutherland, Moloo, Holmes and Peregrine, 1991) showed it to be insensitive to both the prophylactic and therapeutic activity of isometamidium; breakthrough infections

occurred in cattle challenged 28 days following intramuscular (IM) prophylactic isometamidium (1.0 $mgkg^{-1}$), and relapses occurred when the resulting infections were treated with isometamidium at 1.0 or 2.0 $mgkg^{-1}$ IM, or 0.25, 0.5, 0.75, or 1.0 $mgkg^{-1}$ by the intravenous (IV) route. The route of administration has been shown to be of some significance in the therapeutic use of isometamidium against infection with a tsetse-transmitted clone of *T. congolense* exhibiting a low level of drug resistance (Sutherland, Codjia, Moloo, Holmes and Peregrine, 1992). Cattle treated with 1.0 $mgkg^{-1}$ IV all underwent relapse, while the same dose administered IM resulted in cure (follow-up period 6 months).

Interactions Between Trypanocidal Drugs And The Host Immune System

The necessity for a functional immune system for the efficacy of chemotherapy and chemoprophylaxis is well recognised in a variety of parasitic and microbial infections (Doenhoff, Modha, Lambertucci, and McLaren, 1991). The connection between drugs and the immune response was recognised as early as 1909 by Paul Ehrlich (cited by Doenhoff *et al.*, 1991) who conducted pioneering work in the fields of both chemotherapy and immunology. In chicken malaria it was shown experimentally that acquired immunity played a key role in the effectiveness of quinine therapy (Taliaferro, 1948), and in a murine model significantly fewer *Schistosoma mansoni* worms were killed by four chemotherapeutic agents if mice were immunosuppressed prior to infection (Sabah, Fletcher, Webbe and Doenhoff, 1985; Brindlay and Sher, 1987). A similar relationship has been chemotherapy and the immune system has been shown to occur in trypanosomiasis; immunosuppression was shown to substantially reduce the efficiency of trypanocides, including isometamidium and diminazene, in curing *T. evansi* infections in mice (Osman, Jennings and Holmes, 1992).

Drug-stimulated immunity

It has been suggested by many workers that the exposure of cattle to natural trypanosome challenge, either while under chemotherapeutic regimens leads to a type of acquired immunity whereby the animals become increasingly resistant to trypanosome infections, to a degree greater than the could be explained by the action of the trypanocidal drugs alone. This somewhat controversial topic has been discussed by Williamson (1970), Leach and

Roberts (1981), Homes and Scott (1982), Jordan (1986), and Holmes and Torr (1988).

Bevan (1928, 1936) working in Zimbabwe noticed that trypanosome-infected cattle treated with tartar emetic and then re-exposed to challenge in the same area, often subsequently remained uninfected. Later studies in the 1970's in Uganda and Kenya renewed interest in "acquired tolerance" to trypanosomiasis. This work showed that there appeared to be a relationship between the level of challenge and the degree of protection obtained. In the Ugandan study (Wilson, Paris and Dar, 1975), cattle were exposed to a relatively high level of tsetse challenge, and chemotherapy (diminazene) was administered to infected cattle on an individual basis only when the PCV fell below 20%, or when cattle became clinically ill. During the course of the two year study, the number of live calves born increased and calf mortality and the incidence of abortion decreased.

A similar system of chemotherapy produced even better results in an area of medium tsetse challenge in Kenya (Wilson, Le Roux, Paris, Davidson, and Gray, 1975; Wilson, Paris, Luckins, Dar and Gray, 1976). Over the course of the 29 month study, the interval between diminazene treatments increased from approximately 50 days to approximately 130 days, and some steers which were not treated at all during the last six months of the experiment continued to survive and grow as well as those which were treated. In contrast, the inter-treatment interval for animals which were block treated as a group whenever an individual was detected parasitaemic required treatment remained approximately 26 days throughout the study. Nevertheless, this group produced better economic returns than the group in which infections were allowed to develop, because the latter group suffered poor growth during the early stages before immunity could develop. Interestingly, the best economic return was observed in a third group of cattle to which isometamidium ($0.5 mg kg^{-1}$) was administered when any individual was detected parasitaemic, because of a lower trypanocidal treatment requirement and improved growth rates. This group also showed slight evidence of development of immunity, although it appeared to be short-lived.

Leach and Roberts (1981) concluded from these experiments that demonstrable development of immunity is not necessary for successful maintenance of cattle in tsetse areas, provided drug treatment regimens are closely controlled (and significant drug resistance does not develop). In

addition the development of trypanosomiasis in a host before drug treatment was considered necessary to induce a degree of immunity. The level of tsetse challenge was considered significant by these authors; medium tsetse challenge was more conducive than high challenge to the development of drug-stimulated immunity. This relationship was further supported by Jordan (1986) who considered relatively low levels of challenge to be optimal for the development of immunity under chemotherapeutic regimes. He described experiences on government stock farms in Southern Nigeria in the 1950's with small resident populations of *G. p. pallidipes.* Cattle could tolerate the local stains of trypanosomes of cattle if initially they were protected by drugs, although they were susceptible to other strains of trypanosomes if moved into a new area. Nevertheless, Bourn and Scott (1978) also showed that strategic administration of trypanocidal drugs to work oxen in a trypanosomiasis-endemic area of high tsetse challenge in south-western Ethiopia resulted in the development of a degree of non-sterile immunity; when trypanosome-infected animals were treated alternately with isometamidium and diminazene only on the basis of clinical signs of disease, although 50 - 69% of the oxen remained positive on blood-films, only approximately 20% per month required treatment.

It has been further suggested that because of the phenomenon of drug-stimulated immunity the optimum trypanocidal drug regime for the prevention of trypanosomiasis differs depending on the type of herd being treated (Leach and Roberts, 1981). While in ranch cattle the use of block prophylactic treatment with isometamidium at regular intervals, or treatment with diminazene on detection of parasitaemia, is likely to give the best economic return, for small breeding herds individual treatment with diminazene would be preferable in an attempt to allow the development of a degree of immunity. Holmes and Scott (1982) observed that for the successful development of non-sterile immunity in endemic areas high levels of veterinary supervision and cattle husbandry, adequate nutrition and relative freedom from concomitant infections are also required. Finally Holmes and Torr (1988) pointed out that in the absence of suitable drug assays it is not possible to identify the contribution accumulated drug residues may make to apparent immunity in repeatedly treated animals.

Drug-stimulated immunity is thought not to be significant under successful chemoprophylactic regimens (Holmes and Torr, 1988): the prevention of development of infection by the prophylactic drug precludes the development of an immune response. Experimental evidence to this effect has been obtained

in laboratory studies in which cattle under isometamidium prophylaxis were challenged repeatedly with a clone of *T. congolense* using infected tsetse flies or intradermal inoculation of metacyclic forms (Whitelaw, Bell, Holmes, Moloo, Hirumi, Urquhart and Murray (1986); Peregrine, Ogunyemi, Whitelaw, Holmes, Moloo, Hirumi, Urquhart and Murray, 1988). Antibodies to metacyclic trypanosomes did not appear in cattle which remained uninfected following up to seven monthly challenges, except when the highest level of intradermal challenge was used (5×10^5 parasites), in which case antibody titres were low and non-protective.

Trypanocidal drug resistance

The problem of trypanocidal drug resistance was reviewed by Williamson (1962 and 1970), Leach and Roberts (1981), Schillinger (1984), Holmes and Torr (1988), and Sutherland and Holmes (1991). As pointed out by Holmes and Torr (1988), drug resistance is only one reason for apparent failure of trypanocidal drugs.

The phenomenon of the development of resistance by trypanosomes to chemotherapeutic agents was first observed as early as 1907, when Ehrlich (1907) observed that with repeated injections of trypan red, trypanosomes became increasingly insensitive to this aniline dye. The early successes when quinapyramine, homidium, and diminazene were introduced led to mass treatment campaigns, which were initially very effective (Leach and Roberts, 1981). However the usefulness of these campaigns was later diminished by the successive appearance in turn of strains of trypanosomes resistant to each of these drugs. The problem of trypanocidal drug resistance is now seen as a cause for considerable concern as drug resistance has now also been demonstrated to isometamidium (see below), the only subsequently introduced bovine trypanocide.

Stephen (1986) defined two types of drug resistance in the context of trypanocidal chemotherapy and chemoprophylaxis. Natural drug resistance was defined as "abnormal resistance of strains or species of trypanosomes which have had no previous exposure to the drug". *Trypanosoma simiae* which is naturally resistant to most trypanocidal drugs is an example of this phenomenon. Induced drug resistance was defined as "the reduction in susceptibility of a strain of trypanosome to the lethal action of a drug of the same chemical composition, or of a drug of a different chemical composition, following exposure to the original drug". The latter part of this definition,

referring to drugs of different chemical composition is important as there is a widely recognised phenomenon of cross resistance, in which trypanosomes resistant to one trypanocidal drug also express resistance to another drug to which they may never have previously been exposed.

Reasons for development of drug resistance

The acquisition of drug resistance by trypanosomes has been considered to be the result of prolonged exposure to less than trypanocidal drug concentrations and hence drug resistance has been considered to be more likely to occur with prophylactic drugs than therapeutic drugs. This is because following the administration of a prophylactic drug the level of drug in the blood and tissues of a treated animal will gradually decline to a level insufficient to kill all trypanosomes and but enough to induce resistance or exert a selection pressure for the more resistant members of the population. In contrast, following the administration of a therapeutic drug, the entire dose is likely to be eliminated far more rapidly, and the drug will be present at sub-therapeutic concentrations for only a very brief period. The fact that resistance to the therapeutic trypanocide diminazene aceturate is difficult to induce experimentally (Fussgänger and Bauer, 1960; Bauer, 1962), and the early belief that resistance to diminazene did not occur in the field were considered to support this hypothesis. Nevertheless, soon after the initiation of mass treatment campaigns with diminazene, resistance was observed in the field in West Africa (Jones-Davies, 1967) and East Africa (Mwambu and Mayende, 1971). The fact that the widespread use of isometamidium, which has pronounced prophylactic activity, has not often been followed by the clear-cut demonstration of the development drug resistance also fails to confirm the hypothesis. For example Folkers (1966) administered isometamidium repeatedly to cattle in an area of high natural tsetse challenge, at a dose rate of 0.25 $mgkg^{-1}$ IM. Although up to 7 such injections were given over a period of 15 months, when animals were detected parasitaemic, no evidence of the development of drug resistance was found. Similarly, at Mkwaja ranch in Tanzania, a prophylactic regimen with isometamidium was followed for twenty years without evidence of the development of drug resistance (Trail, Sones, Jibbo, Durkin, Light and Murray, 1985).

It has been widely held that it is important to maintain effective trypanocidal levels of the drug in cattle under challenge, as the replication of trypanosomes in animals with less than effective drug levels would lead to the development of

drug-resistant populations (Leach and Roberts, 1981). Stephen (1986) stated that "Drug-resistance is usually considered to arise when inadequate dosages with the same or similar drug is administered repeatedly", and that provided that re-infection could be ruled out, "the persistence of trypanosomes in the blood... subsequent to treatment at the recommended dosage indicates drug resistance".

Osman, Jennings and Holmes (1992) demonstrated that immunosuppression of the host considerably reduces the efficacy of trypanocidal drugs and can lead to the rapid development of high levels of drug resistance. Resistance to trypanocidal drugs, including isometamidium and diminazene, could be readily induced in *T. evansi* in immunosuppressed but not immunocompetant mice.

Mechanisms of drug resistance

The molecular basis of drug resistance has been recently reviewed by Hayes and Wolf (1990), who made the distinction between intrinsic and acquired drug resistance, on the basis of whether the organism or cell in question was resistant at the time treatment began, or whether it was initially sensitive and became resistant only after treatment was commenced. Reasons for intrinsic drug resistance included absence of target site; species-specific structure of target site; high detoxification capacity arising from tissue-specific function, ontogenic variations, sex-specific differences, population polymorphisms, self defence, or high repair capacity; low drug delivery; cell cycle effects; adaptive change; and stress response. Intrinsic resistance may not be possessed by all members of the 'wild type' population, but may exist in only a proportion, an example of this being the polymorphisms associated with certain drug-metabolising enzymes. Intrinsic resistance is likely to be the result of selection pressures entirely independent of the chemical agent against which resistance is observed, and may therefore be present in a significant proportion (10 - 100%) of the population prior to the application of drug selection pressure. The failure of most of the chemotherapeutic agents used in the treatment of other trypanosomiases of domestic animals to cure *T. simiae* infections in pigs (Stephen, 1986) may be considered to be an example of this type of drug resistance.

In contrast to the situation with intrinsic resistance, with acquired resistance the protective feature arises from a spontaneous mutation within the population, a very rare event (10^{-6}), and hence is either absent from, or rarely expressed in

the 'wild type' population before drug selection. this form of resistance can arise by a number of mechanisms (Hayes and Wolf, 1990), although mutation and selection for protective genes are always central to this process. The essential difference between acquired and innate resistance is thus the frequency with which the mutated gene occurs within the 'wild type' population; in both cases the characteristic resulting in resistance arises independently of exposure to the drug and is part of biological variation. For example, antibiotic resistance has been demonstrated in strains of bacteria freeze-dried and stored prior to the commercial development of antibiotics.

The biochemical mechanisms responsible for drug resistance include reductions in drug influx into target cells; increases in drug efflux from target cells; reduced metabolic activation of the drug; sequestration of drug to prevent interaction with the target site; increase in intracellular concentration of target sites; structural alterations in target sites; duplication of the functions of the target site; and increased repair of damaged target sites (Hayes and Wolf, 1990).

Of the possible mechanisms for drug resistance, those affecting drug uptake and efflux have received particular attention from workers on the parasitic protozoa, such as *plasmodium falciparum* (Fitch, Chevli and Gonzalez, 1975; Krogstad, Gluzman, Kyle, Oduola, Martin, Milhous and Schlesinger, 1987), *Entamoeba hystolytica* (Samuelson, Ayala, Orozco and Wirth, 1990), and *T. brucei brucei* and *T. brucei rhodesiense* (Damper and Patton, 1976; Frommel and Balber 1987), who found a relationship between reduced drug accumulation and resistance to the applied compound. Similarly, Sutherland, Peregrine, Lonsdale-Eccles and Holmes (1991) who took advantage of the intrinsic fluorescence of isometamidium to use fluorescence microscopy and flow cytometry to investigate accumulation of this trypanocide by a range of clones of *T. congolense*, found evidence of an inverse relationship between drug accumulation and the degree of resistance expressed by the parasites. Investigations into the rates of ^{14}C-isometamidium accumulation by drug-sensitive and drug resistant *T. congolense* clones showed that drug uptake was significantly more rapid and quantitatively greater in drug sensitive parasites, and there was clear evidence that drug uptake in both sensitive and resistant parasites was by a specific receptor-mediated process (Sutherland, Mounsey and Holmes, 1992). Furthermore, drug uptake by the sensitive clone could be reduced by the addition of the metabolic inhibitor salicyl-hydroxamic acid and

glycerol, which indicated that specific drug transport was energy dependant. Kinetic modelling of isometamidium uptake by *T. congolense* indicated that reduced accumulation of the drug was not likely to be due to reduce drug influx, and was likely to be due to either a reduction in affinity of the intracellular binding site for the drug, or to increase drug efflux (Sutherland, Mounsey, Eisler, and Holmes, 1992).

Cross resistance

Trypanocidal drug resistance and cross resistance were investigated in detail in Kenya by Whiteside (1961; 1962a), using strains of *T. congolense* and *T. vivax*. Earlier confusion surrounding cross resistance between quinapyramine and phenathridines was shown to be due to the degree of resistance expressed by various trypanosome strains, and it was reported that resistant strains could be produced in the laboratory by exposure of infected cattle to repeated subcurative doses of trypanocides. The greater the number of such exposures, the greater the degree of resistance that could be induced. Whiteside (1961) was able to produce a table of cross-resistance, based on strains of *T. congolense* prepared in cattle, which is reproduced with modifications in Table 4.

Table 4. Cross resistance of trypanosomes to trypanocidal drugs (After Whiteside, 1961; and Leach and Roberts, 1981)

Trypanosomes resistant to:	Response of trypanosomes to: Quinapyramine	Homidium	Prothidium	Metamidium	Diminazene
Quinapyramine	R	++	+	+	++
Homidium	+	R	+	+	0
Prothidium	+	++	R	+	?
Metamidium	+	++	+	R	0
Diminazene	0	0	0	0	R

R: Direct resistance
+ : cross resistance to curative dose
++ : cross resistance to higher dose
0 : no cross resistance

Whiteside (1958, 1961, 1962a) introduced the concept of "sanative pairs" of drugs; each of the two drugs comprising a sanative pair would be active against strains of trypanosomes resistant to the other drugs. The use of one member of the pair was recommended until resistant forms were detected, at which point the second member of the pair was substituted for the first, until the resistant strains had disappeared from both the cattle and the tsetse.

The lack of cross reactivity between the diamidine, diminazene aceturate, and the phenanthridines homidium, prothidium, and metamidium suggested the suitability of diminazene with one of the phenanthridines for use as sanative pairs. With the withdrawal from the market of prothidium, the only sanative combinations currently available are homidium and diminazene and isometamidium and diminazene.

Experimental induction of drug-resistant trypanosomes

Whiteside (1962a) was able to induce drug-resistance in strains of *T. congolense* with "astonishing rapidity" by administering three to six successive subcurative treatments to infected steers with most trypanocides, the exception being diminazene aceturate. By this method the median curative dose (CD_{50}) could be increased by 40 - 80 times above that of the original strain.

Stable resistance to mel Cy, diminazene aceturate, and isometamidium chloride was induced in clones of *T. evansi* by frequent passage in immunosuppressed mice given sub-curative drug treatments (Osman, Jennings and Holmes, 1992). Similarly, Ndoutamia, Maloo, Murphy, and Peregrine (1993) induced quinapyramine resistance in a cloned population of *T. congolense* by repeatedly passaging in mice relapse populations which appeared following treatment with increasing dosages of the drug. A 40-fold increase in resistance to quinapyramine was shown to be associated with an eight-fold, 28-fold and 5.5 fold increases in resistance to isometamidium, homidium and diminazene respectively. The resistant populations were shown to be capable of full cyclical development in tsetse flies.

Field Occurrence of Drug-Resistant Trypanosomes

Mass treatment campaigns with quinapyramine and homidium were followed in relatively short time by appearance of refractory infections termed drug-resistant or "drug-fast" strains (Jones-Davies and Folkers, 1966). Following the introduction of diminazene aceturate, resistance to this trypanocide was initially thought not to occur; Williamson (1970) stated that "in

ten years of widespread use it has not given rise to any serious degree of drug resistance". However, this is now also ample evidence that resistance to this trypanocide can develop (Jones-Davies, 1967; Mwambu and Mayende, 1971; Authié, 1984; Codjia, Mulatu, Majiwa, Leak, Rowlands, Authié, d'Iteren and Peregrine, 1993).

Evidence for the development of isometamidium-resistant populations of trypanosomes in the field has been obtained in a number of countries. The earliest report of isometamidium-resistance in the literature is probably one of two cited by Lewis and Thompson (1974), from the 1967 and 1970 Annual Reports of the Branch of Tsetse and Trypanosomiasis Control of the Rhodesian Ministry of Agriculture, in which *T. congolense* and *T. vivax* resistant to isometamidium at dose rates of 0.25 and 0.5 $mgkg^{-1}$ body weight. Lewis and Thompson (1974) themselves described the isolation of isometamidium-resistant *T. congolense* from the Masumo and Logolo Valley region of Rhodesia, where chemoprophylaxis had been practised since 1958 initially using quinapyramine, and since 1967 using isometamidium. Resistance to isometamidium was first suspected from1970 onwards, when the period of protection afforded by the drug was reduced. Stabilates of *T. congolense* were shown to be resistant to the therapeutic use of isometamidium in cattle, which developed relapse infections when kept outside the tsetse infested area following treatment at 1.0 $mgkg^{-1}$ body weight.

Isometamidium resistance was also demonstrated in the 1970's at a resettlement scheme in the Angar-Gutin Valley in south western Ethiopia, where 450 work oxen were kept in an area of high tsetse challenge by the strategic use of trypanocidal drugs (Bourn and Scott, 1978). Drug-resistant *T. congolense* became a problem when attempts were made to rely solely on isometamidium prophylaxis at a dose rate of 1.0 $mgkg^{-1}$ every eight weeks. Thirty-six days after the second treatment (which was in fact delayed until 12 weeks after the first), two thirds of the oxen were found to be infected. Studies in mice on *T. congolense* from Angar-Gutin demonstrated that resistance to homidium (and thus the phenanthridine group) was indeed present (Scott and Pegram, 1974). Thereafter alternate treatments with isometamidium and diminazene were given only to the infected animals when clinically affected; that most remained in surprisingly good condition in spite of a significant infection rate (over 50%) was attributed to the development of non-sterile immunity.

The isolation of a strain of *T. congolense* from the Shimba Hills Settlement Scheme, in Kwale District of Kenya's Coast Province which demonstrated multiple drug resistance against quinapyramine, diminazene aceturate, homidium chloride and bromide, and isometamidium (Gitatha, 1981) was of significance on two counts. Firstly, isometamidium resistance was demonstrated in spite of the fact that isometamidium had never been used at this site; quinapyramine methylsulphate and Prosalt, homidium bromide and diminazene aceturate had all been used between 1958 and 1971. Secondly, resistance to both phenanthridines (isometamidium and homidium) and to diminazene indicated that the usual sanative drug pairs (Whiteside, 1961) might not be effective. As in a similar situation described recently in south western Ethiopia, see below, the main factor implicated in the development of multiple drug resistance was the use, and possibly misuse of quinapyramine; up to 50% of cattle were not presented for treatment on some occasions.

In 1983, Kupper and Wolters (1983) carried out a study in a feedlot in Northern Ivory Coast. Although tsetse were scarce at the feedlot itself, the cattle had previously trekked through tsetse-infested bush. Following treatment with isometamidium at dose rates of 0.5 and 1.0 $mgkg^{-1}$ body weight, relapse infections with *T. congolense* and *T. vivax* were soon detected, and it was concluded that strains resistant to these doses of the drug were present.

Pinder and Authié (1984) investigated trypanocidal drug resistance in cattle in the pastoral zone of Samorogouan, Upper Volta (Burkina Faso), where, under a World Bank-funded programme started in 1978, 9000 head of Zebu cattle had been treated with isometamidium chloride at a dose rate of 0.5 $mgkg^{-1}$ body weight four times a year, and with diminazene aceturate at 3.5 $mgkg^{-1}$ body weight twice a year, in May and November. The doses of both drugs were doubled when a resistance problem was suspected. Trypanosome infections, mainly with *T. congolense*, were detected as little as two weeks following isometamidium treatment at 1.0 $mgkg^{-1}$, suggesting that resistance had developed. Tests in mice showed that *T. congolense* stocks isolated in 1982 and 1983 were four to eight times less sensitive to isometamidium than others isolated three years earlier, prior to the widespread use of the drug.

Seven years later, the continued presence of multiple drug resistance at this location was confirmed when diminazene, homidium and isometamidium were all ineffective in treating *T. congolense* infections at Samorogouan (Clausen, Sidibe, Kaboré and Bauer, 1992). Twenty Zebu steers, naturally

infected with *T. congolense*, were moved into a fly-proof stable and treated with diminazene aceturate at 7.0 $mgkg^{-1}$ body weight, and homidium bromide and isometamidium chloride, both at 1.0 $mgkg^{-1}$ body weight). An isolate of *T. congolense* obtained from one of these animals was used to experimentally infect three previously unexposed Zebu bulls and goats in which infections relapsed after treatment with diminazene aceturate at 7.0 (bulls) and 14 and 17 (goats) $mgkg^{-1}$ body weight. Attempts were also made to treat similarly infected goats infected with intravenously administered isometamidium chloride which was ineffective at dose rates of both 1.0 and 2.0 $mgkg^{-1}$ body weight; five out of six goats treated at the lower dose rate, and two of six goats treated at the higher dose rate relapsed; one and three goats, treated at the lower and higher dose rates respectively, died within 24 hours of treatment.

Röttcher and Schillinger (1985) described isolates of *T. vivax* from cattle possibly (Njogu and Heath, 1986) suffering a haemorrhagic form of the disease at Galana Ranch in Tana River District of Kenya. Stabilates of *T. vivax* prepared from 11 cattle were pooled and used to infect 36 steers from a tsetse-free area which were subsequently treated with isometamidium chloride at 0.5, 1.0 and 2.0 $mgkg^{-1}$ body weight, homidium chloride (at the same dose rates), diminazene aceturate (1.75, 3.5 and 7.0 $mgkg^{-1}$ body weight), or quinapyramine sulphate (1.5, 3.0 or 5.0 $mgkg^{-1}$ body weight). Nine cattle were treated with each drug, three at each dose rate. Isometamidium was ineffective in all cattle treated at all three dose rates; no drop in parasitaemia was observed after treatment. Relapse of infections also followed treatment with quinapyramine and homidium at all three dose rates, and with diminazene at all but the highest dose rate used. Three untreated control steers which appeared likely to die following infection were also treated with isometamidium at 0.5 $mgkg^{-1}$ bodyweight by the intravenous route. After a brief period of remission of parasitaemia, relapses were observed within seven to ten days. The sensitivity of these and a further five *T. vivax* isolates from other sites in Kenya (including four from the Coast Province, and one from Narok District) and one *T. vivax* isolate from Buulobarde District, Somalia) to the same four trypanocides were examined in cattle by Schönefeld, Röttcher and Moloo (1987). The isolates were all resistant to the recommended dose rates of quinapyramine, homidium and isometamidium, but all were sensitive to diminazene aceturate at a dose rate of 7.0 $mgkg^{-1}$. These findings suggested that multiple drug resistance had recently become widespread along the East African coast; in earlier sensitivity testing in 1981, *T. vivax* isolated from cattle in two of the Kenyan Coast

Province sites were fully susceptible to isometamidium (Schönefeld, Röttcher and Moloo, 1987).

Ainanshe, Jennings and Holmes, (1992) isolated *T. congolense* from two areas in the Lower Shabelle Region of southern Somalia by injecting blood from infected cattle into a recipient calf. A novel aspect of this study was the expansion of the relapse infections which developed in the calves following treatment with isometamidium (0.5 $mgkg^{-1}$ bodyweight), in cattle and mice for testing for sensitivity to isometamidium and diminazene aceturate. Both isolates showed high levels of resistance to both trypanocides, minimum curative doses in cattle for both isolates being above 2.0 $mgkg^{-1}$ and 7.5 $mgkg^{-1}$ for isometamidium and diminazene respectively. While this unsophisticated approach precluded assessment of the number of drug-resistant infections at each site, its relatively low cost and simplicity were appropriate for the logistical and financial conditions pertaining in Somalia.

Dolan, Stevenson, Alusha and Okech (1992) reported the apparent failure of isometamidium to prevent infections in cattle at Galana ranch for more than two to three weeks following prophylactic injections of 1.0 $mgkg^{-1}$ body weight during 1987/88. In the previous year (1986/87), a year of low to medium trypanosome challenge, prophylactic injections of homidium bromide (1.0 $mgkg^{-1}$) had apparently protected cattle at Galana for as long as 8 to 17 weeks (Dolan, Okech, Alusha, Mutugi, Stevenson, Sayer and Njogu, 1990). In 1987/88 an attempt was made to compare the prophylactic efficacy of homidium bromide with that of isometamidium chloride, the prophylactic drug usually used in Kenya. However that in that year (1987/88) trypanosome challenge was exceptionally high, and neither drug afforded much of a prophylactic period (mean for isometamidium, 28.4 days; mean for homidium, 25.4 days).

Codjia *et al.*, (1993) characterised the drug sensitivity of field isolates of *T. congolense* from the Ghibe valley in south western Ethiopia, where there appeared to be a rising occurrence of failure to respond to trypanocidal drug treatment. Twelve stabilates were shown to be resistant in calves to diminazene aceturate (7.0 $mgkg^{-1}$ body weight), and of these eleven were also resistant to isometamidium chloride (0.5 $mgkg^{-1}$ body weight) and homidium bromide (1.0 $mgkg^{-1}$ body weight). Five clones derived from one of the isolates all expressed a high level of resistance to all three trypanocides, which indicated the multi-drug resistance phenotype was possessed by individual trypanosomes. This study was therefore significant in that although it was not

the first occasion multi-drug resistant populations of *T. congolense* have been demonstrated, it was shown that individual trypanosomes may express resistance to both phenanthridines (isometamidium and homidium) and diminazene aceturate, and thus infections with such parasites would not be cleared by the usual sanative combinations. Furthermore, the multi-drug-resistance phenotype was shown by molecular karyotyping and isoenzyme studies to be associated with many genetically distinct populations. It is believed that the use, and particularly misuse of quinapyramine may give rise to resistance to both phenanthridines and diminazene. It is understood that the use of quinapyramine was previously widespread in this area, and indeed there may be continued use of black-market stockpiles of the drug in south western Ethiopia as a whole (staff of National Tsetse and Trypanosomiasis Investigation and Control Co-ordination Office, Addis Ababa, personal communication).

Characteristics of Drug-Resistant Trypanosomes

Stability, infectivity and cyclical transmission

The stability of drug resistance in trypanosomes is likely to be important in determining their prevalence in wild populations, and in governing the rate of spread of resistance in the presence and absence of drug pressure. Considerable variability has been observed in the stability of drug resistance; atoxyl resistance was still present in a strain of *T. brucei rhodesiense* maintained for 24 years by serial passage in the absence of this drug (Fulton and Grant), whereas a strain of *T. congolense* showed a rapid loss of quinapyramine resistance after just 65 days.

Gray and Roberts (1971a) carried out a series of experiments which demonstrated that drug resistance (including resistance to isometamidium) in *T. congolense* and *T. vivax* is stable and transmissible on cyclical passage through tsetse flies. Following infection and treatment, breakthrough infections had reduced pathogenicity in their original bovine hosts, but pathogenicity was not necessarily reduced following sub-passage through the fly to other animals. Drug resistance was shown to persist for up to 29 months in the absence of drug treatment, while being passaged cyclically through cattle and tsetse. Drug resistance was altered by neither the length of time between the infection of an animal and transmission of the trypanosomes to a new host, nor by cyclical passage through antelope (Gray and Roberts, 1971b). These last observations were of significance as they suggested that the immune response of the host does not affect drug resistance once established in a trypanosome strain.

Evidence of cyclical transmission of drug-resistant strains has also been obtained in the field (Fiennes, 1953; Williamson and Stephen, 1960; Jones-Davies, 1968). The stability of these resistant strains in the field are however variable. Quinapyramine resistance was detected in the field 5 - 7 months after the removal of all drug-treated cattle (Fiennes, 1953), and homidium-resistant *T. congolense* were reported in tsetse flies in Nigeria two years after the replacement of the drug by diminazene aceturate (Jones-Davies, 1968). However, other workers have found that resistant *T. congolense* and *T. vivax* disappear from tsetse 6 - 9 months after the removal of cattle and the discontinuation of drug use (Whiteside, 1961). These finding would be explicable on the basis that some resistant strains are examples of innate resistance and do not require the presence of drug pressure to maintain detectable levels in the population, while others represent acquired resistance which, in the absence of drug pressure, may be less viable than the non-resistant strains from which they developed.

Viability of drug-resistant Strains

The number of reports from the field of isometamidium-resistant trypanosomes are relatively few in number, when it is considered that the drug has been widely used for over 30 years, and having pronounced prophylactic activity might be expected to give rise readily to resistant forms (Whiteside, 1962a). For example, the in spite of extended use of isometamidium at Mkwaja ranch, Tanzania, there was no evidence of the development of drug resistance for 20 years (Trail *et al.*, 1985). This is perhaps the more surprising considering the treatment regimens used, which involved re-treatment of herds after parasitaemia had been detected in a proportion of animals, and thus trypanosomes were presumably regularly exposed to sub-trypanocidal drug concentrations.

This lower than expected prevalence of resistant forms of trypanosomes could be explained if the isometamidium-resistant strains had reduced viability compared to the isometamidium-sensitive strains, and that in mixed infections the latter tend to overgrow the former, which then die out. Reduced growth of drug resistant bacteria (Mayr-Harting, 1955) and *Plasmodium gallinaceum* (Bishop and McConnachie, 1950) has been observed, compared to drug-sensitive parent strains. In mice, some drug-resistant stocks of *T. equiperdum* appear to be less able to survive in the absence of trypanocidal drugs than the drug-sensitive parent stocks (Cantrell, 1956).

Further evidence for this hypothesis was obtained by Sones, Holmes and Urquhart (1989), who investigated the interference between isometamidium-sensitive and isometamidium-resistant stocks of *T. congolense* when one and then the other was used to infect goats. It was demonstrated that when the goats were infected first with the isometamidium-sensitive and then the isometamidium-resistant stock, the resulting infection could be cleared by isometamidium treatment, indicating the resistant stock had not become established. If the goats were infected with the resistant stock first, followed by the sensitive stock, isometamidium treatment caused temporary remission, followed by relapse. In contrast, following infection with the resistant stock alone, treatment resulted in neither remission nor cure. This suggested that superinfection with the sensitive stock resulted in the establishment of infection which suppressed the resistant stock to below the limit of detection of the haematocrit centrifugation - buffy coat technique (Paris, Murray and McOdimba, 1982), until treatment eliminated the sensitive stock, and allowed the resistant stock to become re-established. In this context, the 'interference phenomenon' in ruminants in which the presence of an active infection with one serodeme of *T. congolense* inhibits the establishment of a subsequent second infection with a different serodeme (Morrison, Wells, Moloo, Paris and Murray 1982; Dwinger, Luckins, Murray and Moloo, 1986) is also of relevance. Although this phenomenon might explain why the resistant stock failed to become established when it followed infection with the drug-sensitive stock, it does not explain why superinfection with the drug-sensitive stock was able to suppress the previously established drug-resistant stock.

Parasitaemia and Pathogenicity

Differences between the course of infections with drug-sensitive and drug-resistant trypanosomes have been observed by many workers. For example, parasitaemia in rats infected with drug-resistant *T. brucei gambiense* was intermittent rather than continuous as in infections with drug-sensitive parasites (Tobie and von Brand, 1953). The time to relapse following sub-curative treatment of trypanosome infections in rodents may also be reduced for drug-resistant strains, a phenomenon which has been proposed as the basis for a test for drug-resistance using sheep (Williamson and Stephen, 1960). In cattle, breakthrough infections with *T. congolense* towards the end of a period of prophylaxis may be characterised by low or irregularly intermittent

parasitaemias, although this may not hold for infections caused by *T. vivax* (Stephen, 1962b).

It has also been suggested by various workers that drug-resistant strains of trypanosomes may be of lesser pathogenicity than their drug-sensitive counterparts (Stephen, 1960; Leach and Roberts, 1981). These suggestions would have important implications for the future use of trypanocides, but have yet to be fully substantiated (Holmes and Torr, 1988).

Tests for trypanocidal drug resistance

It is clearly important in the control of African bovine trypanosomiasis, that information be obtained on the development, distribution and prevalence of trypanosomes resistant to the few extant trypanocidal drugs. The true incidence and practical importance of drug resistance is largely unknown, and a matter of considerable controversy (Holmes and Torr, 1988). Three main approaches have been used for the demonstration of drug-resistance, namely by conducting therapeutic tests either in domestic livestock or in rodents, and testing the drug sensitivities of trypanosome isolates *in-vitro*. Unfortunately each method has particular drawbacks (Sutherland and Holmes, 1991).

Drug Sensitivity testing in Livestock

Use of domestic livestock is time consuming and expensive, particularly in trypanosomiasis endemic areas where fly-proof accommodation is required. Furthermore, resistance to the prophylactic use of drugs may differ from resistance to their therapeutic use (Peregrine, Moloo and Whitelaw, 1987), but prophylaxis experiments are necessarily more difficult and prolonged, and therefore expensive. Cyclical challenge using tsetse flies may also be necessary to ensure experimental conditions realistically approximate natural challenge (Stephen, 1986), particularly for prophylactic drugs which may exert their effects at the level of inoculation of metacyclic trypanosomes into the skin (Whitelaw *et al.*, 1986).

Drug Sensitivity Testing in Rodents

Drug sensitivities in trypanosomes of domestic animals have been evaluated in mice for many years (Hawking, 1963). Mice are normally infected with the trypanosome stock in question, and then treated with various doses of trypanocidal drug on the appearance of patent parasitaemia. They are then monitored parasitologically for a prolonged period to assess the minimum drug doses necessary to achieve either a temporary clearance of parasites, the

minimum effective dose (MED) or a permanent cure, the minimum curative dose (MCD). It has been suggested that Hawking's (1963) results showed a close relationship between mouse effective dose and cattle curative dose, with mouse curative doses being at least ten times those for cattle (Pinder and Authié, 1984). However, Sones, Njogu and Holmes (1988), comparing the doses of isometamidium necessary to cure mice and cattle of infection with various *T. congolense* stocks concluded that although the result of a mouse test may give a broad indication of the sensitivity of a strain, it cannot be used reliably to predict curative dose for cattle. In addition many trypanosomes, notably *T. vivax* but also many *T. congolense* isolates, are non-infective for rodents. In addition large numbers of laboratory rodents must be sacrificed, and long observation periods of up to 100 days are involved.

Drug Sensitivity Testing in Vitro

In vitro techniques for assessment of drug resistance in trypanosomes have been reviewed by Kaminsky (1990) and Kaminsky and Brun (1993). Significant advances have been made in techniques of *in-vitro* culture of bloodstream forms of *T. brucei* (Hirumi, Doyle and Hirumi, 1977), *T. vivax* (Brun and Moloo, 1982), and *T. congolense* (Hirumi and Hirumi, 1984; Gray, Ross, Taylor, Tetley and Luckins, 1985). Of particular importance in the development of tests for drug resistance has been the culture of bloodstream forms of *T. brucei* (Baltz, Baltz, Giroud and Crocket, 1985), *T. congolense* (Hirumi and Hirumi, 1991) and *T. vivax* (Zweygarth, Gray and Kaminsky, 1991) in cell-free systems.

In vitro tests for drug sensitivity may require adaptation of trypanosomes to culture, and possibly the prior expansion of the population in laboratory rodents. Successful initiation of *in vitro* culture of *T. brucei* usually requires a minimum inoculum of 2×10^3 to 1×10^5 trypanosomes per ml from mouse blood during a rising parasitaemia (Hirumi et al, 1977; Baltz et al, 1985). Expansion of a trypanosome population in laboratory animals involves the possibility of a change in its drug sensitivity (Kaminsky and Brun 1993). However, Zweygarth and Kaminsky (1990) described a method of initiating cultures of bloodstream form *T. brucei* directly from disease hosts with low parasitaemia. Stocks of *T. congolense* have been shown to have similar sensitivity to the therapeutic action of isometamidium in mice before and after adaptation to culture (Brown, Ross, Holmes, Luckins, and Taylor, 1987), and there was no significant change in susceptibility to a number of trypanocidal drugs, including isometamidium, quinapyramine, and diminazene aceturate following *in vitro* maintenance of a

multi-drug-resistant *T. brucei* stock (Kaminsky and Zweygarth, 1989a). However if strains are kept in laboratories for long periods *in vitro* drug sensitivity patterns may become altered (Kaminsky and Brun, 1993).

Drug Incubation Infectivity Test

The drug incubation infectivity test (DIIT) is a combination of *in vivo* and *in vitro* techniques which does not require the adaptation of trypanosomes to culture. Trypanosomes are incubated for 24 hours in culture media containing various concentrations of trypanocidal drugs and subsequently inoculated into mice which are observed for 20 to 30 days for the development of parasitaemia. The technique is therefore limited to mouse infective trypanosomes, and has been used for *T. brucei* (Kaminsky, Gumm, Zweygarth and Chuma, 1990) and *T. congolense* (Hill, 1965; Sutherland, Mounsey and Holmes, 1991).

^{3}H-hypoxanthine Incorporation Assay

In this assay trypanosomes are incubated in culture medium lacking hypoxanthine, but containing various concentrations of trypanocidal drug for 24 hours, after which tritiated hypoxanthine is added. After a further 16 hours incubation the incorporation of hypoxanthine by the trypanosomes is determined by scintillation counting. It has been used successfully with *T. brucei* (Brun and Kunz, 1989) and *T. congolense* (Ross and Taylor, 1990), but expensive equipment is required for cell harvesting and scintillation counting.

Growth Inhibition and Viability Assays

In the 24-hour growth inhibition assay, continuously growing bloodstream forms may be incubated in various concentrations of trypanocide for 24 (or 48) hours and simply counted to observe growth inhibition as compared to the controls. The test is relatively fast, but the 24 hour incubation may not be long enough for some drugs to show their trypanocidal effects (Kaminsky and Brun, 1993). In long-term *in vitro* viability assay, trypanosomes are cultured in the presence of drug for 10 days, with replenishment of the drug containing medium as necessary, after which trypanosome growth and morphology are assessed. This test has been used for *T. brucei*, *T. congolense* and *T. vivax* (Kaminsky and Brun, 1993).

In Vitro Derived Metacyclic Incubation Test

Metacyclic trypanosomes may be derived readily from most *T. congolense* stocks and also *T. congolense* infected tsetse flies, whereas to date bloodstream forms of this trypanosome species from naturally infected animals

have not been adapted to culture. In this test metacyclic trypanosomes are incubated without feeder cells for 48 hours in the presence of various drug concentrations, after which they are transferred to bovine aortic endothelial monolayers to which they attach, enabling the medium to be replaced with fresh medium without drug. After 5 days these cultures are examined and the minimum inhibitory concentration (MIC) of drug which kills all the trypanosome population is determined (Gray and Peregrine, 1993). Although the development of metacyclic forms in culture may take as long as 12 weeks, the method has the advantage that populations of *T. congolense* incapable of growth in rodents may be investigated.

Photometric assays

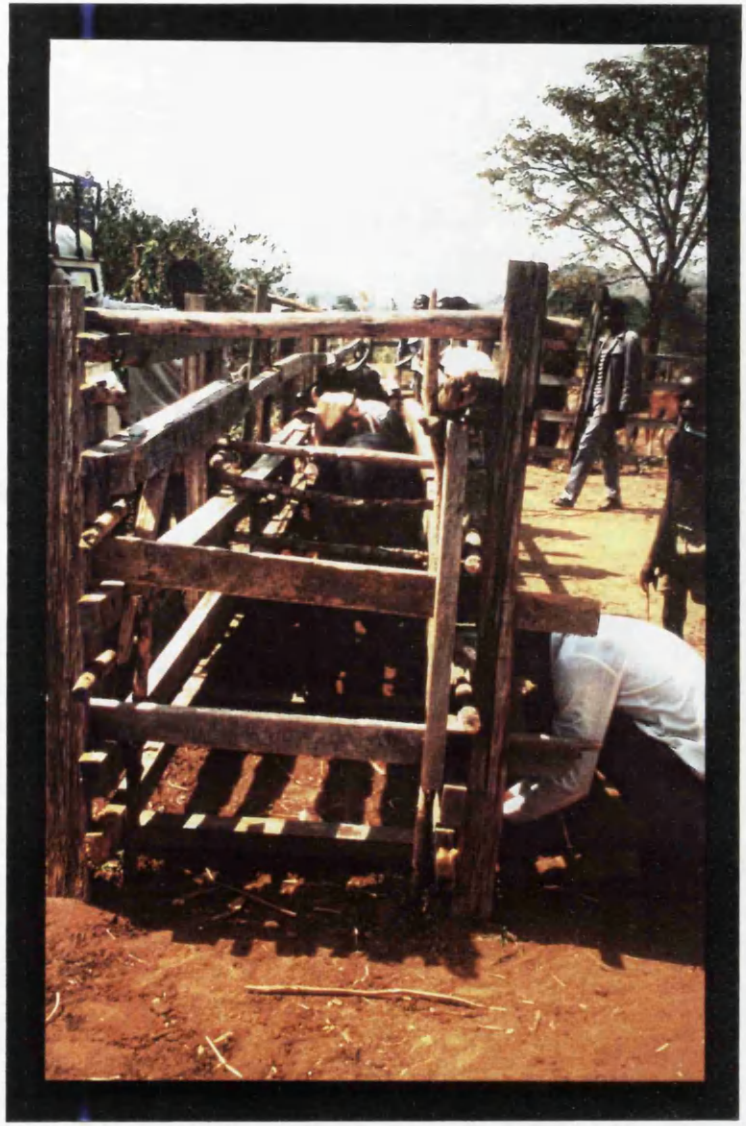
Two photometric assays have been described for *in vitro* trypanosome drug sensitivity which may be conducted in microtitre plates and read using a multichannel spectrophotometer. In one system the production of pyruvate by intact trypanosomes with consequent lowering of the pH of the culture medium and a colour change in phenol red indicator. Non-viable cultures, inhibited by the presence of trypanocide result in correspondingly less colour change (Zinsstag, Brun and Gessler, 1991). Alternatively, with *T. congolense* cultures, the trypanosomes are adherent to the culture wells, and may be stained *in situ* at the end of the incubation period with sulforhodamine B, so that the absorbance measures at 540 nm is proportional to the number of trypanosomes present (Kaminsky and Wasike, 1992). A similar fluorometric assay which utilises an esterase substrate which is cleaved by intact trypanosomes to produce a fluorescent product has also been described (Obexer, Schmid and Brun, 1992).

Akinetoplastic Induction Test

Chitambo and Arakawa (1992) described a test which was used to assess the relative sensitivities of *T. congolense* isolates to diminazene and isometamidium, in which drug sensitive isolates developed a higher proportion of akinetoplastic forms during incubation with the drug, and at lower drug concentrations than resistant isolates.

Unfortunately, very few laboratories in Africa have the resources for such demanding techniques. Furthermore, the correlation between in vitro and in vivo susceptibility of trypanosomes to drugs has yet to be clearly established.

Plate 5. Well-organised facilities for accurately weighing cattle for calculation of trypanocidal drug doses and estimating productivity under African field conditions. Portable equipment may be easily transported by four-wheel drive vehicle, and set up in minutes in a new location. Unfortunately such facilities are seldom available in Africa, with the result that underdosing is common.
a) Portable electronic weigh-bars being placed under a cattle crush.
b) Portable wooden weigh platform in position on top of weigh bars. Electronic read-out fastened to bonnet of Land-Rover.



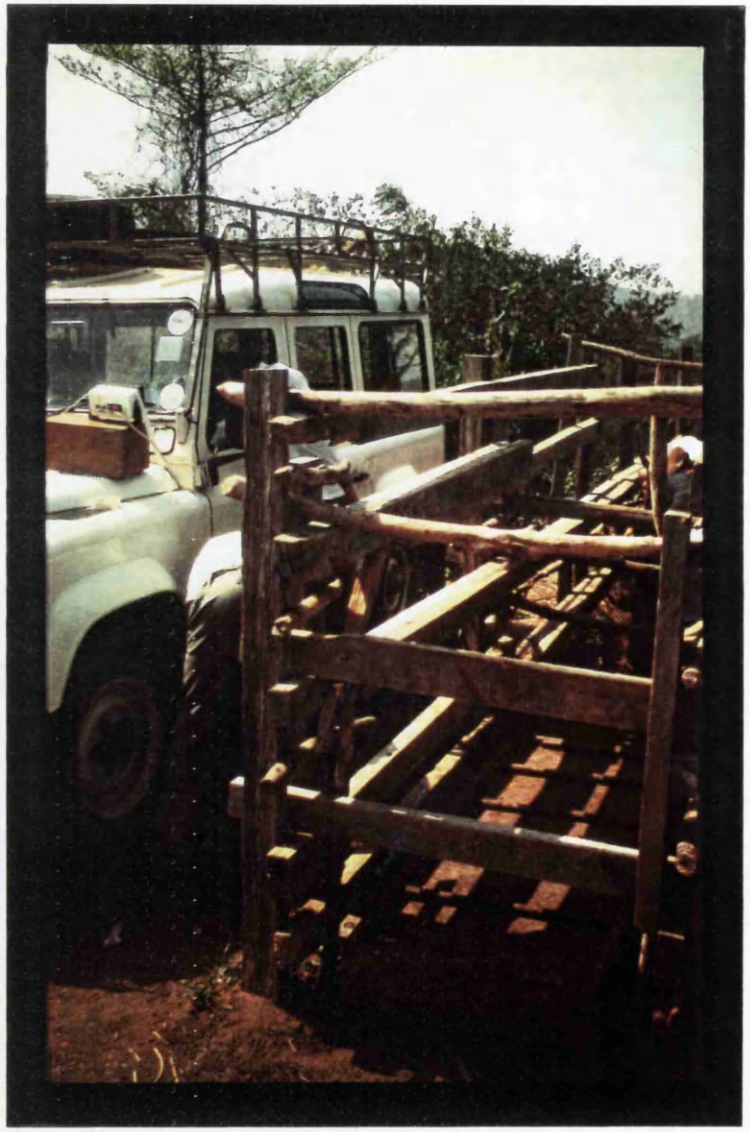
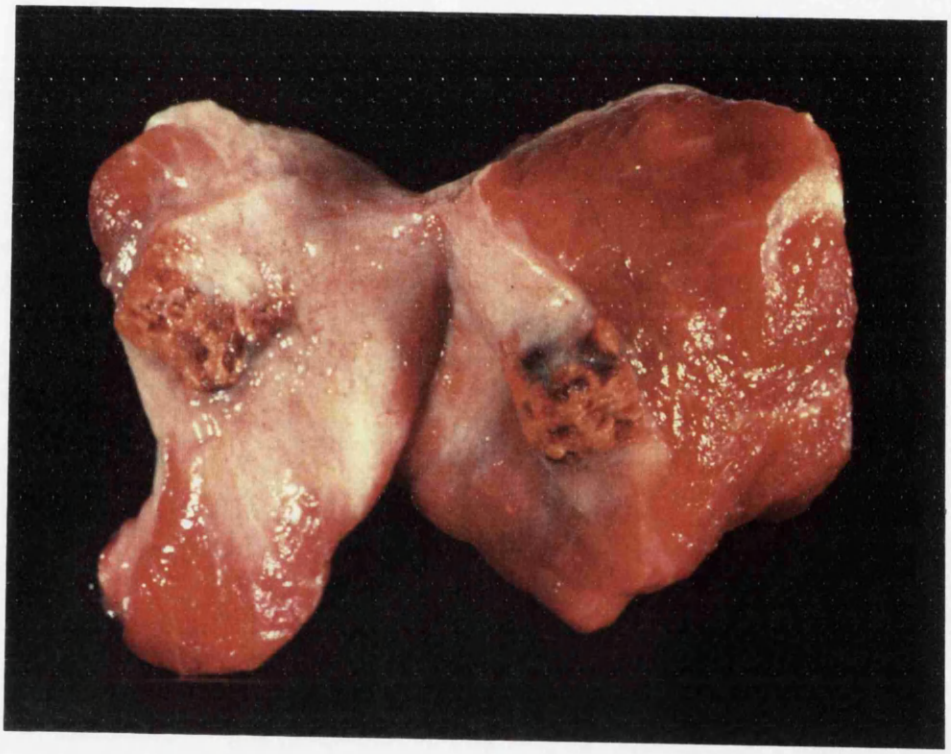


Plate 6. Intramuscular isometamidium chloride injection site, showing extensive fibrosis and central area of coagulative necrosis, within which dark purplish-brown deposits of the drug are visible.



Constraints on the use of trypanocidal drugs

Drug Failure

One of the main constraints on the use of trypanocidal drugs is clearly drug failure due to trypanocidal drug resistance which has been discussed extensively above. However, there are other important reasons why the use of trypanocidal drugs may fail to achieve a satisfactory level of control of the disease, and these are especially important in the field assessment of whether drug resistance has in fact developed at a particular location. There has been a tendency for field workers to equate failure of drug regimens with resistance, but this may not always be the case, and the true importance and distribution of resistance is controversial (Holmes and Torr, 1988). Some possible reasons for drug failure in the field are listed in Table 5.

As well as being a factor in the development of drug resistance, underdosing *per se* may be one of the most frequent causes of apparent drug failure. In considering the role of underdosing in the development of drug resistance, Jordan (1986), considered the most important to be the underestimation of body weight (see Plate 5), and hence of the dose required, underestimation of the level of trypanosome challenge in establishing a chemoprophylactic regime, and irregular treatment with prophylactic drugs. The same author considered the problem of trypanocidal drugs appearing on the open market or through the black market, rather than through the appropriate veterinary authorities. This may lead to the dividing of doses intended for a single animal between a number of animals, either in an effort to save money, or as blatant profiteering on the part of an illicit vendor. Drug fraud is also considered to be a serious problem in many parts of Africa (Verhulst, A., personal communication), and may reach a level of considerable sophistication, so that the ineffective counterfeit products and their packaging are virtually indistinguishable from the genuine article, and may only be detected by examination of batch numbers (Bado, A., personal communication).

Errors in preparation and administration of trypanocidal drugs may also lead to underdosing, particularly where drugs are administered by inexperienced and untrained personnel. Trypanocidal drugs are generally sold in the form of powders for dissolution in water; sterile distilled water may not be available, and the means to measure volumes of water accurately may be lacking. A balance capable of accurately weighing small quantities of drug is not usually available, but the measured amount of drug as presented in the manufacturers original

packaging may be greater than required on any particular occasion. Although, once prepared, any unused drug in solution should be discarded if not required for immediate use, in practice the drug is often likely to be saved for the next occasion it is required. This is understandable, considering the economic constraints pertaining in the countries affected by trypanosomiasis, but may result in prolonged storage of solutions under adverse conditions, such as exposure to high temperature and strong sunlight, and the efficacy of the product may have deteriorated to an unknown extent. Another problem of drug administration was suggested by Lewis and Thompson (1974) who considered that isometamidium solution might escape from the injection site along the needle track when the injection was in a severely fibrosed location. Finally, where large numbers of animals are to be treated it is possible that individual animals will be missed, either through problems of identification, difficulties in adequately restraining ranch cattle unused to handling, or straight forward human error.

Another important consideration in the failure of trypanocidal drug treatment is the possibility of relapse due to the persistence of infections in privileged sites or cryptic foci inaccessible to the drug. Jennings, Whitelaw and Urquhart (1979a,b) showed a relationship between the duration of infection with *T. brucei* and the efficacy of chemotherapy with a variety of drugs. Treatment of mice three days after infection resulted in permanent cure, but if treatment with the same drugs was delayed until 14 to 21 days after infection, none of the drugs cured the infections, even if dose rates were increased far in excess of those recommended; a period of aparasitaemia was followed by reappearance of trypanosomes up to seven months later. Jennings, Whitelaw, Holmes, Chizyuka, and Urquhart (1979) demonstrated that during this aparasitaemic period the only tissue of the treated mice capable of transferring infection to uninfected recipients was the brain. The failure of treatment is now considered to be due to the persistence of extravascular trypanosomes in the central nervous system (CNS) which are unaffected by drugs which are unable used to pass the blood brain barrier. When, later, the trypanocidal drug has been eliminated, these trypanosomes may re-invade the circulation. The treatment of late stage human African trypanosomiasis caused by *T. brucei* group parasites still relies mainly upon the trivalent arsenical melarsoprol which is able to cross the blood-brain barrier in sufficient quantity to eliminate trypanosomes from the CNS (Jennings, 1993).

The *T. brucei*-group trypanosomes are not generally considered to be as important as *T. congolense* and *T. vivax* in cattle, which are generally considered to be confined to the vasculature, apart from a brief period of development at the site of inoculation of metacyclic forms by the tsetse fly into the skin (Luckins and Gray, 1978). However, extravascular development of *T. congolense* has been reported to occur in mixed infections with *T. brucei* (Masake, Nantulya, Akol and Musoke, 1984). *T. congolense* were detected in cerebrospinal fluid of Boran steers in mixed experimental infections with *T. brucei*, but not in single infections. Mixed infections may be more common than is generally recognised; *T. brucei* may frequently detected if mouse subinoculation (Robson and Ashkar, 1972) or Ag-ELISA (IAEA, 1993) are used, but in practice the HCT-BCT, the sensitivity of which is considerably lower for *T. brucei* than *T. congolense* or *T. vivax* (Paris, Murray and McOdimba, 1982), is more commonly used. *T. vivax* has also been reported in extravascular locations such as the marginal sinuses of lymph nodes and cardiac tissues (Bungener and Mehlitz, 1977; Masake, 1980). McLennan (1973) failed to infect cattle by subinoculation of blood from *T. vivax*-infected cattle undergoing remission of parasitaemia following treatment with diminazene aceturate; this suggested the persistence of parasites during the period of remission in an extravascular site, possibly inaccessible to the drug. Similarly, Haase, Bernard and Guidot (1981) demonstrated infectivity for rats of brain tissue from cattle in remission from *T. congolense* parasitaemia following diminazene treatment, whereas blood from the same animals was unable to establish infection. The possibility of relapse infections of *T. congolense* and *T. vivax* due to cryptic foci should therefore not be discounted, and requires further investigation.

Re-infection represents another possible explanation for the reappearance of parasitaemia following trypanocidal drug therapy. If the response to treatment with a trypanocidal drug is to be used as an index of the drug-sensitivity of the infecting trypanosomes, it is essential the animals be removed from the possibility of further challenge during the follow-up of the infection.

Table 5: Reasons for apparent drug failure (After Holmes and Torr, 1988)

Underdosing
Preparation
Administration
Fraud
Not treated
Cryptic foci
Re-infection

Logistical and Economic constraints

The effective use of trypanocidal drugs is constrained in much of sub-Saharan Africa by severe logistical and economic restraints (Leach and Roberts, 1981). These include the difficulty in accessing extensively kept livestock distributed over large distances, frequently under nomadic systems of husbandry. Mass treatment campaigns may therefore be the most effective method of minimising the cost of individual treatments. Lack of communications, such as all weather roads, vehicles and fuel, facilities such as diagnostic laboratories and equipment, and trypanocidal drugs, needles, syringes all hamper control schemes (Holmes and Scott, 1982). The services of adequately paid and motivated personnel are also essential, but trained staff are rarely available in adequate numbers.

Adverse Effects

A significant constraint on the use of most trypanocidal drugs, both for use in human African trypanosomiasis and trypanosomiases of domestic livestock has been the problem of trypanocidal drug toxicity. Trypanocidal drugs, like other antimicrobial agents are effective because of a differential between the toxicity of the drug for the pathogenic micro-organism and that for the host. The ratio of the level of toxicity for the parasite to that for the host, may be expressed as the therapeutic index, the ratio of the maximum dose tolerated by the host, to the minimum dose curative of the parasite (Stephen, 1986). The therapeutic index may be very small for many trypanocidal drugs. This has the effect that the simple expedient of raising the dose of drug may not be possible as a means of countering the development of trypanocidal drug resistance.

Adverse reactions to isometamidium can be classified as either systemic or local to the injection site (see Plate 6). Following the conventional intramuscular injections in cattle systemic reactions are not generally reported (Toro, Léon, López, Pallota, Garcia and Ruiz, 1983), although local reactions

are considered almost inevitable (Williamson, 1970). To prevent lameness and damage to the hindquarters it is usually recommended that the drug be injected into the neck muscles, although this may cause special problems in draught oxen (Holmes and Scott, 1982). Histopathological examinations have shown that coagulative necrosis with inflammation, fibrosis and indurations cause damage at intramuscular injection sites (Braid and Isoun, 1980), and leakage of the drug to subcutaneous tissues may result in sloughing of the skin (Kinabo and Bogan, 1988d). The reaction, as well as being undesirable on animal welfare grounds, may result in an erratic and unpredictable pattern of drug absorption (Kinabo and Bogan, 1988d). Nevertheless, in twenty years of use at Mkwaja Ranch, Tanzania, intramuscular isometamidium had no deleterious effects on reproductive performance in terms of calving intervals, and multiple inoculation of the drug did not raise any serious problems of local reactions, increased requirement for treatment, or any decline in productivity (Trail *et al.*, 1985).

The problem of local tissue reactions has led some workers to investigate the use of the drug by the intravenous route (Touré, 1973; Dowler, Schillinger and Connor, 1989; Münstermann, Mbura, Maloo, and Löhr, 1992). However, intravenous injections may be associated with signs of acute systemic toxicity. Schillinger, Maloo and Rötcher (1985) investigated the toxicity of intravenous isometamidium in cattle, goats, dogs and camels, with a view to establishing the maximum tolerated dose in each species. Immature Boran steers, experimentally infected with *T. vivax*, were administered isometamidium chloride as a 1% solution at three dose rates. Four steers given the drug at $mgkg^{-1}$ body weight showed signs of tachycardia, salivation and lachrimation, and frequent urination and defecation. In addition to these signs, another steer given 1.5 $mgkg^{-1}$ body weight showed muscle tremors and diarrhoea. A further steer, given 2.0 $mgkg^{-1}$ body weight, showed additionally, recumbency, convulsions and diminished reflex responses, and thus 1.5 $mgkg^{-1}$ was considered to be the maximum tolerated dose.

Sutherland, Moloo, Holmes and Peregrine (1991) injected Boran steers experimentally infected with a drug-resistant clone of *T. congolense* isometamidium intravenously at doses of 0.25 - 1.0 $mgkg^{-1}$. At 0.25 and 0.5 $mgkg^{-1}$ no adverse effects were observed, but various acute systemic reactions were observed in 10 steers administered the drug at 0.75 $mgkg^{-1}$: six steers exhibited hyperlachrimation, three hypersalivated, and an excessive nasal

discharge was observed in one steer. Eight of the cattle also exhibited immediate urination and diarrhoea. Four out of five steers given 1.0 $mgkg^{-1}$ exhibited all these signs. Unsteady and shuffling gaits were also observed in three steers. The signs described appeared within ten minutes of treatment, and disappeared within 15 - 20 minutes following treatment. Unexpectedly, there was no advantage to intravenous administration over intramuscular administration in its ability to clear the *T. congolense* infection; in fact there was some evidence to the contrary.

Marked, dose-dependent reductions in levels of the serum cations calcium, sodium and potassium to below normal values have been shown to occur within one hour of intravenous isometamidium treatment (Schillinger, Maloo and Rötcher, 1985), possibly due to the diarrhoea and hypersalivation; these ions do not combine with isometamidium which is itself a cation. (Schillinger, Maloo and Rötcher, 1985).

Philips, Sternberg, Cronin, Sodergren and Vidal (1967) considered the acute responses to isometamidium to be typical of agents containing quarternary ammonium groups and which involve stimulation or blockade of cholinergic effects. Typical of such signs are the vagal effects of salivation and intestinal hyperperistalisis (Schillinger, Maloo and Rötcher, 1985). Further evidence for this was the finding that the acute toxic effects of isometamidium in mice were reduced by atropine, which suggested at least some of its effects are mediated cholinergic activity, possibly through the inhibition of cholinesterase (Gimbi and Kinabo, 1992). Potentiation of histamine responses through the degranulation of mast cells is also a possible component of the acute toxicity response (Philips *et al.*, 1967).

More long-term adverse effects of phenanthridinium drugs, including isometamidium were reviewed by Williamson (1970). The use of dimidium in particular caused a high incidence of delayed toxicity, and symptoms resembling photosensitization frequently appeared. Periportal fatty infiltration of the liver was thought to be of central importance in the development of this condition, but external factors such as climate, nutritional status and dietary photodynamic substances such as the chlorophyll derivative phylloerythrin were also considered significant. Homidium, pyrithidium, and metamidium were also considered capable of producing liver lesions, but only at doses considerably higher than the therapeutic level; delayed toxicity and photosensitization had not followed their use in the field. However, a severe wasting condition with

high mortality and substantial liver damage evident *post mortem* which occurred in a group of experimental cattle on Galana ranch, Kenya, was attributed to use of isometamidium at a frequency that exceeded the manufacturer's recommendation (Dolan *et al.*, 1992). Isometamidium had been administered intramuscularly at 1.0 $mgkg^{-1}$ body weight at approximately monthly intervals over a period of eight months, together with another trypanocide, diminazene aceturate, in an attempt to control trypanosomiasis in a situation of very high challenge; *T. vivax* infections were frequently detected within a few weeks of treatment.

Drug Residues

There has been increasing concern in recent years about residues of veterinary drugs used in animals used for human consumption, and this applies particularly to prophylactic trypanocidal drugs which are likely to persist in the tissues of treated animals (Braide and Eghianruwa, 1980; Kinabo and Bogan, 1988d). The Joint FAO/WHO Expert Committee on Food Additives (WHO, 1993) has established international criteria to be applied in evaluating toxicological and residue data for use in assessing the safety of veterinary products present in human food. This is intended to set international standards for their safe use in human food, and to promote harmonisation and reduce technical barriers to international trade. Particularly in relation to older veterinary drugs with a long history of use, such as the trypanocides, the Committee decided that in order to establish maximum residue levels (MRLs) and acceptable daily intakes (ADIs) the following issues must be adequately addressed: pharmacological effects, general toxicity, reproductive toxicity, embryo-toxicity/fetotoxicity, genotoxicity, carcinogenicity, other effects identified as being of importance, metabolism, tissue residues, and analytical methodology. Isometamidium was evaluated for the first time by the Committee in 1989 (WHO, 1989; WHO, 1990) which was unable to establish and MRL or ADI for the drug because of a lack of available information in many of these areas. However, in 1992, the Committee evaluated new data from studies of the uptake of radiolabelled isometamidium from lyophilised bovine tissues, short term and teratogenicity studies in rats, and a range of genotoxicity assays. An ADI of 0 - 100μgkg^{-1} body weight was established, based on the non-toxic dose level of 50 $mgkg^{-1}$ body weight per day in a 13 week rat study and a 500-fold safety factor. Similarly, the Committee evaluated new residue data from a metabolism study in lactating cows injected with ^{14}C-labelled isometamidium, a

bioavailability study in rats which were fed lyophilised calf tissues containing ^{14}C-labelled isometamidium, and a residue study in young bulls given an intramuscular injection of isometamidium at 1.0mgkg^{-1} body weight, with analysis of tissues by high performance liquid chromatography (HPLC) at 1, 3 and 6 months after treatment. Based on these studies the Committee recommended an MRL of 0.1 mgkg^{-1} for parent isometamidium in muscle and fat, 0.5 mgkg^{-1} in liver, 1.0 mgkg^{-1} in kidney and 0.1mgkg^{-1} in milk.

Methods for the determination of trypanocidal drug concentrations

Any evaluation of the factors which influence the duration of chemoprophylaxis is particularly dependant upon a knowledge of the drug concentrations present in treated animals. In particular, the quantification of drug levels in treated cattle could be useful as a field method of assessing the presence of drug resistance. The presence of trypanosomes in prophylactically treated cattle in which the drug was above the minimum level normally considered to be trypanocidal, would be a valuable index of resistance (Holmes and Torr, 1988).

Colorimetric and spectrophotometric methods

Initial attempts to quantify isometamidium included the spectroscopic method of Philips *et al.*, (1967), which was subsequently used in studies in goats (Braid and Eghianruwa, 1980) and camels (Ali and Hassan 1984). However this method was not sufficiently sensitive or specific to detect concentrations of less than 1 μgml^{-1} in plasma, and later studies (Kinabo and McKellar, 1990) cast doubts on its validity. Use of radio-isotope labelled drugs

High Performance Liquid Chromatography (HPLC)

Perschke and Vollner (1985) developed more sensitive methods for the detection of isometamidium, homidium and quinapyramine, using HPLC, although the method for isometamidium was indirect in that the drug needed to be converted to homidium before detection, and large volumes of plasma (10 ml) were required. An improved ion-pair reversed-phase HPLC method developed by Kinabo and Bogan (1988a) overcame some of these disadvantages, but was still insufficiently sensitive to detect isometamidium more than a few hours following treatment of cattle with 0.5 mgkg^{-1} IM (Kinabo and Bogan 1988b).

Radioimmunoassay

The development of immunoassay methods has revolutionised the detection of small molecules including drugs, and such methods have now been applied to trypanocidal compounds with considerable success. Kinabo and Bogan (1988c) developed a radioimmunoassay method for isometamidium, which was limited in sensitivity principally because of the low specific activity of the ^{14}C-labelled isometamidium tracer.

Enzyme-linked immunosorbent assay (ELISA)

Since its development, the use of enzyme labels has steadily replaced the use of radioactive labels in the field of immunoassay (Chard, 1990). As with the detection of anti-trypanosomal antibodies and of trypanosome antigens in the diagnosis of infection, the ELISA is an attractive option for the detection and quantification of trypanocidal drugs. With a modest amount of suitable equipment, a high throughput of test samples in suitably equipped laboratories is possible (Luckins 1992). The feasibility of the use of microplate ELISAs in African institutes involved in tsetse and trypanosomiasis control was demonstrated during the Co-ordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, from 1987 to 1992, in which ELISA-based diagnostics were successfully implemented in ten sub-Saharan countries (Ooijen, 1993).

Maes, Vanderveken, Hamers, Doua and Cattand (1988) developed an ELISA method for measuring and monitoring levels of the trypanocide melarsoprol, an organic arsenical used in the treatment of late-stage human African trypanosomiasis, in serum and cerebrospinal fluid. The assay had a working range of 200 to 2000 nannogrammes melarsoprol per ml of human serum, and was capable of detecting the drug in patients serum following treatment.

An early attempt at the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of isometamidium, was reported by Whitelaw, Gault, Holmes, Sutherland, Rowell, Phillips and Urquhart, (1991). The work described in this thesis was initiated using this methodology as a starting point for the development of reliable, repeatable, reproducible and robust ELISAs for the determination of isometamidium concentrations in bovine serum with a view to the improvement of chemoprophylactic strategies for the control of African bovine trypanosomiasis.

Chapter 2:
General Materials and Methods

Reagents

Phosphate Buffered Saline (150 mM, pH 7.2)

The following salts were weighed out and distilled water added to a volume of 1L, and mixed well.

- NaCl 8.0g
- Na_2HPO_4 1.48g
- KH_2PO_4 0.43g

The pH of the resulting buffer was checked with a pH meter, and the buffer stored at 4°C until use.

If large volumes of PBS were required it was found convenient to make up this solution at ten times the normal concentration, and to store it at 4°C. Before use, this 10 x concentrated stock solution was diluted 1/9 in distilled water and mixed well.

Washing buffer for ELISA

Buffer for washing ELISA plate wells was prepared by diluting PBS 1/5 in distilled water. Tween 20 (Sigma, Poole, UK) was added to a final concentration 0.05% (v/v) and mixed well. To aid the dispersion of Tween 20, it was first added to a small volume of PBS, mixed well, and then added to the remainder of the PBS and mixed again.

Carbonate/bicarbonate buffer (50 mM, pH 9.2)

To 900 ml of a solution of 4.2 g sodium bicarbonate made up to one litre in distilled water was added to 100 ml of a solution of 1.325 g sodium carbonate made up to 250 ml in distilled water. The buffer was mixed well, and by adding dropwise sodium bicarbonate solution to lower, or sodium carbonate solution to raise, the pH was adjusted to 9.2, using a pH meter.

5% (v/v) Acetic acid

One hundred and fifty millilitres of glacial acetic acid (BDH, Poole, UK) was added to 2850 ml of distilled water.

Dextran-charcoal

Two and a half grams (2.5g) decolourising charcoal (BDH) and 0.25 g dextran (Sigma) were added to 1L distilled water. The resulting suspension was mixed well and stored at 4°C until required for use.

Acetate buffers

Anhydrous sodium acetate (BDH) and glacial acetic acid (BDH) were dissolved in distilled water to various concentrations, and the two solutions added together in various proportions to produce the following buffers:

0.015 M, pH 5.7

4.88 g sodium acetate made up to 4 l in distilled water.

0.86 ml glacial acetic acid made up to 1 l in distilled water.

380 ml acetic acid solution added to 4 l sodium acetate solution

0.06 M, pH 4.0

2.46 g sodium acetate made up to 500 ml in distilled water.

1.72 ml glacial acetic acid made up to 500 ml in distilled water.

500 ml acetic acid solution added to 500 ml sodium acetate solution

0.1 M, pH 5.7

8.20 g sodium acetate made up to 1 l in distilled water.

5.73 ml glacial acetic acid made up to 1 l in distilled water.

95 ml acetic acid solution added to 1 l sodium acetate solution

Potassium phosphate buffers, 0.3 M and 0.01 M, pH 8

Potassium dihydrogen orthophosphate (KH_2PO_4, BDH) and dipotassium hydrogen orthophosphate (K_2HPO_4, BDH) solutions in distilled water were added together at a ratio of 2.2 : 17.8 (v/v). For 0.3 M buffer, KH_2PO_4 (40.8 gl^{-1}) was added to K_2HPO_4 (52.3 gl^{-1}). For 0.01 M buffer, 0.3 M buffer was diluted 1/30 in distilled water.

Preparation of isometamidium conjugates

Dialysis Procedure

A length of dialysis tubing (BDH) of approximately 250 mm was cut from the roll with scissors. The tubing was hydrated by boiling in distilled water for thirty minutes. One end of the tubing was closed using a plastic clip, and a measured volume (usually 1 - 5 ml) of the material to be dialysed was placed into the tubing via the open end, using a Pasteur pipette. The open end of the dialysis tubing was then closed with a second plastic clip, ensuring no air remained within, and the tubing placed in a beaker containing 2.5 litres of the solution against which dialysis was to take place, usually PBS, for 24 hours at room temperature (18 - 22°C). During this incubation the dialysis solution was changed at least three times for fresh solution and agitated continuously using a magnetic stirrer. At the end of the period of dialysis the tubing was removed from the beaker, one of the plastic clips removed, and the dialysed material collected using a Pasteur pipette.

Preparation and Use of G25 Sephadex Column

A 300 mm x 20 mm glass chromatography column (Pharmacia Biosystems Ltd., Milton Keynes, UK) was packed with G25 Sephadex (Sigma) pre-soaked overnight in PBS (pH 7.2), and equilibrated by three sequential applications of one bed volume (30 ml) of the same buffer. The mixture to be separated was applied to the top of the column, and buffer pumped onto the column at a rate of 60 ml per hour. The eluate from the column was collected in glass test tubes which were changed every two minutes, either using a fraction collector (Pharmacia) or manually. The optical density (OD) at 280 nm of all the fractions collected was recorded using a spectrophotometer (Perkin Elmer 550A, Beaconsfield, Bucks), and the approximate protein concentration calculated by multiplying the OD by 0.75. Fractions comprising individual peaks in OD were pooled, and their volumes and protein concentrations recorded.

When the material being separated was a crude preparation of an isometamidium-horseradish peroxidase conjugate, the coloured conjugate could be observed as it passed down the column. A dark brown band, consisting of unconjugated isometamidium was retained at the top of the column, while a paler but distinct brown band consisting of conjugated isometamidium moved rapidly down the column; this was collected after the passage of the void volume (approximately 9 ml). With experience it was possible to identify the

fraction(s) containing purified conjugate on the basis of the passage of the coloured band alone, rather than using the ODs.

Diazotisation Procedure

Isometamidium was conjugated to two protein carriers, either porcine thyroglobulin (PTG) or chicken egg albumin (ovalbumin; both Sigma). All incubations were carried out in light-proof containers. Fifty milligrams isometamidium was dissolved in 1 ml hydrochloric acid (1 in 3 dilution concentrated HCl in water) to which 1 ml sodium nitrite (6.95 $mgml^{-1}$) was added, and shaken gently for 10 minutes at 4°C. The isometamidium solution was then added to protein (PTG or ovalbumin) in 200mM phosphate buffer pH 7.0, to a ratio of 0.2:1 (w/w) isometamidium:PTG or 0.0125:1 (w/w) isometamidium:ovalbumin, and incubated in the dark with stirring at 4°C for two hours. The conjugate was dialysed overnight against 5% acetic acid, and stored in aliquots at 4°C. Some aliquots of isometamidium-ovalbumin conjugate were mixed for 15 minutes with 0.5 ml dextran charcoal suspension, and centrifuged (Eppendorf) at 14,000 rpm to remove the charcoal; the supernatant was stored in aliquots at -20°C after mixing well with an equal volume of glycerol (Sigma).

Immunisation of experimental sheep.

Experimental sheep

Scottish Blackface wethers aged six months to one year were obtained from a commercial farm, and loose housed in an indoor pen. They were fed on hay and concentrate and allowed access to water *ad libitum*.

Emulsification of immunogen in adjuvant

The immunising conjugate was emulsified in Freund's complete adjuvant (Sigma) or Freund's incomplete adjuvant (Sigma) as required, by adding together adjuvant and immunogen at a ratio of 2:1 (by volume), and repeatedly passing the mixture back and forth between two 2 ml syringes until a water-in-oil emulsion was obtained. This was evaluated by placing a drop of the emulsion on the surface of a beaker of distilled water: if the droplet did not disperse across the surface, but remained as an intact globule, it was considered to be a satisfactory water-in-oil emulsion (Goding, 1983).

Immunisation Protocol

Primary immunisation of Scottish Blackface wethers was by sub-cutaneous injection of conjugate emulsified in Freund's complete adjuvant. A total of 2 mg in a volume of 3 ml was divided between two sites in the inguinal region and the post-scapular flank, on the opposite sides of the body (i.e. right flank and left inguinal region).

Three to five subsequent sub-cutaneous booster immunisations of the same conjugate emulsified in Freund's incomplete adjuvant were administered over the following six to nine months. At each occasion of booster immunisation, a total of 1 mg in a volume of 3 ml was again divided between two sites in the inguinal region and the flank. Injection sites were used on the opposite sides of the body to those used in the previous immunisation.

Collection of hyperimmune serum

Blood samples were collected by jugular venipuncture into Vacutainers (Beckton-Dickinson, Meylan Cedex, France) without anti-coagulant, and placed in an incubator at 37°C for four hours to optimise clotting. After incubation overnight at 4°C to maximise clot retraction, the clots were removed, the serum centrifuged (Chilspin, MSE, Fisons, Crawley, UK) at 1000 rpm for 15 minutes, and the supernatant stored at -20°C until required.

Purification of immunoglobulin.

Ammonium sulphate precipitation of immunoglobulin

To 20 ml hyperimmune sheep serum, 20 ml saturated ammonium sulphate was added slowly, dropwise, on a magnetic stirrer. The mixture was centrifuged (Chilspin) at 3000 rpm for 20 minutes, and the pellet resuspended in PBS. Residual ammonium sulphate was removed by dialysis against PBS for 24 hours at 4°C.

Caprylic acid separation of immunoglobulin

Ten millilitres of hyperimmune sheep serum were added to 20 ml 0.6 M acetate buffer (pH 4.0), in a beaker with magnetic stirring. Three-hundred and thirty millilitres of caprylic acid (n-octanoic acid, BDH) were added and shaken vigorously. After a further thirty minutes stirring, the mixture was centrifuged (Chilspin) for 10 minutes at 3000 rpm. The supernatant was adjusted to pH 5.7 using dilute sodium hydroxide solution, and then dialysed against 0.015 M acetate buffer (pH 5.7) for 24 hours at 4°C. Finally the immunoglobulin was centrifuged again to remove any precipitate formed during the dialysis stage.

Anion-exchange chromatography of immunoglobulin G

Immunoglobulin G (IgG) was purified from crude immunoglobulin fractions obtained by ammonium sulphate precipitation or caprylic acid separation by anion-exchange chromatography using diethylamino-ethyl (DEAE) cellulose, using either a batch separation method or a column method.

Batch separation method

DEAE-cellulose (DE 52, Whatman) was equilibrated by mixing into 0.1 M acetate buffer (pH 5.7), allowing the particles to settle for ten minutes and decanting and discarding the supernatant. The process was repeated once using the same buffer, and then a few more times using 0.015 M acetate buffer (pH 5.7) until the discarded supernatant was pH 5.7. The immunoglobulin solution was then stirred into the sedimented particles, and incubated at room temperature for two hours with occasional stirring. The mixture was then centrifuged (Chilspin) at 3000 rpm for five minutes, and the supernatant dialysed against PBS for 24 hours at 4°C.

Column method

DEAE-cellulose (DEAE-Sephacel, Pharmacia) was added to 0.01 M potassium phosphate buffer (pH 8.0) and allowed to swell at room temperature overnight. A 300 mm x 20 mm glass chromatography column (Pharmacia) was packed with DEAE-cellulose, and equilibrated by the sequential application of three bed volumes (30 ml) of the same buffer. Twenty millilitres of crude immunoglobulin preparation were applied to the column and allowed to soak in for one hour. A linear elution gradient of potassium phosphate buffer (pH 8.0), starting molarity 0.01 M, final molarity 0.3 M, was applied using a gradient mixer (Pharmacia), pump speed 25 ml per hour. The eluate was collected in glass tubes changed every ten minutes using a fraction collector (Pharmacia). The optical density (OD) at 280 nm of all the fractions collected was recorded using a spectrophotometer (Perkin Elmer 550A), and the approximate protein concentration calculated by multiplying the OD by 0.75. Fractions comprising individual peaks in OD were pooled, and their volumes and protein concentrations recorded. Purified IgG was dialysed against PBS for 24 hours at 4°C.

Immunoglobulin G preparation

Immunoglobulin was purified from hyperimmune sheep anti-isometamidium serum initially by ammonium sulphate precipitation, or caprylic acid separation. IgG was purified by anion-exchange chromatography of ammonium sulphate precipitated immunoglobulin using the column method, and of caprylic acid separated immunoglobulin using the batch method.

Following anion-exchange chromatographic separation by either method, the purity of the IgG was checked using immunoelectrophoresis against anti-whole sheep serum (Sigma) and anti-sheep IgG (Sigma). IgG separation was considered satisfactory if a single precipitation line was observed in the same position with either anti-globulin reagent.

Before storage, IgG was concentrated to at least 2.0 $mgml^{-1}$ using an immersible vacuum ultrafilter (CX 10, Millipore); the immunoglobulin concentration was checked by measuring the OD at 280 nm. Aliquots of purified IgG were stored at -20°C in polypropylene vials (Cryovials, Nunc).

Chapter 3:
Evaluation and Improvement of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Isometamidium in Bovine Serum

Introduction

In spite of decades of control programmes, bovine trypanosomiasis remains one of the greatest constraints to agricultural production in sub-Saharan Africa. The few available chemoprophylactic and chemotherapeutic agents, of which isometamidium chloride (Samorin®) is one of the most widely used (Sutherland and Holmes, 1991; Leach and Roberts, 1981), remain the major means of control in most endemic countries. However, there are an increasing number of reports of suspected drug-resistant trypanosomes (Kupper and Walters, 1983; Pinder and Authié, 1984; Schönefeld, Röttcher, Moloo 1987; Holmes and Torr, 1988), and with little prospect of new drugs becoming available, control schemes which optimise the usage of existing drugs are required.

A sensitive assay for quantifying isometamidium in bovine serum could provide a valuable tool for measuring the duration of protective drug levels under a variety of field conditions, and make a major contribution to rational drug usage. In conjunction with tests for the presence of trypanosome infection, such an assay could rapidly provide indirect evidence for drug resistance in the field, thereby enabling rationalisation of chemoprophylactic and chemotherapeutic regimes.

An enzyme-linked immunosorbent assay (ELISA) for isometamidium has been reported (Whitelaw, Gault, Holmes, Sutherland, Rowell, Phillips and Urquhart, 1991), although with increasing experience of its use, the following constraints on the method have become apparent. Firstly, dispensing small volumes (5 µl) of test sera directly into ELISA plate wells was inaccurate with commonly available air-displacement pipettes. Secondly, significant background reactions, observed with many bovine sera, lead to difficulty in interpreting results. Thirdly, quality assurance was not considered; assay calibration by linear interpolation of a limited number of standards was liable to considerable bias, methods for determining precision and the lower limit of detection were not considered, and the assay was not validated for use in Africa.

To overcome these difficulties a new isometamidium ELISA method has been developed and evaluated using a statistically based approach which is intended as the basis of a quality assurance programme for routine use with the assay. The new ELISA includes use of:

- pre-dilution of sera prior to addition to the wells of the ELISA plate

- lower dilutions of test sample
- the most appropriate microtitre plates
- the biotin-streptavidin system for the detection of specific antibody
- peroxidase and tetramethylbenzidine for colour development
- four-parameter logistic curve-fitting for assay calibration.

The new ELISA has been optimised by investigating and minimising sources of response variance. Hence, assay precision and the limits of detection have been defined and improved.

Materials and methods

Untreated cattle

Twenty-four 3 to 5-month old castrated male and female Friesian (Bos taurus) calves were kept loose housed and fed on hay and concentrates in Glasgow, U.K. Twenty-one 3 to 9 month old Boran (Bos indicus) calves, kept in a similar manner, and 20 adult Boran cattle, extensively managed, were maintained in a tsetse-free area of Kenya. These cattle were not treated with isometamidium.

Trypanosome-infected steer

A single 5-month old Boran steer, maintained as described above in a tsetse-free area of Kenya, was experimentally infected with *Trypanosoma congolense* KETRI 3211. Six weeks later, when the animal had been parasitaemic for 5 weeks, serum was collected. The animal was not treated with isometamidium.

Cattle treated with isometamidium chloride

Ten 9-month old Boran cattle, maintained as described above, were treated with isometamidium chloride (Samorin®, RMB Animal Health Ltd.) by deep intramuscular injection in the middle third of the neck at a dose rate of either 0.5 (5 cattle) or 1.0 (5 cattle) $mgkg^{-1}$ body weight. None of the cattle had previously been treated with isometamidium.

Serum samples

Blood samples were collected by jugular venipuncture into Vacutainers (Beckton-Dickinson) without anti-coagulant, and the serum separated by centrifugation and stored at -20°C until tested by ELISA.

Isometamidium-ovalbumin conjugate

An isometamidium-ovalbumin conjugate was prepared by diazotization at a ratio of isometamidium to ovalbumin of 0.0125:1, as described in Chapter 1.

Sheep anti-isometamidium IgG

Sheep anti-isometamidium IgG was prepared and purified as described in Chapter 1. To 1 mg aliquots of IgG was added 20 μg N-hydroxy-succinimidobiotin (Sigma) dissolved in 120 μl dimethyl sulphoxide (DMSO; Sigma). The mixture was incubated at room temperature for 4 hours and dialysed against two changes of phosphate buffered saline (PBS), pH 7.2.

Biotinylated IgG was lyophilised in 400 µl aliquots and stored at +4°C for up to 6 months until use. Prior to use, aliquots were reconstituted with sterile distilled water to the original volume, and mixed thoroughly with an equal volume of glycerol. In this form aliquots of biotinylated sheep anti-isometamidium IgG were stored at -20°C between assays for up to 6 weeks without loss of activity.

Bovine serum standards spiked with isometamidium chloride

Normal bovine serum (NBS) was obtained from cattle prior to treatment with isometamidium chloride, or from a commercial source (Gibco).

A 500 $\mu g ml^{-1}$ solution of isometamidium chloride (Samorin®, RMB Animal Health Ltd.) in distilled water was prepared using volumetric glassware, allowing time for the compound to dissolve fully, and stored in a dark glass bottle at room temperature for not more than 3 days. Before use this solution was further diluted 1/10 in distilled water, and diluted again in NBS to a final concentration of 1.5 $\mu g ml^{-1}$. From this dilution a threefold serial dilution was performed in NBS, with vortex mixing after each transfer and a change of pipette tip after every third transfer.

The resulting spiked "standards" contained a range of isometamidium concentrations from 500 $ng ml^{-1}$ to 0.025 $ng ml^{-1}$, and contained not less than 99% NBS. Standards were stored at 4°C for up to 7 days.

Enzyme-linked immunosorbent assay (ELISA)

Immulon 4 (Dynatech Laboratories Inc., Billingshurst, UK) 96-well microtitre ELISA plates were coated overnight at 4°C with 100 µl per well isometamidium-ovalbumin conjugate diluted in carbonate-bicarbonate buffer (pH 9.2). Plates were washed 5 times with a 1/5 dilution of PBS in distilled water containing 0.05% (v/v) Tween 20 (Sigma), and blotted dry.

Bovine test sera or spiked NBS standards were pre-diluted 1/20, 1/10, or 1/6 in biotinylated anti-isometamidium IgG optimally diluted (see below) in PBS containing 0.05% Tween 20 (PBST). Pre-dilutions were made in 1.0 ml polypropylene tubes racked in an 8 x 12 matrix with identical dimensions to a 96-well microtitre plate (Microtubes, Alpha Laboratories). Racks of tubes were vortex mixed and the contents transferred to the coated microtitre plates by multichannel pipetting. Plates were incubated at 37°C on a shaker incubator (Varishaker, Dynatech) for one hour and washed as described above.

A 1/3000 dilution in PBST of streptavidin-horseradish peroxidase (SA-HRP; Sigma) was dispensed into every well (100 µl/well), incubated with shaking for 40 minutes at 37°C, and washed as described above. ELISAs were developed using 100 µl per well of substrate/chromogen solution prepared immediately before use by slowly adding 120 µl of a 42 mM solution of tetramethylbenzidine (TMB; Sigma) in DMSO, to 12 ml of 0.1 M sodium acetate/citric acid buffer (pH 6.0), and adding hydrogen peroxide to a final concentration of 1.3 mM (17.5 µl of a 3% v/v solution). Ten minutes after adding this solution, the reaction was quenched with 100 µl per well of 2 M sulphuric acid.

Absorbances were read in a multichannel spectrophotometer (Multiskan Plus Mk 11, Labsystems, Oy, Finland) connected to a personal computer (Personal System/2 Model 50 Z, IBM). Calculations were performed using spreadsheet software (1-2-3 version 3.1, Lotus Development Corporation) for which various ELISA data analysis macro-language programs were written (M.C.E., unpublished).

Optimal dilutions of isometamidium-ovalbumin conjugate and biotinylated anti-isometamidium IgG were determined by titration using NBS (isometamidium-free) in place of test serum to end-points not less than 0.8 optical density units.

The principle of this ELISA is illustrated in Plate 7.

Calibration curves

Programmed microcomputer spreadsheets were used for assay calibration and calculation of unknowns, using four-parameter logistic curve fitting (Healy, 1972; Rodbard and Hutt, 1974; Ukraincik and Picknosh, 1981).

Parallelism assays

Serum samples from isometamidium-treated cattle, or isometamidium-spiked serum, were serially diluted in NBS, or in serum from a trypanosome-infected Boran steer. The concentration of each dilution, determined by ELISA, multiplied by the dilution factor (abscissa), was plotted against dilution (ordinate axis). Sera were considered to show parallelism when a horizontal line parallel with the ordinate axis was obtained.

Intra-sample variance

Intra-sample (intra-assay) response variance (σ_w^2) was estimated using at least 20 replicate determinations of a single serum, or using the mean of the

variances of duplicate wells for at least 20 individual sera whose absorbance results fell in a local segment of the calibration curve (Rodbard and Hutt, 1974).

Intra-sample variance associated with pre-dilution of sera

Individual pre-dilutions of NBS were tested in duplicate wells of an ELISA plate. Intra-predilution and inter-predilution (intra-assay) variance were compared by one way analysis of variance.

Intra-sample variance associated with the solid-phase

Variance associated with the solid-phase was assessed by measuring intra-sample, intra-pre-dilution, (intra-assay) variance of pooled untreated bovine serum at zero-dose. All 96 wells of ELISA plates were treated identically through the coating, competition reaction, enzyme-conjugate and colour development stages. A single pre-dilution sufficient for all 96 wells (100 µl per well) was used.

The mean, standard deviation and coefficient of variation (CV) of the absorbances of entire plates, individual rows, columns, and quadrants of wells of plates, and non-edge and edge wells were calculated and compared. This procedure which was termed a "whole-plate B_0 CV test", B_0 representing the absorbance at zero-dose.

Inter-sample variance

Inter-sample (intra-assay) variance of the mean absorbances of duplicate determinations, σ_x^2, was measured at zero-dose by testing untreated Friesian and Boran sera in the isometamidium ELISA in duplicate wells. Using σ_w^2, calculated as described above from the mean of the variances of duplicate wells, σ_b^2, the true inter-sample (intra-assay) variance was calculated from the equation:

$$\sigma_x^2 = \sigma_b^2 + \sigma_w^2/r \qquad 1.$$

(Rodbard, 1974) where r is the number of replicate wells for each serum sample.

The significance of the difference between σ_b^2 and σ_w^2 was tested by one way analysis of variance.

Plate 7. The four stages of indirect competition ELISA for the detection of isometamidium. Between each stage, unbound reagents are removed by washing.

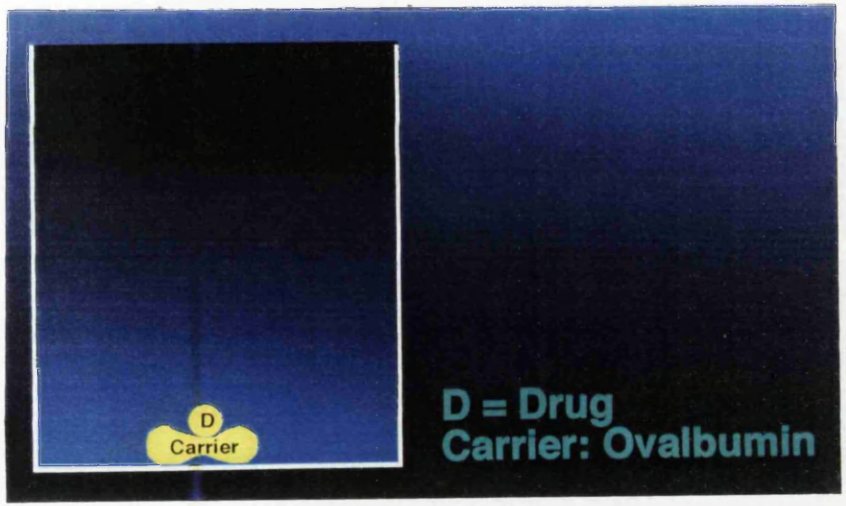
a) The wells of a 96-well polystyrene microtitre ELISA plate are coated by passive adsorption of drug (isometamidium) conjugated to a carrier protein (ovalbumin).

b) The sample, containing an unknown quantity of drug, is incubated in the ELISA plate wells, together with a fixed quantity of biotinylated specific anti-drug IgG. Sample drug competes for specific antibody, reducing the amount available to bind to the immobilised drug-conjugate.

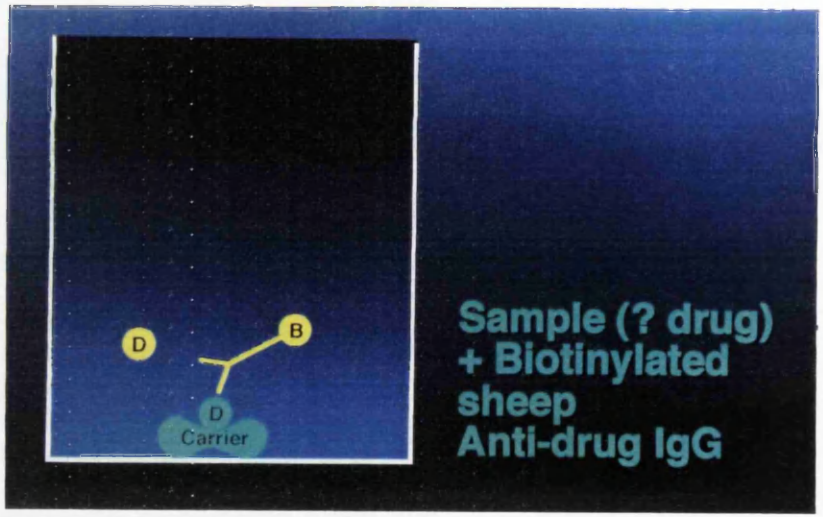
c) Streptavidin-horseradish peroxidase is used to detect biotinylated antibody.

d) In the presence of peroxidase and hydrogen peroxide, colourless tetramethylbenzidine is converted into a coloured product.

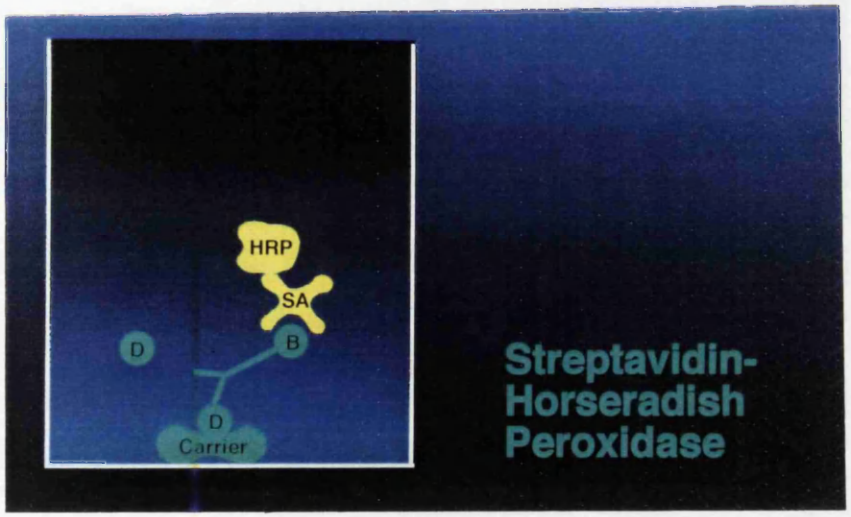
a


D
Carrier
D = Drug
Carrier: Ovalbumin

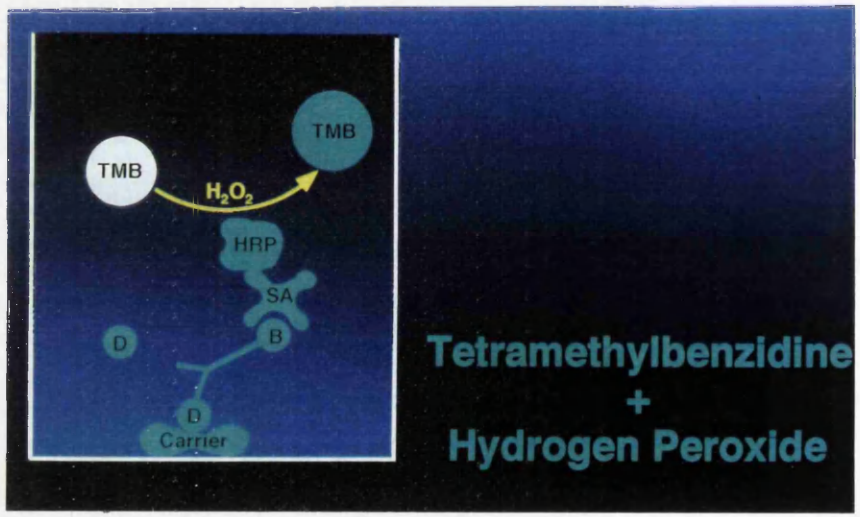
b


D
B
D
Carrier
Sample (? drug)
+ Biotinylated
sheep
Anti-drug IgG

c


HRP
SA
B
D
D
Carrier
Streptavidin-
Horseradish
Peroxidase

d


TMB
TMB
H_2O_2
HRP
SA
D
B
D
Carrier
Tetramethylbenzidine
+
Hydrogen Peroxide

Results

Background reactions

Absorbances of background control wells from which specific IgG had been omitted from the PBST used to predilute test serum, and absorbances obtained using spiked standard sera containing excess isometamidium (500 $ngml^{-1}$) were at most 10% of the zero-dose absorbance (B_0); usually background reactions were 5% B_0, or less.

Reagent titrations

The biotin-streptavidin system with TMB resulted in significantly higher absorbances than were obtained with the earlier ELISA method (Whitelaw *et al.*, 1991). This allowed the use of higher dilutions of isometamidium-ovalbumin conjugate and anti-isometamidium IgG which significantly reduced the concentration of isometamidium at which B/B_0 = 50% (B/B_0 represents absorbance divided by B_0, x 100%, after correction for background).

Calibration curves

Good curve fitting was obtained using the four-parameter logistic method, at all 3 dilutions of serum tested (1/6, 1/10, 1/20). A typical calibration curve obtained by this method is shown in Figure 1. The calibration curve varied with the dilution of serum used, so that the concentration at B/B_0 = 50% increased with increasing serum dilution.

Parallelism assays

The assay showed parallelism when a) sera from isometamidium-treated Boran cattle, b) isometamidium-spiked untreated Boran calf sera, or c) isometamidium-spiked untreated Friesian calf sera, were diluted in commercial NBS (Gibco). Parallelism was also obtained when isometamidium-spiked untreated Boran calf serum was diluted in serum from a trypanosome-infected Boran steer.

Intra-sample variance

The numerical value of σ_w^2 at zero-dose varied between individual assays, as did the mean zero-dose absorbances (B_0). However, the corresponding CVs (σ_w/B_0 x 100%) were consistently between 5% and 10%. Results for sera from 40 untreated Friesian calves, tested at 3 dilutions in the isometamidium ELISA, are shown in Table 6.

At dose levels other than zero, σ_w^2 varied with absorbance, so that intra-sample CVs were similar over most of the response range of the assay (data not shown). With the highest isometamidium concentrations σ_w^2 was very small, although the corresponding CVs were sometimes greater than 10%.

Intra-sample variance associated with pre-dilution of sera

No significant difference was found between intra-predilution and inter-predilution variance ($p > 0.1$). Hence the preparation of pre-dilutions did not make a significant contribution to intra-sample variance.

Intra-sample variance associated with the solid-phase

Whole plate B_0 CVs between 5% and 10% were obtained. Very occasionally larger CVs occurred, in which case examination of the mean, standard deviation and CV of absorbances over various parts of the plate usually allowed identification of non-Gaussian error suggestive of pipetting error.

Inter-sample variance

Zero-dose values of σ_x^2 varied between assays. However, the corresponding CVs (σ_x/B_0 x 100%) remained about 10%. Values for 24 untreated Friesian calves, 21 untreated Boran calves, and 20 untreated adult Boran cattle are shown in Table 7. CVs derived from the variance components σ_w^2 and σ_b^2 (σ_w/B_0 x 100% and σ_b/B_0 x 100%, respectively), calculated for 40 untreated Friesian calf sera tested in duplicate at 3 different dilutions, are shown in Table 6. The difference between σ_w^2 and σ_b^2 was shown by analysis of variance to be significant ($p < 0.01$) at all 3 serum dilutions.

Samples from isometamidium-treated cattle

Mean serum isometamidium concentration profiles for two groups of Kenyan Boran cattle injected intramuscularly with isometamidium at 0.5 $mgkg^{-1}$ ($n = 5$) and 1.0 $mgkg^{-1}$ ($n = 5$) body weight are shown in Figure 2. An initial peak within the first 24 hours following treatment was followed by a smooth pattern of disappearance of drug which was similar at both dose rates. The rate of drug disappearance varied with time; the initial peak was followed firstly by a phase of relatively rapid reduction in concentration, then by a phase of more gradual decline. Isometamidium could be detected in sera for at least 140 days after treatment.

Table 6. Intra-sample (intra-assay) and inter-sample (intra-assay) variance components, expressed as coefficients of variation, CV_w and CV_b , calculated for 40 isometamidium-free sera from normal Friesian calves tested in duplicate at 3 dilutions in the isometamidium ELISA; and inter-sample (intra-assay) coefficients of variation of the mean response for r replicates (CV_x).

	Serum Dilution		
	1/6	1/10	1/20
CV_w	7.2%	8.2%	7.8%
CV_b	9.5%	9.3%	6.0%
CV_x			
r = 1	11.9%	12.4%	9.8%
2	10.8%	11.0%	8.1%
3	10.4%	10.4%	7.5%
4	10.2%	10.2%	7.2%
5	10%	10%	6.9%
Infinity	9.5%	9.3%	6.0%

CV_w, CV_b , CV_x were calculated from the corresponding standard deviations σ_w, σ_b, σ_x using $CV = \sigma / B_0 \times 100\%$ where B_0 is the overall mean response for isometamidium-free sera calculated for each ELISA plate.
CV_x was calculated using $\sigma_x^2 = \sigma_b^2 + \sigma_w^2/r$.

Table 7. Inter-sample (intra-assay) coefficients of variation (CV_X) for the means of duplicate determinations for sera from untreated Boran and Friesian cattle.

	No.	CV_x (%)
Friesian calves	24	9.3%
Boran calves	21	9.4%
Boran adults	20	8.9%

Figure 1. Calibration curve fitted by four-parameter logistic method.

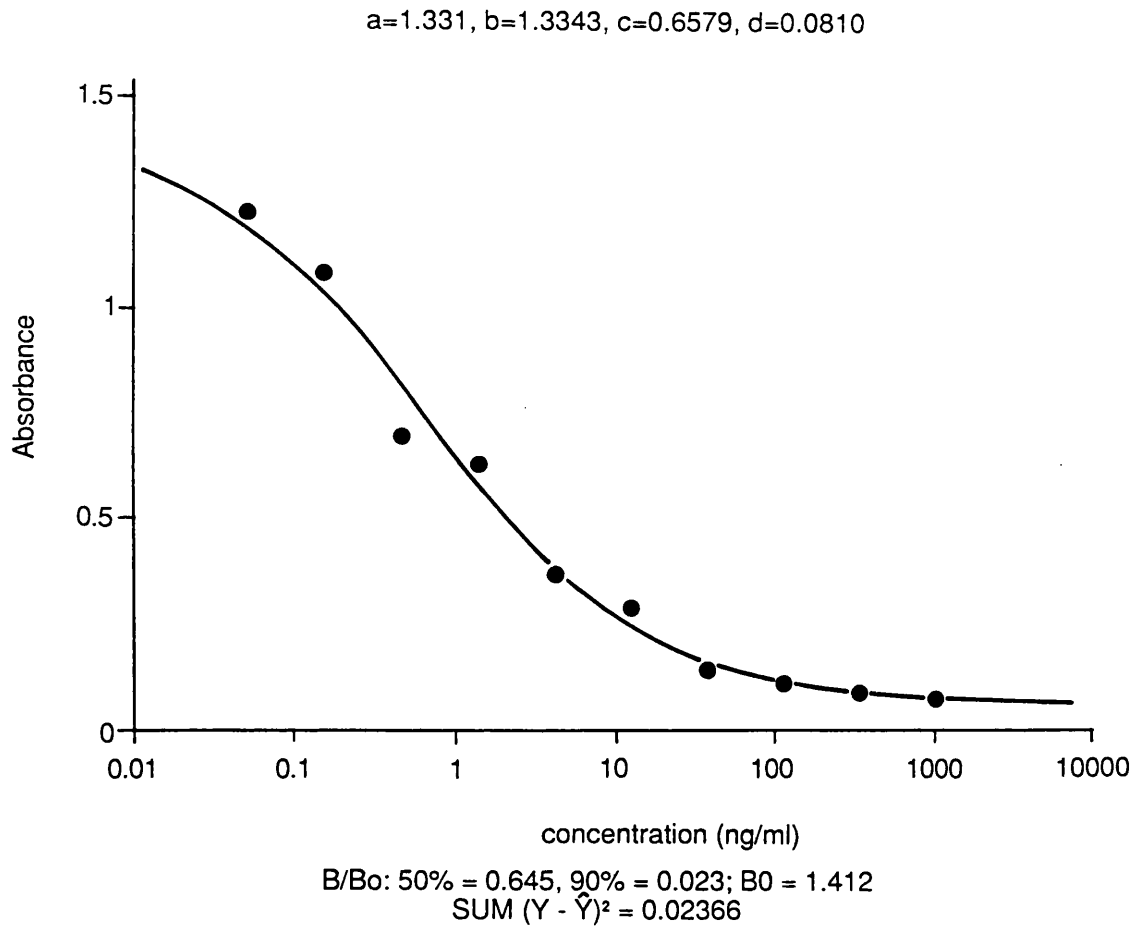
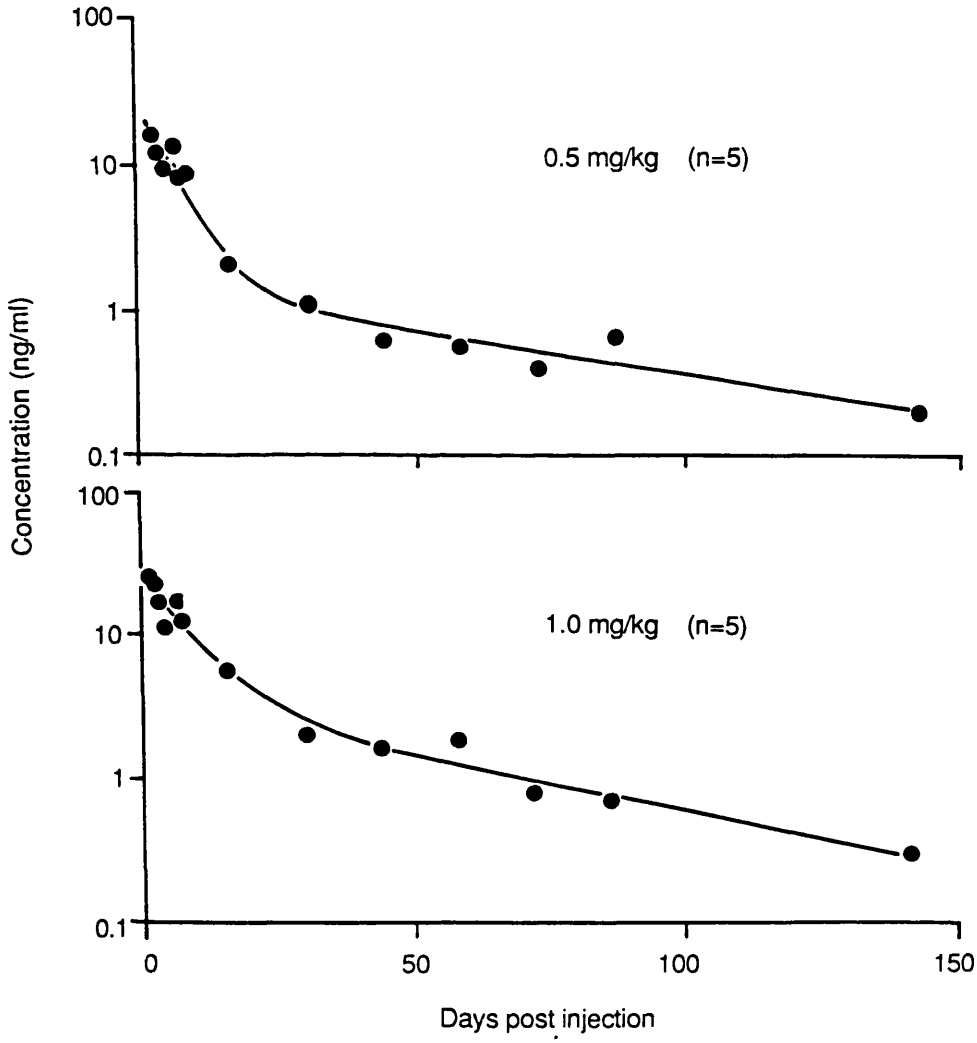


Figure 2. Pharmacokinetic profiles of healthy Boran cattle treated with 0.5 and 1.0 $mgkg^{-1}$ isometamidium chloride (Samorin®) by deep intramuscular injection.



Discussion

The development of a new isometamidium ELISA, described here, has enabled many constraints on an earlier method (Whitelaw *et al.*, 1991) to be overcome. Furthermore, it has also allowed statistical parameters of assay performance to be defined, enabling the new ELISA to be validated for use on field samples in Africa.

Two years of experience with isometamidium ELISA development, both in the United Kingdom and in Africa, has revealed a number of serious limitations to the earlier method. Three aspects particularly unsuitable for large-scale testing of field samples were the methods of dispensing and dilution of serum samples and standards; the occurrence of unacceptably high background reactions; and the methods of assay calibration and data reduction. In the new assay, these 3 aspects have been modified appropriately, and in addition, quality assurance has been addressed by statistical analysis.

The isometamidium ELISA method described in this Chapter may be referred to as an indirect competition ELISA (ICE) by analogy with the indirect ELISA, of which it is essentially a modification. In the indirect ELISA, specific antibody is quantified by incubation with a predetermined quantity of antigen is adsorbed onto a solid-phase, unbound non-specific antibody removed by washing, and subsequent incubation first with an suitable enzyme-conjugated anti-globulin reagent, and secondly with a chromogenic substrate solution (Tijssen, 1985).

In the indirect competition ELISA (see Plate 7) the total quantity of specific antibody is also fixed, but the amount available to bind to the immobilised antigen may be modulated by the presence of analyte - essentially the same (or closely related) antigen in the liquid phase. Because the specific antibody is added in a fixed quantity from a defined source, rather than in unknown quantity from a biological sample, it is also possible to label the specific antibody reagent, in this case with biotin. Enzyme-conjugated streptavidin, rather than a conjugated anti-globulin reagent may then be used as the antibody detection system.

The biotin-streptavidin system for detection of specific antibody has a number of advantages over the use of an anti-globulin reagent. Firstly, the system is extremely sensitive and efficient, as the dissociation constant for the biotin-streptavidin interaction is one of the smallest known, at approximately 10^{-}

^{15} (Tijssen, 1985). Secondly, streptavidin is more specific than anti-globulin reagents, which may cross react with immunoglobulins of animal species other than that against which they were raised. Finally, unlike anti-globulin reagents, which may vary between batches (unless monoclonal), streptavidin is a single defined molecule of consistent affinity and specificity.

The cause of unacceptably high background reactions observed with the earlier isometamidium ELISA method (Whitelaw *et al.*, 1991) was probably detection by anti-sheep IgG conjugate of bovine IgG adsorbed on the solid-phase. Evidence for this is that when PBST or foetal calf serum was substituted for bovine serum samples in the earlier isometamidium ELISA method, background reactions remained below 10% B_0; additionally, polyclonal anti-sheep IgG enzyme conjugates from a variety of sources cross-reacted strongly with bovine IgG (unpublished observations). Use of streptavidin enzyme-conjugate to detect biotinylated anti-isometamidium IgG overcame this problem. Biotinylated IgG was stable at 4°C for at least 6 months after lyophilisation, which is important in the context of use in, and delivery to, African laboratories where cold chain continuity may be unreliable.

Four-parameter logistic curve-fitting was found to be a suitable method of assay calibration, and is preferable to linear interpolation or the logit-log method (Ukraincik and Picknosh, 1981; Rodbard,1974). The use of a three-fold dilution series for the standards resulted in a greater number of data points within the dynamic range of the assay, compared to the 10-fold dilutions used with the earlier method (Whitelaw *et al.*, 1991). This improved the reliability of the calibration curve.

The ELISA plates chosen for use in the new isometamidium ELISA (Immulon 4; Dynatech) were those which consistently had the lowest overall CV (5-10%), out of 9 types of plate from 4 major manufacturers (unpublished data). The plates chosen carried a manufacturer's guarantee of CV, suggesting future batches could be expected to be of similar quality to those tested. They were also physically robust, an advantage for transportation in developing countries.

The variance of the means, σ_x^2, of replicate determinations of a number of different untreated sera comprised an inter-sample component, σ_b^2, attributable to non-specific effects of serum matrix components, and an intra-sample component, σ_w^2, attributable to pipetting error and variation between wells of ELISA plates. Estimates of σ_x^2 and σ_w^2 were calculated, respectively, as the variance of mean responses, and the mean of variances of duplicates, of a

representative sample of the untreated cattle population. Using Equation 1, σ_b^2 was calculated from σ_x^2 and σ_w^2 (Rodbard, 1974).

From σ_b^2 and σ_w^2, the variance of the mean response σ_x^2 could be predicted for any number of replicates (r) by substituting new values for r in Equation 1 (Rodbard, 1974). Increasing r diminishes the term σ_w^2/r, and as $r \rightarrow \infty$, σ_x^2 tends to its limiting value σ_b^2. Unless σ_b^2 is considerably less than σ_w^2, increasing r will have limited effect on σ_x^2, and hence on assay precision. From Table 6 it may be observed that only a small decrease in CV of the mean ($\sigma_x/B_0 \times 100\%$) would be achieved by increasing the number of replicates from one to two; with further increases in r, increases in CV would be progressively smaller. Even with an infinite number of replicates only a limited decrease in CV would be possible. The use of duplicate wells is nevertheless worthwhile as it enables σ_w^2 to be calculated as an ongoing, in-built quality control, and it facilitates identification of non-Gaussian or "catastrophic" errors (Chard, 1990), which may occur as a result of pipetting mistakes.

Using σ_x, r, and the assay calibration curve, the least detectable dose may be derived from the appropriate percentile of Student's t-distribution at the desired probability level. Using a modification of Rodbard's (1978) method, to allow for inter-sample variance, the least detectable dose for cattle within the populations tested in this work was approximately 0.1 $ngml^{-1}$.

A single dose of isometamidium may afford up to 6 months prophylaxis to cattle challenged by fully susceptible isolates of *T. congolense* (Whitelaw, Bell, Holmes, Moloo, Hirumi, Urquhart and Murray, 1986), although drug-resistant trypanosome stocks may result in break-through infections as little as one month following isometamidium treatment (Sutherland, Moloo, Holmes and Peregrine, 1991). Ion-pair high performance liquid chromatography, the most sensitive technique described to date, detected isometamidium for only a few hours in treated cattle (Kinabo and Bogan, 1988). Hence in the investigation of isometamidium resistance the ELISA, which quantified drug levels for at least 140 days, represents a significant improvement over earlier methods.

These investigations have shown the new isometamidium ELISA to be a powerful, sensitive and reproducible technique for the quantification of the drug in bovine sera. An integral calibration and quality assurance software package incorporating the data reduction and statistical analyses described here is envisaged for use with the assay. Potential uses of the assay include the development of rational chemoprophylactic drug regimens; the detection of

isometamidium resistance in the field, by showing levels of drug normally considered to be prophylactic in the serum of parasitaemic animals; laboratory investigations on chemoprophylaxis in vivo; the extension of its use to other species and other biological fluids, enabling detection of isometamidium residues in meat and animal products; and investigation of suspected inappropriate use of trypanocides. Further work to validate the assay using field samples from trypanosomiasis endemic areas of Africa is described in later chapters.

Chapter 4:
Isometamidium concentrations in the sera of Boran cattle: correlation with prophylaxis against tsetse-transmitted *Trypanosoma congolense*

Introduction

More than thirty years after the introduction of the trypanocidal drug isometamidium chloride (Berg, 1960) for prophylaxis and therapy of tsetse-transmitted trypanosomiasis in domestic livestock, its use remains the most widespread control strategy for this disease (Leach and Roberts, 1981; Holmes and Torr, 1988; Sones *et al.*, 1988; Jordan, 1992). In recent years, there has been increasing concern regarding the potential development of drug resistance in pathogenic trypanosomes (Sutherland and Holmes, 1991), and although this has been shown to occur in a number of situations (Kupper and Wolters, 1983; Pinder and Authié, 1984; Ainanshe *et al.*, 1992), the true prevalence of resistance remains largely unknown because of the lack of appropriate field tests.

A wide range has been reported in the prophylactic period afforded by intramuscular isometamidium chloride under field conditions of use. In studies conducted in the decade following the introduction of the drug, doses of 0.5 to 1.0 $mgkg^{-1}$ body weight afforded prophylaxis against natural tsetse challenge for periods as long as 14 to 36 weeks (Robson, 1962; Fairclough, 1963; Kirkby, 1964; Wiesenhutter *et al.*, 1968). More recently, however, prophylactic periods as short as 2 to 3 weeks have been observed (Dolan *et al.*, 1992, Münstermann *et al.*, 1992). When prophylactic periods are reduced in the field, the emergence of drug-resistant strains of trypanosomes is frequently suspected. However, when studying the variation in the duration of prophylaxis, it has not previously been possible to assess the relative contributions of either drug-resistance levels in trypanosomes, or of variability in drug levels. Measurement of drug resistance levels in trypanosomes is rarely possible under field conditions, for economic and logistical reasons (Sutherland and Holmes, 1991), and variability in drug levels has not been determined due to the lack of a method capable of measuring serum isometamidium concentrations during the prophylactic period.

The development of an enzyme-linked immunosorbent assay (ELISA) for the measurement of isometamidium concentrations in the sera of treated cattle (Chapter 3) has therefore provided a method which may contribute to the assessment of the relative importance of these factors. In order to evaluate data obtained using the ELISA on samples from the field, base-line data on the relationship between circulating drug concentrations and susceptibility to trypanosome infection is clearly required. This chapter describes an

experiment in which the isometamidium ELISA described in Chapter three was used to investigate concentrations of the drug in the sera of Boran cattle under controlled laboratory conditions, which were challenged with stocks of *T. congolense* of varying susceptibility to the drug, by the application of experimentally-infected tsetse flies at regular intervals following administration of a single prophylactic dose.

Materials and methods

Cattle

Boran (*Bos indicus*) cattle were obtained from a trypanosomiasis-free area of Kenya, housed in fly-proof accommodation and fed on hay and concentrate as described by Whitelaw *et al.* (1986), and allowed water *ad libitum*.

Isometamidium administration

Isometamidium chloride (Samorin®) was administered at a dose rate of 1.0 $mgkg^{-1}$ body weight, by deep intramuscular injection of a 2% (w/v) aqueous solution into the middle third of the neck.

Blood samples

Blood samples for use in the isometamidium-ELISA were collected by jugular venipuncture into Vacutainers (Becton-Dickinson) prior to isometamidium treatment; daily for the first week after treatment, twice weekly for a further 2 weeks, and weekly thereafter. Sera were separated and stored in borosilicate glass tubes (Vial Files, Jencons Scientific Ltd., Leighton Buzzard, UK) at -60°C. Blood samples for monitoring of infections were collected three times weekly, as described above, into Vacutainers (Becton-Dickinson) containing potassium ethylenediamine tetra-acetate.

Trypanosomes

Three isolates of *T. congolense* were used to challenge cattle. *T. congolense* IL 3893 is a stock derived from an Ethiopian isolate: IL 3330 (Codjia *et al.*, 1993). *T. congolense* IL 3889 is a derivative of KETRI 3302/E36, isolated at Nguruman, Kenya (Gray, Kimarua, Peregrine and Stevenson, 1993). *T. congolense* IL 1180 is a well-characterised, doubly cloned, derivative of an isolate made from a lion in the Serengeti National Park, Tanzania (Geigy and Kauffmann, 1973; Nantulya *et al.*, 1984; Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988).

Infected tsetse flies

Six weeks before each challenge, goats were inoculated intramuscularly with one of the 3 isolates of *T. congolense*. Twelve to 14 days later, trypanosomes were detected in peripheral blood. Commencing a further 7 days later, teneral *Glossina morsitans centralis* were fed daily on the infected goats for 25 days. Flies were then starved for 2 days, and monitored for infection by

microscopic examination of salivary probes. Tsetse flies with metacyclic trypanosomes in their saliva were used to challenge cattle (see Plate 4).

Parasitological monitoring

Following challenge, cattle were monitored for the appearance of trypanosomes using the phase-contrast buffy-coat technique (Murray *et al.*, 1977).

Isometamidium standards

Sera were collected from 33 Boran cattle (including the 25 experimental cattle, see below), prior to isometamidium treatment of any animal. Sera were pooled to provide a large batch for preparation of isometamidium-spiked standards for use in the ELISA. The pool was considered suitable for preparing standards as the mean absorbance for the pool in the isometamidium ELISA (see below) was similar to the mean of the absorbances for the individual sera. Standards were prepared as described previously (Chapter 3), aliquoted into microplate-format racked tubes (Microtubes, Alpha Laboratories), and stored at 4°C until use.

Isometamidium determinations

Sera collected following isometamidium treatment were tested using the indirect competition ELISA for isometamidium (Chapter 3). Briefly, microtitre plates (Immulon 4, Dynatech) were coated by overnight incubation of 100 µl per well of a 1/2000 dilution of isometamidium-ovalbumin conjugate in carbonate-bicarbonate buffer (pH 9.2). Plates were washed 5 times using a 1/5 dilution of phosphate-buffered saline (PBS), pH 7.2, in distilled water containing 0.05% Tween 20 (Sigma), and blotted dry. Test sera, standards, and quality control sera were pre-diluted 1/10 in a 1/2400 dilution of biotinylated sheep anti-isometamidium IgG in PBS containing 0.05% Tween 20 (PBST), and vortex mixed. Each pre-dilution was added to duplicate wells of microtitre plates (100 µl per well) which were incubated and shaken at 37°C for 1 h. Plates were then washed 5 times as described above, 100 µl of a 1/3000 dilution of streptavidin:horseradish peroxidase conjugate (Sigma) in PBST was added to every well, and plates were incubated and shaken at 37°C for 45 minutes. Plates were thereafter washed 5 times as described above, and 100 µl per well added of a solution containing 120 µl of 42 mM tetramethylbenzidine (TMB; Sigma) in dimethyl sulphoxide (Sigma), 12 ml of 0.1 M sodium acetate/citric acid buffer (pH 6.0), and hydrogen peroxide to a final concentration of 1.3 mM. Ten

minutes later the reaction was quenched with 100 µl per well of 2 M sulphuric acid.

Absorbances were read using a multichannel spectrophotometer (Multiskan MCC Mk II, Labsystems), connected to a personal computer (386SX, AST).

Data analysis

Isometamidium concentrations in sera of treated cattle were calculated from the ELISA optical densities, with reference to the optical densities of isometamidium standards, using 4-parameter logistic curve fitting. Four-parameter logistic calibration curve-fitting, precision profiling, and calculation of drug concentrations of unknown samples, were performed using spreadsheet software (Lotus 1-2-3 R3.1) specifically written for this purpose (M.C.E., unpublished).

Drug profiles were fitted to a bi-exponential model by iterative curve stripping (Dunne, 1986), using the computer programme JANA (SCI Software, Lexington, KY, USA). Prepatent periods of infection in cattle were compared using the Wilcoxon rank sum test (Ott, 1988).

Experimental design

Twenty-five cattle were divided into 3 groups of 5 animals each (groups 1, 2 and 3), and a further group of 10 animals (challenge controls). At the start of the experiment all cattle in groups 1, 2 and 3 received isometamidium. Isometamidium-treated cattle were then challenged repeatedly with one of 3 different *T. congolense* populations at monthly intervals until they were detected parasitaemic; animals with patent infections were not challenged further. Cattle were challenged by the application of 5 *T. congolense*-infected tsetse flies (see Table 8). Cattle in Group 1 were challenged with *T. congolense* IL 3893, Group 2 cattle were challenged with *T. congolense* IL 3889, and Group 3 cattle were challenged with *T. congolense* IL 1180. Challenge controls were used to confirm the infectivity of the tsetse flies: one for each challenge with each *T. congolense* population. Details of the experimental design are summarised in Table 8.

Results

Parasitological findings

All cattle challenged with *T. congolense* IL 3893 (Group 1 and challenge control no. 16) and all cattle challenged with *T. congolense* IL 3889 (Group 2 and challenge control no. 17) became parasitaemic following the initial monthly challenge (Table 9). By contrast, all cattle in Group 3, challenged with *T. congolense* IL 1180, remained aparasitaemic following the first 5 monthly challenges, although challenge control animals (nos. 18 - 22) became infected after every challenge (Table 9).

The first trypanosomes detected in a Group 3 animal (no. 11) occurred following the sixth monthly challenge (Table 9). Two further animals in Group 3 (nos. 12 and 13) became parasitaemic following the seventh monthly challenge. The remaining 2 cattle (nos. 14 and 15) became parasitaemic following the eighth monthly challenge (Table 9).

There were notable differences in the prepatent periods of infection (PPI) between animals in groups 1, 2 and 3 (Table 9); i.e., the time between challenge and first detection of trypanosomes. Group 1 animals were first detected parasitaemic 16 to 18 days following challenge. Group 2 animals exhibited significantly longer PPIs ($p < 0.025$), with trypanosomes not detected until 25 to 35 days following challenge. All cattle in groups 1 and 2 exhibited longer PPIs than the corresponding challenge controls, no. 16 (14 days) and no. 17 (11 days), respectively.

In Group 3 animals, challenged with *T. congolense* IL 1180, PPIs (mean, 20.2 days) were significantly longer ($p < 0.025$) than the PPIs (mean,13.9 days) of the corresponding challenge controls (Group 6; Table 9). There was, however, some overlap in the range of PPIs for Group 3 (range, 15 - 32 days) and the corresponding controls (range, 13 - 15 days). Finally, the lengths of the PPIs for animals in Group 3 appeared to be related to the number of challenges an animal received before trypanosomes were detected; earlier break-through infections were associated with longer PPIs.

Drug profiles

The highest isometamidium concentrations, approximately 40 - 50 $ngml^{-1}$, were observed at the time of the first sampling, 24 hours following isometamidium administration. Serum concentrations then fell relatively rapidly

for about 8 days post-treatment, and thereafter rather more gradually (Figures 1 and 2). The mean serum drug concentrations in animals in all 3 groups were similar for the first 6 weeks following treatment, after which drug concentrations fell more rapidly in those cattle which succumbed to infection following the first tsetse challenge (groups 1 and 2). At the time of the first monthly challenge the mean isometamidium concentration in all three groups of treated cattle (groups 1, 2 and 3) was 6.1 $ngml^{-1}$ (range, 2.8 - 12 $ngml^{-1}$). By 100 days after treatment, serum drug concentrations in groups 1 and 2 had reached the limit of detection of the assay (0.1 - 0.2 $ngml^{-1}$). However, at that time, the mean drug concentration in Group 3 cattle was 0.75 $ngml^{-1}$ (range, 0.51 - 0.89 $ngml^{-1}$), which was still well within the dynamic range of the assay.

The results of bi-exponential curve-fitting using the mean results for each of the 3 isometamidium-treated groups of cattle are shown in Table 10. Prior to the detection of parasites (days 0 - 45, 0 - 59 and 0 - 104 for groups 1, 2 and 3, respectively), isometamidium concentrations in all treated groups closely fitted the bi-exponential model (R^2 greater or equal to 0.98). Half-lives for the second phase were 29.4 days for Group 1, 22.5 days for Group 2, and 23.9 days for Group 3. Isometamidium concentrations in Group 3 cattle closely fitted the bi-exponential model (R^2, 0.99) over the entire period of measurement (days 0 - 104, Figure 3). However, groups 1 and 2 showed divergence from the bi-exponential model after times which corresponded with the appearance of trypanosomes (days 48 and 60 for groups 1 and 2, respectively [Figure 4]). Following the development of patent parasitaemias, therefore, the bi-exponential model was no longer considered appropriate. An estimate of the new rate of disappearance of isometamidium over this period (days 52 - 104 and days 62 - 104 for groups 1 and 2, respectively) was obtained by performing linear regression (log mean concentrations on time post-treatment). This gave apparent drug elimination half-lives of approximately 14 days for both groups (R^2 values both 0.92; Table 10 and Figure 4).

Table 8. Experimental design: challenge groups

i.m. = intramuscular treatment.

Group	Number of Cattle	Isometamidium Prophylaxis (1 $mgkg^{-1}$ i.m.)	Challenge: *T. congolense* stock	Number of cattle challenged each month*
1	5	+	IL 3893	5
2	5	+	IL3893	5
3	5	+	IL 1180	5
Challenge control	1	-	IL3893	1
Challenge control	1	-	IL 3889	1
Challenge control	8	-	IL 1180	1

*Cattle challenged at monthly intervals until trypanosomes detected.

Table 9. Outcome of tsetse challenge of isometamidium-treated Boran cattle

CC = challenge control. PPI = prepatent period of infection; n.a. = not applicable

Group	Animal number	*T. congolense* challenge	PPI (days)	Month of challenge*
1	1	IL 3893	16	1
1	2	IL 3893	16	1
1	3	IL 3893	16	1
1	4	IL 3893	18	1
1	5	IL 3893	18	1
		mean	16.8	1
2	6	IL 3889	25	1
2	7	IL 3889	28	1
2	8	IL 3889	28	1
2	9	IL 3889	28	1
2	10	IL 3889	35	1
		mean	28.8	1
3	11	IL 1180	32	6
3	12	IL 1180	18	7
3	13	IL 1180	21	7
3	14	IL 1180	15	8
3	15	IL 1180	15	8
		mean	20.2	7.2
CC	16	IL 3893	14	1
CC	17	IL 3889	11	1
CC	18	IL 1180	14	1
CC	19	IL 1180	13	2
CC	20	IL 1180	15	3
CC	21	IL 1180	14	4
CC	22	IL 1180	13	5
CC	23	IL 1180	15	6
CC	24	IL 1180	14	7
CC	25	IL 1180	13	8
		mean	13.9	n.a.

*Month of challenge resulting in infection.

Table 10. Bi-exponential curve fitting and linear regression results for isometamidium concentration data.

Days = period of observations; n = number of observations; $t_{½}$ = half life; R^2 = correlation co-efficient; ND = not determined.

	Group 1	Group 2	Group 3
Bi-exponential curve fitting:			
Days	0 - 45	0 - 59	0 - 104
n	15	17	24
$t_{½}$	29.4	22.5	23.9
R^2	0.98	0.98	0.99
Linear regression:			
Days	52 - 104	62 - 104	ND
n	9	7	ND
$t_{½}$	14.1	13.8	ND
R^2	0.92	0.92	ND

Figure 3. Mean isometamidium concentrations ($ngml^{-1}$) in the sera of cattle challenged with *T. congolense* IL 1180 (Group 3). Line represents bi-exponential curve-fit. Tsetse fly symbols: *T. congolense* challenge.

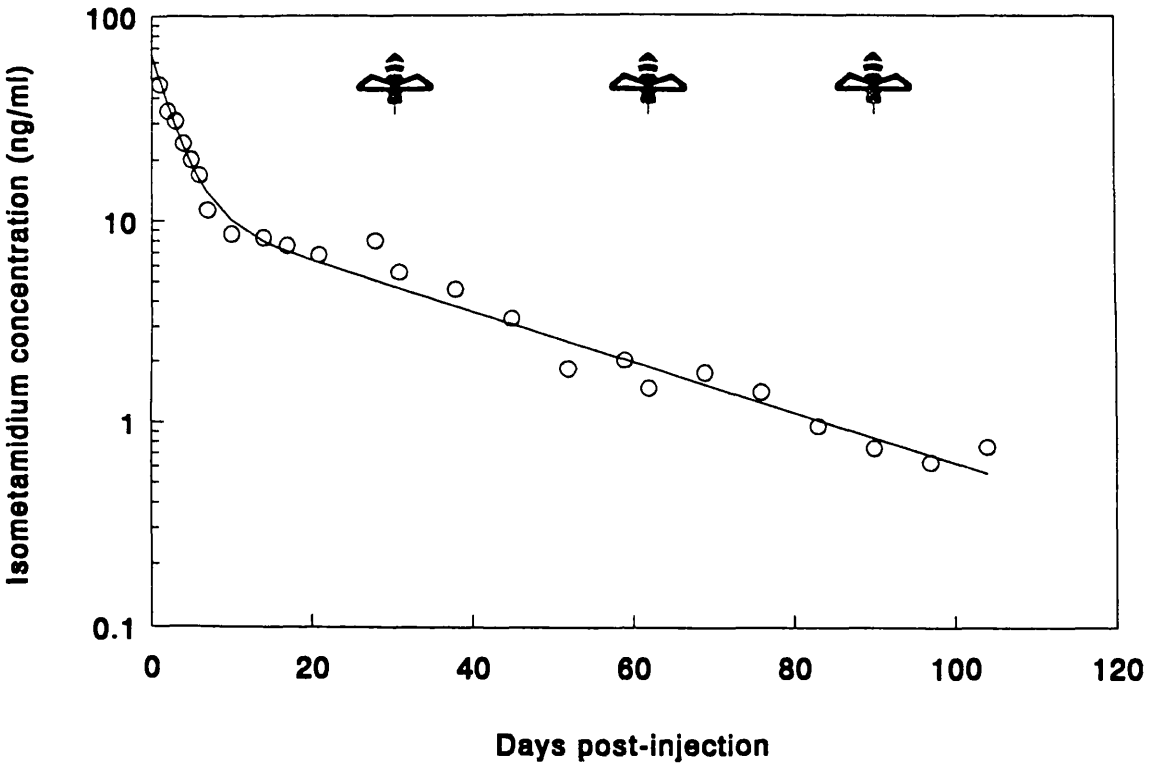
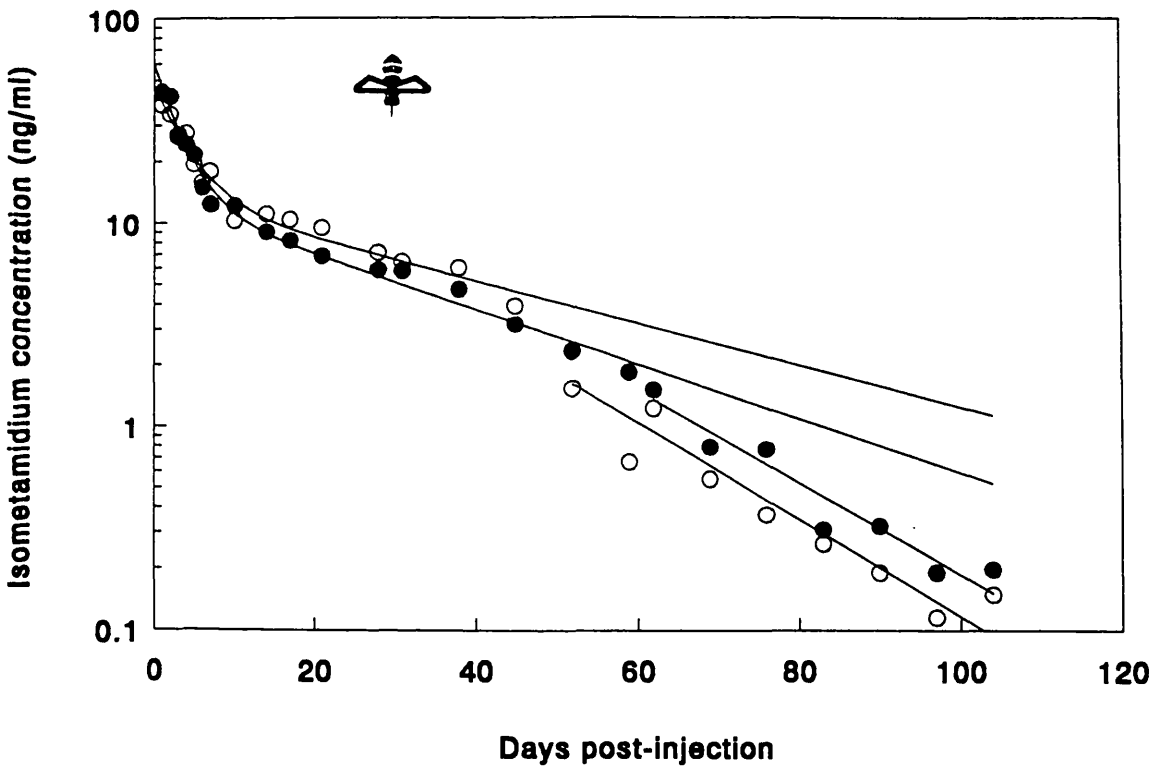


Figure 4. Mean isometamidium concentrations ($ngml^{-1}$) in the sera of cattle challenged with *T. congolense* IL 3893 (Group 1), open symbols, and *T. congolense* IL 3889 (Group 2), closed symbols. Long lines: bi-exponential curve-fits. Short lines: linear regression. Lower long line and lower short line: Group 1. Upper long line and upper short line: Group 2. Tsetse fly symbol: *T. congolense* challenge.



Discussion

In this experiment, all isometamidium-treated cattle challenged with *T. congolense* IL 3893 or *T. congolense* IL 3889 became infected following the first monthly challenge, whilst those challenged with *T. congolense* IL 1180 resisted at least 5 monthly challenges. Following treatment, isometamidium was detected in the serum of all the treated cattle for up to 3 months. Analysis of the pharmacokinetic profiles indicated that the development of parasitaemia was associated with accelerated drug elimination. The results therefore show that the isometamidium-ELISA is useful in the determination of isometamidium concentrations in the sera of treated cattle, and in correlating these concentrations with protection against isolates of *T. congolense* with various levels of drug resistance.

The three isolates of *T. congolense* used to challenge cattle were chosen on the basis that they possessed differing levels of resistance to isometamidium. *T. congolense* IL 3893, which has been shown to possess a high level of resistance to a number of trypanocides, including isometamidium (Codjia *et al.*, 1993), infected all challenged animals one month following treatment. *T. congolense* IL 3889, isolated at a field site (Nguruman, Kenya) where resistant trypanosomes are believed to occur, was expected to show an intermediate level of resistance on the basis of in vitro tests (Gray *et al.*, 1993), but behaved similarly to *T. congolense* IL 3893 in that no animal withstood challenge one month post-treatment. Finally, intramuscular doses of isometamidium chloride at 1.0 $mgkg^{-1}$ body weight have been shown to protect cattle against tsetse challenge with *T. congolense* IL 1180 for up to 6 months (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988); the population behaved similarly in this experiment.

Although break-through infections with both *T. congolense* IL 3893 and *T. congolense* IL 3889 occurred at the same stage of the experiment in isometamidium-treated cattle, differences in the PPIs indicated a difference in the resistance of these isolates. The prolonged PPI in treated cattle challenged with *T. congolense* IL 3889 (mean 28.8 days), compared with *T. congolense* IL 3893 (mean 16.8 days), may suggest that although both parasite populations were resistant, the growth rate of the former population was initially relatively low due to a high drug concentration present at the time of challenge. Thereafter, the rate may have gradually increased as drug concentrations declined. Alternatively, differences in PPI may reflect elimination of different

percentages of the initial infective metacyclic inoculum by isometamidium. The absence of antibody responses, skin reactions or local lymph node enlargement at the site of application of tsetse, following experimental *T. congolense* challenge of cattle rendered refractory to infection by isometamidium (Whitelaw *et al.*, 1986), suggests that the drug exerts its effect on the parasites at the level of the skin.

The drug profiles in isometamidium-treated cattle were similar to those obtained previously in uninfected cattle (Chapter 3). Prior to the development of parasitaemias, the half-life (approximately 25 days) of the second phase of the disposition curves was similar to that previously described. The ELISA was thus shown to be reproducible and reliable.

Studies in cattle using the ELISA (M.C.E., unpublished), and high-performance liquid chromatography (Kinabo and Bogan, 1988), have shown that the maximum drug concentration occurs within 45 minutes of intramuscular administration of isometamidium. In the experiment described here, drug concentrations decreased with time after treatment. This suggested that the maximum drug concentration had preceded the first sampling at 24 h.

In cattle which were detected parasitaemic following the first tsetse challenge (groups 1 and 2), disappearance of drug was more rapid following the onset of parasitaemia than in cattle which resisted challenge over the same period (Group 3). The apparent half-life of the drug in animals detected parasitaemic with either *T. congolense* IL 3893 or *T. congolense* IL 3889 was approximately 14 days. (Drug concentrations at the time of, or following, break-through infections with *T. congolense* IL 1180 were not determined). The increase in the rate of drug elimination following the development of patent infections may have resulted from direct interaction between the drug and the parasites. For example, in other work, one h after *T. congolense*-infected calves had been treated with ^{14}C-labelled homidium bromide, when blood counts had reached a peak, 80% of the radioactivity was bound to trypanosomes (Gilbert, Curtis and Newton, 1979). However, since the tissue, blood levels, and excretion of radioactivity were similar in calves whether or not they were infected at the time of drug administration this would suggest that the trypanosomes did not have a significant effect on the drug pharmacokinetics. Similarly, isometamidium's period of prophylaxis in cattle was shown to be independent of the presence or absence of *T. congolense* at the time of administration (Peregrine *et al.*, 1988). However, the pharmacodynamics of the

trypanocides in these experiments, in which pre-existing infections were rapidly cleared by trypanocide, would have been very different to the present experiment, in which infections with drug-resistant parasites became established in spite of significant serum drug concentrations.

Increased drug elimination following patent infection may also have occurred as a result of changes in the pathophysiological state of the host. For example, anaemia, increased circulating fluid volume, and changes in the composition of plasma proteins and lipids, which could alter the pharmacokinetics of isometamidium through changes in drug binding and distribution, have been documented in *T. congolense* infections in ruminants (Katunguka-Rwakishaya, Murray and Holmes, 1992a, 1992b).

The mean isometamidium concentration in serum of cattle in groups 1, 2 and 3 on the day of the first monthly challenge was 6.1 $ngml^{-1}$. This concentration was insufficient to protect cattle against tsetse challenge with either *T. congolense* IL 3893 or *T. congolense* IL 3889. However, the same concentration of drug was protective against challenge with *T. congolense* IL 1180, since cattle remained refractory to infection for a minimum of 4 further monthly challenges. Three months after isometamidium injection, the mean serum drug concentration in uninfected cattle was 0.75 $ngml^{-1}$, and on the basis of the elimination profiles obtained over the first 3 months, drug concentrations protective at the time of the fourth and fifth monthly challenges would have been even lower. The difference between the lowest mean drug concentration (0.75 $ngml^{-1}$) determined protective against the sensitive isolate (*T. congolense* IL 1180), and the highest mean concentration (6.1 $ngml^{-1}$) withstood by resistant populations (*T. congolense* IL 3893 and *T. congolense* IL 3889) was approximately eight-fold.

In this study, the ELISA has permitted the relationship between serum concentrations of isometamidium and susceptibility to tsetse challenge with *T. congolense* to be investigated in cattle. The results indicate that trypanosomes occurring in the presence of serum concentrations of 6 $ngml^{-1}$ or higher are as resistant to isometamidium as *T. congolense* IL 3893 and IL 3889, and that they therefore express marked drug resistance. Trypanosomes occurring where serum isometamidium concentrations are greater than 0.75 $ngml^{-1}$, but lower than 6 $ngml^{-1}$, are more resistant to isometamidium than *T. congolense* IL 1180, and would be an indication of moderate drug resistance. Finally, the presence of trypanosomes where serum concentrations are below 0.75 $ngml^{-1}$ should not

at present be regarded as evidence of a significant level of drug resistance. However, it should be noted that *T. congolense* IL 1180 is not the most isometamidium-sensitive population that has been described (Peregrine, Knowles, Ibitayo, Scott, Moloo and Murphy, 1991).

The information obtained in the studies described in this chapter will enable interpretation of data arising from use of the isometamidium ELISA on serum samples taken from cattle under chemoprophylactic regimens in the field, thereby enabling investigations on the prevalence of trypanosomes of various degrees of resistance to isometamidium in such circumstances. The following chapter describes further work to investigate the relationship between circulating isometamidium concentrations and prophylaxis against *T. congolense*, using a cloned stock with a level of sensitivity to isometamidium intermediate between that of *T. congolense* IL 1180, and those of *T. congolense* IL 3893 and *T. congolense* IL 3889.

Chapter 5:
Concentrations in the Serum of Boran Cattle of the Trypanocidal Drug Isometamidium Chloride Protective Against Tsetse Challenge with a *Trypanosoma congolense* Clone Possessing an Intermediate Level of Drug Resistance

Introduction

The ELISA for the detection and measurement of concentrations of the trypanocidal drug isometamidium chloride in the serum of treated cattle described in Chapter 3 has provided a valuable new method to enable the relationship between drug concentrations and prophylaxis against tsetse transmitted trypanosomiasis to be investigated. In an experiment, using tsetse challenge of Boran cattle with three stocks of *Trypanosoma congolense* under controlled laboratory conditions, an eight-fold difference was shown between the highest serum concentrations of isometamidium in the presence of which infections with drug-resistant trypanosomes could become established, and the lowest concentrations which were still protective against drug-sensitive parasites (Chapter 4). It had been hoped that all three trypanosome stocks would differ in their drug sensitivity, and that the experiment would include parasites of low, intermediate and high levels of drug-resistance. However, two of the populations, IL3893 and IL3889, established infection just one month following administration of isometamidium prophylaxis, while the third population, *T. congolense* IL 1180, required between six and eight monthly challenges before infection was established. The experiment may have investigated trypanosome populations at the two extremes of the drug sensitivity spectrum, but no parasites of intermediate drug sensitivity.

In the work described in this chapter, a similar experiment was conducted to investigate the relationship between circulating concentrations of isometamidium and protection against tsetse challenge of Boran cattle with two cloned populations of *T. congolense*: the drug sensitive population *T. congolense* IL 1180, and another population which possess a level of drug resistance intermediate between that of *T. congolense* IL 1180 and the drug-resistant parasites used previously (*T. congolense* IL3893 and 3889).

Materials and methods

Cattle

Boran cattle (*Bos indicus*) were obtained from an area of Kenya free of tsetse-transmitted trypanosomiasis, and fed on hay and concentrate as described by Whitelaw *et al.*, (1986), and allowed water *ad libitum*.

Isometamidium administration

Isometamidium chloride (Samorin®) was administered at a dose rate of 1.0 $mgkg^{-1}$ body weight by deep intramuscular injection of a 2% (w/v) aqueous solution into the middle third of the neck.

Blood samples

Blood samples for use in the isometamidium-ELISA were collected by jugular venipuncture into Vacutainers (Becton-Dickinson) prior to isometamidium treatment, daily for the first week after treatment and twice weekly for a further 2 weeks, and weekly thereafter. Sera were separated and stored in borosilicate glass tubes (Vial Files, Jencons) at -60°C. Blood samples for monitoring of infections were collected three times weekly, as described above, into Vacutainers (Becton-Dickinson) containing potassium ethylenediamine tetra-acetate.

Trypanosomes

Two cloned populations of *T. congolense* were use to challenge cattle. *T. congolense* IL 1180 is a well-characterised, doubly cloned, derivative of an isolate obtained from a lion in the Serengeti National Park, Tanzania (Geigy and Kauffmann, 1973; Nantulya *et al.*, 1984; Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988). *T. congolense* IL 3343 is a derivative of *T. congolense* IL 1180, in which a moderate level of isometamidium resistance was induced in the laboratory by repeated subcurative treatment of infected mice, and has been shown to possess reductions both in sensitivity to the drug in mice, and in fluorescence following incubation with the drug *in vitro*, compared to the parent population (Sutherland, Peregrine, Lonsdale-Eccles and Holmes, 1991).

Infected tsetse flies and challenge of cattle.

Glossina morsitans centralis were infected with each of the two *T. congolense* populations as described previously (Chapter 4). Cattle were challenged by the application of five tsetse flies, which had the presence of

metacyclic trypanosomes in their saliva confirmed by microscopic examination, to a shaved area of the abdominal flank (see Plate 4).

Parasitological monitoring

Parasitological monitoring of challenged cattle was conducted using the phase-contrast buffy-coat technique (Murray *et al.*, 1977).

Isometamidium determinations

The concentrations of isometamidium in bovine sera were determined using the indirect competition ELISA (Chapter 3). Standards prepared from isometamidium "spiked" pooled Boran cattle serum and incurred quality assurance samples were included on every ELISA plate. Assay calibration and calculation of concentrations of unknowns was by four-parameter curve-fitting, using in-house software (M.C.E., unpublished). Results from individual ELISA plates were only used if the dose-response curves and quality assurance samples were within limits of acceptability. Absorbances were read using a multichannel spectrophotometer (Multiskan MCC Mk II, Labsystems) connected to an IBM-compatible personal computer.

Data analysis

Drug profiles were fitted to a bi-exponential model by iterative curve-stripping (Dunne, 1986) using the computer program JANA (SCI Software). Prepatent periods of infection were compared using Student's t-test. Differences between mean drug concentrations were analysed by Student's t-tests on $\log_{10}$ concentrations (because variances were proportional to the square of concentrations), except in cases where any of the values comprising the means to be compared were zero, in which case the Wilcoxon rank sum test was used (Ott, 1988).

Experimental Design

Twenty-five cattle were allocated into four groups. At the start of the experiment (day 0), Group 1(ten cattle) and Group 2 (five cattle) were administered prophylactic intramuscular injections of isometamidium chloride. Group 3 (five cattle) and Group 4 (five cattle) were untreated challenge controls. One month later, and at monthly intervals thereafter, cattle were challenged by the application of five *Glossina morsitans centralis*. All Group 1 and Group 2 cattle were repeatedly challenged each month until shown to be parasitaemic, while a single animal in each of Groups 3 and 4 was used to

prove the infectivity of each monthly challenge. Group 1 and Group 3 were challenged with *T. congolense* IL 3343. Group 2 and Group 4 were challenged with *T. congolense* IL 1180. Animals were removed from the experiment when their packed red cell volume (PCV) decreased to below 15%. Details of the experimental design are summarised in Table 11.

Results

Parasitological findings

The parasitological findings are shown in Table 12. One month following prophylactic administration of isometamidium, all cattle (Group 1) challenged with *T. congolense* IL 3343 were refractory to infection. However, between 16 and 30 days after the second monthly challenge, trypanosomes were detected in the blood of seven of these cattle (Group 1). The remaining three cattle became parasitaemic 18 to 21 days following the third monthly challenge. The mean prepatent period of those cattle (Group 1) which became infected following the second monthly challenge (20.1 days) was not significantly different ($p = 0.84$) from that of those which became infected following the third monthly challenge (mean 20.0 days).

By contrast, all five cattle (Group 2) challenged with *T. congolense* IL 1180 resisted four monthly challenges. As all cattle (Group 1) challenged with drug-resistant *T. congolense* (IL 3343) had become infected by this time, further challenges of cattle (Group 2) with drug-sensitive *T. congolense* (IL 1180) were considered unnecessary; the duration of the period of prophylaxis that isometamidium at a dose rate of 1.0 $mgkg^{-1}$ body weight confers against *T. congolense* IL 1180 had been determined in several previous studies (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988; and Chapter 4), and hence monthly challenge with this *T. congolense* population was discontinued after the fourth occasion.

All challenge control cattle (Groups 3 and 4) became infected with either *T. congolense* IL 3343 or *T. congolense* IL 1180 as expected, 14 to 18 days after challenge (Table 12), confirming the infectivity of the tsetse flies that were used for each challenge. The virulence of infections with *T. congolense* IL 3343 was demonstrated by the drop in packed cell volumes of the three infected control cattle (Group 3), which occurred by days 37, 43, and 67 of infection. The virulence of *T. congolense* IL 1180 has been documented previously (Nantulya *et al.*, 1984; Whitelaw *et al.*, 1986).

Drug profiles

The isometamidium ELISA results are shown in Figure 5, Figure 6 and Table 13. As on previous occasions bi-phasic drug disappearance curves were obtained, and the results of bi-exponential curve fitting using JANA are shown

in Table 14. The mean apparent half-life of the terminal elimination phase was approximately 23 days. Mean drug concentrations at monthly intervals following treatment, predicted using the JANA results are shown in Figure 7.

Serum isometamidium concentrations measured on the occasion of the first tsetse challenge were similar in Group 1 (mean 5.9 $ngml^{-1}$) and Group 2 (mean 4.9 $ngml^{-1}$), and the difference between them was not statistically significant ($p = 0.79$). Thereafter, drug concentrations remained similar in Group 1 and Group 2 cattle, both at the time of the second monthly challenge (means 2.2 and 1.7 $ngml^{-1}$ respectively; $p= 0.42$), and at the time of the third monthly challenge (means 0.75 and 0.64 $ngml^{-1}$ respectively; $p = 0.87$). One month later, at the time of the fourth monthly tsetse challenge (applied only to Group 2 cattle, as all Group 1 cattle were by this stage infected), serum concentrations of the drug were lower in Group 1 cattle (mean 0.12 $ngml^{-1}$) than Group 2 cattle (mean 0.40 $ngml^{-1}$). Using the Wilcoxon rank sum test, because of zero values for some of the concentrations in Group 1, this difference in concentrations was statistically significant ($T\ 9, 5 = 56$; $p < 0.5$). Hence it appeared that in spite of an initially higher mean value, drug concentrations in cattle which became infected with *T. congolense* IL 3343 fell more rapidly than those which resisted infection with *T. congolense* IL 1180.

At the time of the second tsetse challenge, the mean serum isometamidium concentration in the seven cattle challenged with *T. congolense* IL 3343 which succumbed to challenge (2.1 $ngml^{-1}$) was lower than that in the three which resisted (2.4 $ngml^{-1}$). However the difference was not statistically significant, and there was some overlap in the ranges of the individual concentrations, which are shown in Table 15.

One month after the third challenge, the mean concentration (0.05 $ngml^{-1}$) in the six Group 1 cattle surviving of the seven which had become infected following the second monthly challenge, was lower than that of the three cattle which had not become infected until after the third monthly challenge (0.25 $ngml^{-1}$), and although the difference was not statistically significant ($T\ 6, 3 = 21$; $p > 0.05$), the number of animals being compared was small. The mean concentration in these six cattle was significantly smaller that in cattle (Group 2) resisting challenge with *T. congolense* IL 1180 ($T\ 6,5 = 44$; $p < 0.05$).

Table 11. Experimental design: challenge groups.

Group	Number of cattle	Isometamidium prophylaxis (1 $mgkg^{-1}$ i.m.)	Challenge: *T. congolense* clone	Number of cattle challenged each month
1	10	+	IL 3343	10*
2	5	+	IL 1180	5*
3	5	-	IL 3343	1
4	5	-	IL 1180	1

i.m. = intramuscular injection
*Cattle challenged at monthly intervals until trypanosomes detected

Table 12. Outcome of tsetse challenge of isometamidium treated Boran cattle.

Group	Animal number	*T. congolense* challenge	[1]PPI (days)	Month of challenge[2]
1	1	IL 3343	16	2
1	2	IL 3343	21	3
1	3	IL 3343	16	2
1	4	IL 3343	16	2
1	5	IL 3343	21	3
1	6	IL 3343	16	2
1	7	IL 3343	18	3
1	8	IL 3343	30	2
1	9	IL 3343	16	2
1	10	IL 3343	25	2
	mean		20.1	
2	11	IL 1180	[3]NA	NA
2	12	IL 1180	NA	NA
2	13	IL 1180	NA	NA
2	14	IL 1180	NA	NA
2	15	IL 1180	NA	NA
3	16	IL 3343	18	1
3	17	IL 3343	14	2
3	18	IL 3343	16	3
	mean		16	
4	19	IL 1180	15	1
4	20	IL 1180	14	2
4	21	IL 1180	16	3
	mean		15	

[1]Pre-patent period of infection
[2]Month of challenge resulting in infection
[3]Not applicable as Group 2 cattle remained uninfected

Table 13. Mean serum drug concentrations obtained using the indirect competition ELISA for isometamidium

	All cattle	Group 1 (2 month)[1]	Group 1 (3 month)[2]	Group 2	Groups 1 & 2
DAY 28:					
n	10	7	3	5	15
mean	5.94	7.11	3.19	4.94	5.60
sd	3.29	3.25	0.89	1.68	2.83
DAY 60:					
n	10	7	3	5	15
mean	2.18	2.08	2.40	1.69	2.01
sd	0.90	1.07	0.32	0.75	0.86
DAY 88:					
n	9	6	3	4	13
mean	0.75	0.54	1.17	0.64	0.72
sd	0.56	0.53	0.38	0.24	0.48
DAY 199:					
n	9	6	3	5	14
mean	0.12	0.05	0.25	0.40	0.22
sd	0.17	0.08	0.25	0.18	0.22

n = number of samples.
mean = isometamidium concentration in $ngml^{-1}$.
sd = standard deviation.
[1]Group 1 cattle which became infected following the second challenge.
[2]Group 1 cattle which became infected following the third challenge.

Table 14. Bi-exponential curve-fitting of isometamidium concentration vs. time data using JANA

	Group 1 All cattle	Group 1 (2 month)[1]	Group 1 (3 month)[2]	Group 2	Groups 1 & 2
n	10	7	3	5	15
constant B	11.1	12.5	7.7	9.0	10.4
constant A	43.8	48.7	32.2	31.7	39.8
exponent b	0.0321	0.0361	0.0228	0.0262	0.0301
exponent a	0.9302	0.8911	1.0216	0.8461	0.9022
$T_{½el}$	21.6	19.2	30.4	26.4	23.0

Constants A and B and exponents a and b are fitted to the model: concentration = $Ae^{-at} + Be^{-bt}$
$T_{½el}$ = half-life of terminal phase.
[1] Group 1 cattle which became infected following the second challenge.
[2] Group 1 cattle which became infected following the third challenge.

Table 15. Isometamidium concentrations in the serum of cattle challenged with *T. congolense* IL 3433 two months after prophylactic treatment.

Two month breakthrough*		Three month breakthrough*	
Animal number	$ngml^{-1}$	Animal number	$ngml^{-1}$
1	3.67	2	2.08
3	0.58	5	2.39
4	1.95	7	2.73
6	1.44		
8	1.33		
9	2.73		
10	2.86		
Mean	2.08		2.40
s.e.	0.40		0.19

* Month of challenge with *T. congolense* IL 3343 resulting in infection.
s.e. = standard error of the mean.

Figure 5. Mean isometamidium concentrations in the serum of cattle after intramuscular treatment at 1.0 $mgkg^{-1}$; Group 1 cattle, challenged with *T. congolense* IL 3343. Challenge with *T. congolense* represented by tsetse fly symbols. Error bars: standard deviation.

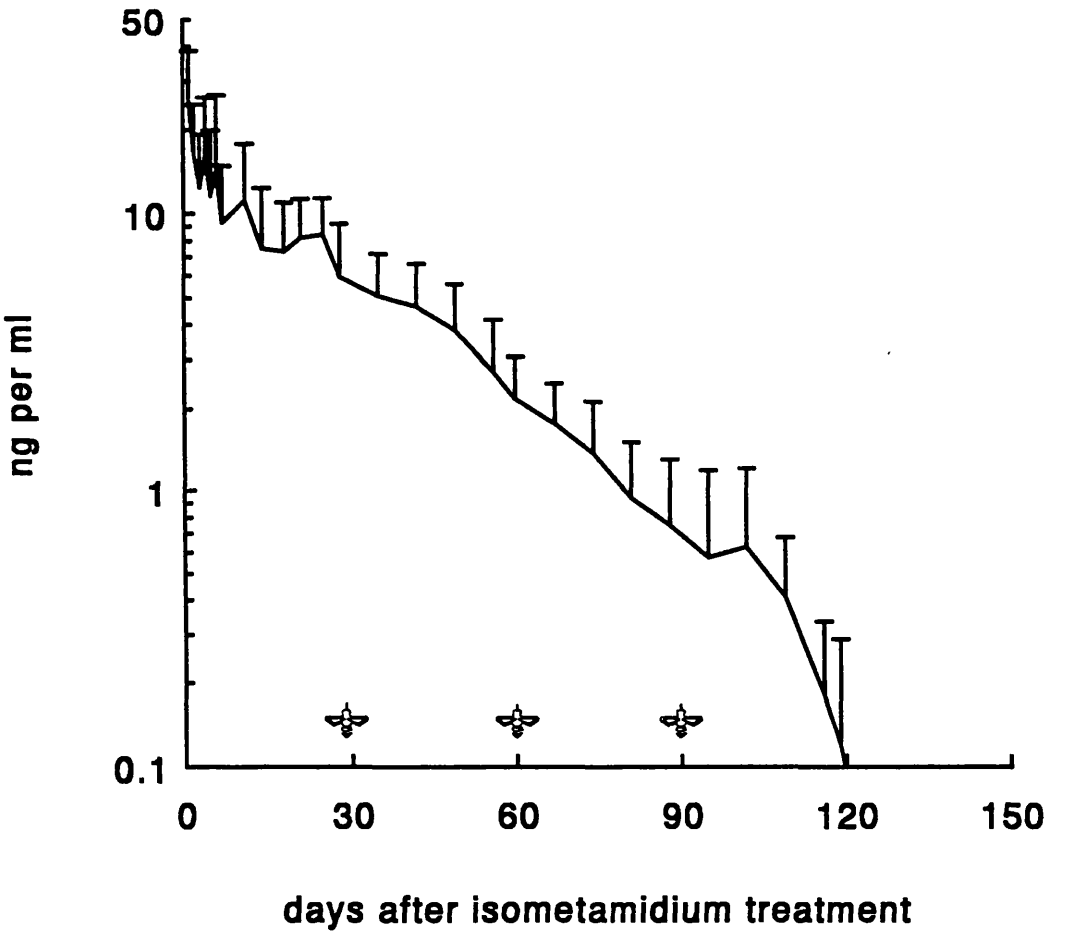


Figure 6. Mean isometamidium concentrations in the serum of cattle after intramuscular treatment at 1.0 $mgkg^{-1}$; Group 2 cattle, challenged with *T. congolense* IL 1180. Challenge with *T. congolense* represented by tsetse fly symbols. Error bars: standard deviation

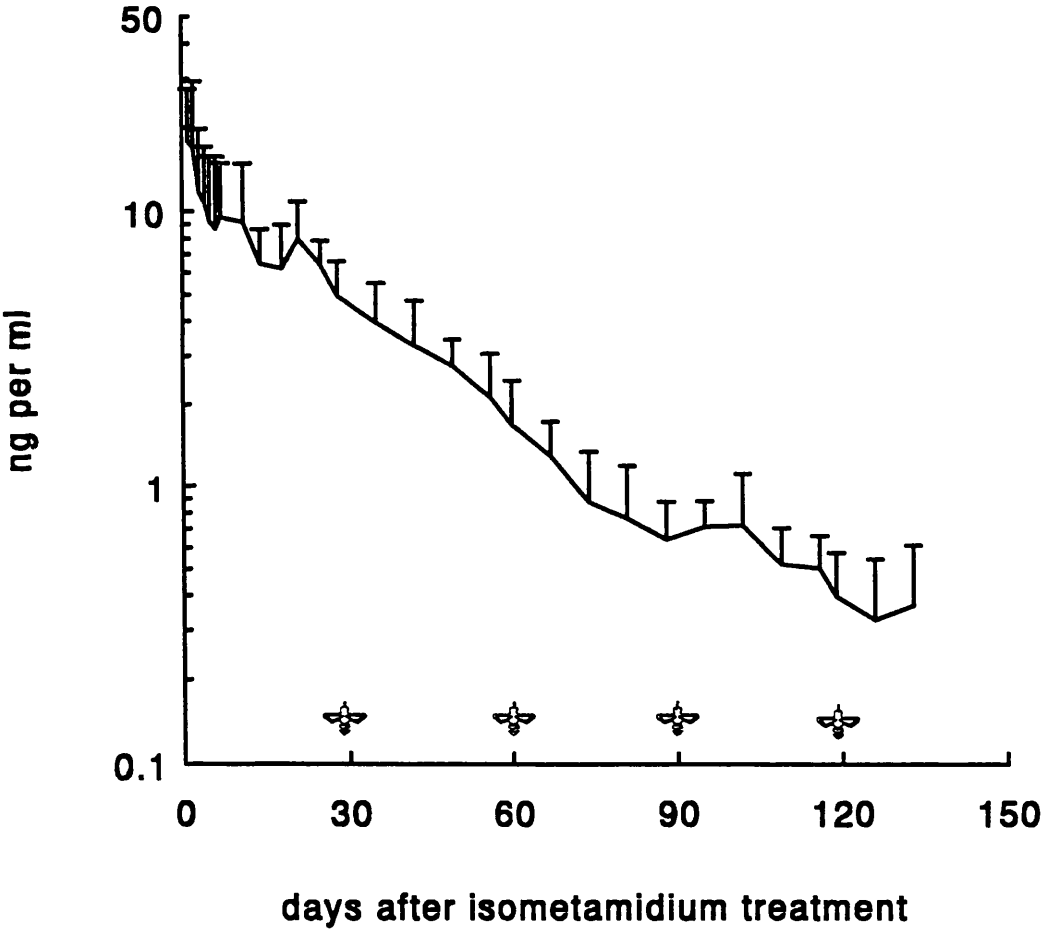


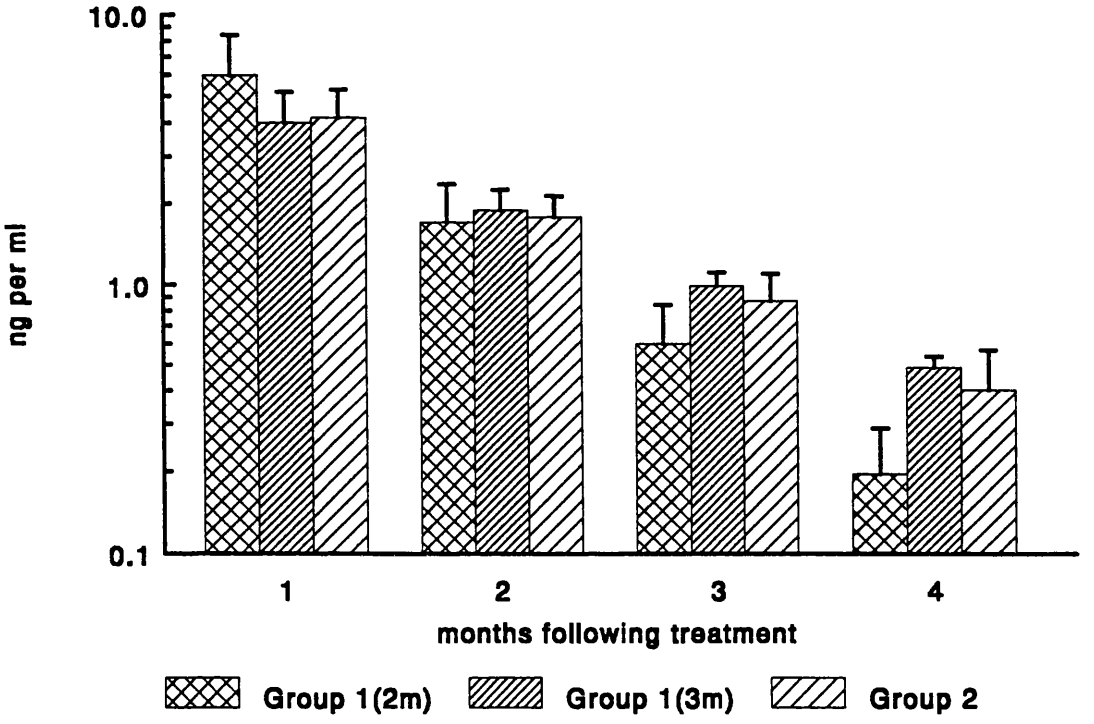
Figure 7. Isometamidium concentrations at monthly intervals following treatment, predicted from a bi-exponential model fitted by iterative curve-stripping using JANA

Group 1 (2M): cattle which became infected following the second tsetse challenge with *T. congolense* IL 3343

Group 1 (3M): cattle which became infected following the second tsetse challenge with *T. congolense* IL 3343

Group 2: cattle challenged with *T. congolense* IL 1180

Error bars: standard deviation.



Discussion

In this study, significant new information has been obtained about the relationship between serum concentrations of the chemoprophylactic drug isometamidium chloride and protection of cattle against tsetse challenge with *T. congolense* possessing an intermediate level of drug resistance. Cattle which had been treated with a single prophylactic intramuscular injection of isometamidium were subsequently challenged at monthly intervals with *T. congolense* infected tsetse flies, and the relationship between susceptibility to infection and the circulating drug concentration investigated using the isometamidium-ELISA.

When Boran cattle, which had not received prophylactic isometamidium chloride, were challenged with cloned populations of *T. congolense* (either IL 3343 or IL 1180) by the application of five infected *Glossina morsitans centralis*, all developed patent and virulent infections, with the onset of parasitaemia between 14 and 18 days following challenge. However, the outcome of *T. congolense* challenge of cattle to which isometamidium chloride had been administered at a dose rate of 1.0 $mgkg^{-1}$ body weight by intramuscular injection depended upon the parasite population and the interval between prophylaxis and challenge. Ten cattle challenged with *T. congolense* IL3343, a cloned population possessing an intermediate level of drug resistance (as judged by experiments in mice and *in vitro* [Sutherland *et al.*, 1991]), were refractory to infection one month after isometamidium administration, but seven cattle became infected when challenged again one month later. The remaining three cattle became infected after a third challenge, three months after drug treatment. In contrast to *T. congolense* IL3343, isometamidium-treated cattle challenged with a drug-sensitive clone of *T. congolense*, IL 1180 (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988; and Chapter 4) were refractory to infection for at least four monthly challenges.

The concentration of isometamidium in the sera of treated cattle (Groups 1 and 2), measured by ELISA, at the time of the first monthly challenge was 5.6 (± 2.83) $ngml^{-1}$. This value is similar to that found previously at this time after treatment at the same dose rate (Chapter 4). In the work described here, the overall mean concentration in treated cattle had fallen to 2.0 (± 0.86) $ngml^{-1}$ by the time of the second monthly challenge. Among those cattle which were challenged with the drug-resistant *T. congolense* clone, IL 3343, there was no obvious relationship between the serum isometamidium concentration in

individual cattle, and the outcome of the second challenge (Table 15). Nevertheless some observations could be made about these concentrations. Firstly, none of the three cattle which were refractory to infection at this challenge (nos. 2, 5 and 7) had concentrations below 2.0 $ngml^{-1}$. Secondly concentrations in all but one of the cattle (nos. 3, 4, 6, 8, 9) which became infected were similar to, or below the mean of those which did not (2.4 $ngml^{-1}$).

There are two considerations in interpreting these data. One is the degree of inherent variability in the ELISA results. Construction of the response-error relationship (Ekins, 1983), using data for duplicate measurements pooled from all the assays conducted on these samples, showed the coefficient of variation in response (absorbance) at this concentration to be as little as 4.4%. As the assay is capable of measuring isometamidium over a fairly wide range of concentrations (0.1 to 10 $ngml^{-1}$), the gradient of the dose-response curve is relatively small, and using this gradient to construct a precision profile (Ekins, 1983) showed that at 2.0 $ngml^{-1}$ the expected coefficient of variation in concentration was 10.7%. In an attempt to allow for some of the variability in individual measurements, concentrations were predicted for each individual animal at the time of the second challenge, using bi-exponential curve fitting of all the data points for that animal (data not shown). Again there was no significant difference ($p = 0.92$) between the modelled serum isometamidium concentrations in cattle succumbing to the second challenge with *T. congolense* IL 3343, and those refractory.

The second of these considerations is the fact that protection against tsetse-challenge with *T. congolense* may involve factors other than the serum isometamidium concentration. While there is some evidence that isometamidium prophylaxis is believed to prevent the development of metacyclic trypanosomes at the site of inoculation (Whitelaw *et al.*, 1986), the relationship between the drug concentration in serum and the concentration at the tsetse bite site is unknown. In addition, there is a growing body of evidence that host resistance factors contribute to the efficacy of chemotherapeutic agents (Doenhoff *et al.*, 1991); for example, drug doses required to clear trypanosome infections may be greater in immunosuppressed than in immunocompetent mice (Frommel, 1988). Similarly, in the present work, individual variation in host resistance factors may have contributed to the variation in the efficacy of isometamidium chemoprophylaxis against the second challenge of cattle with the drug-resistant *T. congolense* clone, IL 3343.

Gray, Kimarua, Peregrine and Stevenson (1993) have shown that metacyclic forms of *T. congolense* derived *in vitro* from various field isolates possess a range of susceptibilities to isometamidium chloride in the incubation medium; a 200-fold difference was demonstrated between the minimum drug concentration to which some isolates were sensitive (5 $ngml^{-1}$) and the maximum drug concentration to which others were resistant (1000 $ngml^{-1}$), and two levels of drug sensitivity were observed between these extremes. In the previous experiment (Chapter 4), an eight-fold difference was demonstrated between the highest mean isometamidium concentrations (6.1 $ngml^{-1}$) measured in sera of cattle susceptible to tsetse challenge with drug-resistant populations of *T. congolense* (IL 3889 and IL 3893), and the lowest mean concentration (0.75 $ngml^{-1}$) measured in sera of cattle protected against tsetse challenge with a drug sensitive population (*T. congolense* IL 1180). In the experiment described in this chapter, the lowest mean isometamidium concentration which could be measured in sera of cattle protected against tsetse challenge with *T. congolense* IL 1180 was 0.4 $ngml^{-1}$ (at the time of the fourth monthly challenge), approximately 15-fold less than the highest concentration (6.0 $ngml^{-1}$) withstood by drug resistant parasites in the previous experiment.

The concentrations of isometamidium to which *T. congolense* IL 3889 and *T. congolense* IL 1180 were either resistant or sensitive (IL 3889 [≡ KE 3302] resistant to 100 $ngml^{-1}$; IL 1180 sensitive to 10 $ngml^{-1}$) *in vivo* (Gray *et al.*, 1993) were over ten times greater than those observed in cattle. The difference between these upper and lower "critical concentrations" for the two parasite stocks observed *in vitro* (10-fold) was, however, similar to that observed in cattle in the experiments described in this and the previous chapter (8 to 15-fold). Further experiments would be required to test whether this relationship holds for stocks of *T. congolense* at other levels of drug sensitivity, such as IL 3898. On the basis of experiments which used three strains of *T. congolense*, which differed in sensitivity to the drug, isometamidium doses in mice were considered to give only a broad indication of the sensitivity of a strain in cattle (Sones *et al.*, 1988).

The absolute difference in these "critical concentrations" between the *in vitro* system and cattle (over 10-fold) was a similar to the 10 to 100-fold difference between doses of isometamidium curative for *T. congolense* infections in mice and those curative for infections with the same parasites in cattle (Sones *et al.*, 1988). The striking difference in the magnitude of drug

concentrations of significance in cattle and *in vitro* might be explained in part by the fact that, *in vivo*, other host factors may contribute to the efficacy of trypanocidal drugs (Doenhoff *et al.*, 1991). For example, immunosuppression substantially reduces the efficiency of isometamidium in curing *T. evansi* infections in mice (Osman, Jennings and Holmes, 1992). Furthermore, drug concentrations at the tsetse bite site are probably related to, but not necessarily the same as those in the circulation.

T. congolense have been demonstrated in extravascular sites within the skin (Luckins and Gray, 1978), where localised proliferation may occur before dissemination into the bloodstream (Akol and Murray, 1982), and there is evidence that isometamidium may exert its prophylactic effect before trypanosomes enter the circulation; Whitelaw *et al.* (1986) found skin reactions (i.e. development of a chancre), local lymph node enlargement, and detectable antibody responses, only in cattle which succumbed to experimental tsetse challenge with *T. congolense*, but not in challenged cattle which were protected successfully by isometamidium.

Whereas the levels of isometamidium present in organs such as liver, kidney and spleen, and at the intramuscular injection site have been the subject of a number of investigations (Hill and McFadzean, 1963; Philips *et al.*, 1967; Kinabo and Bogan, 1988b; Kinabo and McKellar, 1990; Kinabo, McKellar and Eckersall, 1991), investigators have generally neglected to consider the concentrations present in the skin. The rate of entry of a drug from the circulation into any particular tissues of the body will depend upon the rate of blood flow through the capillary beds supplying that tissue, and the permeability of the capillaries for the particular drug molecules. The greater part of the isometamidium present in the circulation is probably bound to plasma macromolecules, especially albumin (Philips *et al.*, 1967; Smith *et al.*, 1991), and this fraction of the total drug is probably unable to pass through the capillary endothelium.

In the absence of specific channels or carriers, unbound isometamidium, a quarternary ammonium compound, is unlikely to pass through the cellular membranes which form the capillary endothelial surface. In considering the accumulation of isometamidium in hepatocytes, Phillips *et al.* (1967) proposed the existence of a mechanism capable of transporting isometamidium into cells, to explain the rapid entry of a cationic substance which is firmly bound to serum proteins. Such a mechanism appears to operate in the accumulation of

isometamidium in trypanosomes (Sutherland, Mounsey and Holmes, 1992; Zilberstein, Wilkes, Hirumi and Peregrine, 1993). Whether or not such a mechanism exists to permit or assist the passage of the drug through the cellular membranes of capillary endothelial cells, unbound isometamidium, like other small, water-soluble ionic species, is probably able to pass readily through the junctions between these cells (Goldstein, Aranow and Kalman, 1974).

Having escaped the capillary bed as unbound drug, isometamidium may once again bind strongly to tissue macromolecules and ground matrix components, as suggested by the importance of the intramuscular injection site as the primary depot of isometamidium resulting in the prolonged period of prophylaxis (Hill and McFadzean, 1963), and the low bioavailability of isometamidium in goats following intramuscular injection (Kinabo and McKellar, 1990).

The relationship between circulating concentrations of isometamidium measured in serum samples using the ELISA, and concentrations in cutaneous tissue fluid, where the drug appears to exert its prophylactic effect, may therefore be fairly complex, and involve dissociation constants for binding of the drug to macromolecules in both serum and cutaneous tissues. Additional investigations of this relationship would be desirable, possibly by comparing drug levels in serum with those in interstitial fluid. Techniques such as implantation of Silastic tissue cages have been used for the study of the concentration of antibacterial agents in interstitial tissue fluid (Chisolm, Waterworth, Calnan, and Garrod, 1973), and attempts have been made recently to use lymphatic cannulation in the study of the distribution of the trypanocide diminazene aceturate in tissue fluid (Mamman, McKeever and Peregrine, 1993).

Although *T. congolense* IL 1180 is not necessarily representative of the most drug-sensitive naturally occurring *T. congolense* populations, as five out of seven field isolates possessed greater sensitivity to the drug *in vitro* (Gray *et al.*, 1993), its inability to establish infection for over five months following a single prophylactic dose of isometamidium (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988; and Chapter 4) supports its use as a standard "sensitive" population in these experiments.

These investigations have significantly added to the information available regarding circulating isometamidium concentrations and protection of cattle against tsetse-transmitted trypanosomiasis. While previous work demonstrated

that *T. congolense* occurring in the presence of serum concentrations above 6 $ngml^{-1}$ express marked drug resistance, this work has shown that trypanosome infections occurring in the presence of serum concentrations between 2 and 6 $ngml^{-1}$ are at least as resistant to isometamidium prophylaxis as *T. congolense* IL 3343, and therefore possess moderate drug resistance. Infections occurring in the presence of between 0.4 and 2 $ngml^{-1}$ can be considered more resistant than *T. congolense* IL 1180, and these too may be considered to possess a modest level of resistance. Finally, the serum isometamidium level below which no inference regarding drug-resistance in *T. congolense* populations infecting cattle can be made can be considered to be 0.4 $ngml^{-1}$.

In the work described in this and the previous chapter (Chapter 4), the relationship between serum isometamidium concentrations and prophylaxis against tsetse-transmitted trypanosomiasis has now been investigated using three populations of *T. congolense* of differing levels of sensitivity to the drug. Further investigations will be required to investigate the relationship between isometamidium concentrations in serum and prophylaxis of cattle against the other tsetse-transmitted species of trypanosomes which affect them, particularly *Trypanosoma vivax*.

Chapter 6:
Efficacy of Prophylactic and Therapeutic Trypanocidal drugs in Dairy Cattle under Natural Tsetse Challenge in Coastal Kenya; Use of the Isometamidium ELISA to measure Serum Concentrations of the Drug

Introduction

Isometamidium chloride is the only drug with clearly established chemoprophylactic activity against tsetse-transmitted bovine trypanosomiasis, and its use is one of the principal methods of trypanosomiasis control in most countries in sub-Saharan Africa. Following a single isometamidium treatment, up to six months prophylaxis has been demonstrated in carefully controlled laboratory experiments using defined tsetse challenge of cattle with stocks of *Trypanosoma congolense* (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988). In well managed chemoprophylactic situations in the field, isometamidium enabled cattle to survive in areas of heavy tsetse challenge (Trail *et al.*, 1985). There are however reports of apparent failure of isometamidium prophylaxis (Pinder and Authié, 1984; Schönefeld *et al.*, 1987; Fox, Mbando and Wilson, 1991; Dolan *et al.*, 1992; Münstermann *et al.*, 1992), although the reason for this failure has not always been clearly established.

Whereas in a successful chemoprophylactic situation circulating drug levels are by definition adequate to protect against trypanosome challenge, break-through infections may occur where drug levels are inadequate, possibly as a result of an inappropriate dosage regimen. Alternatively, infections may occur where the challenge population of trypanosomes expresses drug resistance (Holmes and Torr, 1988). Both causes have important implications for the management of cattle in tsetse-infested areas, but their relative importance may be difficult to evaluate in field situations because, until recently, there has been no method available to measure drug levels in treated animals.

The development of an enzyme-linked immunosorbent assay (ELISA) for the measurement of isometamidium chloride in the sera of treated cattle was described in Chapter 3, and its evaluation in cattle under experimental tsetse challenge was described in Chapters 4 and 5. The assay was shown to be capable of quantifying circulating levels of isometamidium for at least three months after treatment.

In coastal Kenya and neighbouring regions, trypanosomiasis has a serious effect on cattle productivity both through direct effects of infections on production (Trail *et al.*, 1985; Dowler *et al.*, 1989; Münstermann *et al.*, 1992), and through indirect effects resulting from the limitations on the cattle breeds which may be utilised in the area; the current population is mainly Small East African Zebu cattle, managed in traditional smallholder systems (Maloo, Chema,

Connor, Durkin, Kimotho, Maehl, Mukendi, Murray, Rarieya and Trail, 1988). Although isometamidium prophylaxis has been shown to significantly reduce the prevalence of trypanosomiasis and the associated losses in productivity in cattle in this region (Trail *et al.*, 1985; Maloo *et al.*, 1988), more recent reports from the field in coastal Kenya and Tanzania have suggested a reduction in the efficacy of chemoprophylaxis (Dolan *et al.*, 1992; Fox *et al.*, 1993; Maloo, Thorpe and Nantulya, 1993).

This chapter describes the results of use of the isometamidium ELISA in a study conducted in coastal Kenya, in which serum samples were collected on a regular basis from a herd of Jersey cattle in which trypanosome infections occurred in spite of an isometamidium chemoprophylactic regimen. The study provided an opportunity to use the ELISA on sera from cattle under natural tsetse challenge, and to evaluate its potential as a tool for discriminating genuine break-through infections by drug-resistant trypanosomes from failure of prophylaxis due to inadequate drug levels.

Materials and methods

The study was conducted between July 1990 and January 1992 on the Jersey herd of the Regional Research Centre of the Kenya Agricultural Research Institute, at Mtwapa, located in the coconut-cassava agro-ecological zone of the high rainfall coastal strip (Jaetzold and Schmidt, 1983) of the Kenya's Coast Province. The area is approximately 15 m above sea level, and has a mean annual rainfall of 1,100 mm which reaches its peak between April and June. Mean monthly temperatures reach 27 to 32°C, with a corresponding minimum of 20 to 24°C.

A general description of the herd and its management practices are given by Njubi, Rege, Thorpe, Collins-Lusweti and Nyambaka (1992). The breeding herd consisted of lactating and dry cows (see Plate 8), grazing day and night on natural pastures with shade trees, or under cashew nut and coconut palm tree crops. Cattle were hand milked twice daily, with recording of all milk yields (calves were fed from buckets), and the animals were weighed monthly, thus enabling determination of productivity. Cattle were vaccinated annually against rinderpest, foot and mouth disease, lumpy skin disease, anthrax and blackquarter, and sprayed weekly with acaricide. The herd was immunised against East Coast fever by the infection and treatment method in 1987.

Prior to this study, chemoprophylaxis and treatment of trypanosomiasis was conducted using isometamidium chloride and diminazene aceturate respectively. Nevertheless fatalities were common and ten cows died from trypanosomiasis during the three months prior to the study. Isometamidium was administered intramuscularly at a dose rate of 0.5 $mgkg^{-1}$ body weight between one and four times a year, depending on an assessment of trypanosomiasis risk; when the prevalence of infections diagnosed on a clinical basis reached approximately 10%, isometamidium was administered to the entire herd.

The tsetse challenge at Mtwapa was considered to be low, with seasonal peaks. *Glossina pallidipes* and *G. austeni* were the main species of tsetse present, although biting flies (*Stomoxys* sp. and Tabanids) were possibly more abundant (Maloo, 1993). Increasing pressure from human settlement was believed to be responsible for a recent reduction in tsetse challenge, through the destruction of fly habitat.

Study design and procedures

A full description of the study design and procedures, and of herd health, trypanosome incidence and milk production was given by Maloo (1993). At the beginning of the study in June 1990, 78 post-weaning female Jersey cattle were treated with diminazene aceturate (Berenil®) at a dose rate of 7.0 $mgkg^{-1}$ body weight. In July 1990 these animals were divided at random within age and lactational status groups into two equal sized groups: one Group received regular chemoprophylactic treatments, the other served as non-prophylaxis controls. Isometamidium chloride (Samorin®) was administered every 90 days to the chemoprophylaxis group by intramuscular injection at a dose rate of 0.5 $mgkg^{-1}$ body weight, with the last treatment administered in October 1991. Any animal found to be parasitaemic in either the chemoprophylaxis group or the control group was treated on the same day, or the following day, by intramuscular injection of diminazene aceturate (7.0 $mgkg^{-1}$ body weight). Blood samples were taken once weekly for detection of trypanosomes by the buffy-coat technique (Murray, Murray and McIntyre, 1977) and for measurement of the packed cell volume. Serum samples were collected monthly from September 1990 until December 1991, and stored at -20°C until analysis using the isometamidium ELISA.

Trypanosomiasis was found to be the most frequent cause of ill-health in the study herd. The majority of trypanosome infections occurred in the first and third prophylactic periods (i.e. the 90-day periods following the first and third prophylactic isometamidium injections), and all trypanosome parasitaemias detected by the buffy-coat method were found to be due to *T. vivax*. The incidence of parasitaemia in the isometamidium-treated group did not differ significantly from the incidence in the non-prophylactic group. Finally, the occurrence of trypanosome parasitaemia was shown to be associated with significant depression in packed red cell volumes and in daily milk yield, but not in live-weight gains.

For analysis using the isometamidium ELISA, the sera were divided into four groups. Sera from cattle in the non-prophylaxis group were allocated into Groups UN and UP. Sera from cattle in the prophylaxis group were allocated into Groups PN and PP. Groups UN and PN comprised sera from cattle which were never found to be parasitaemic during the study. Groups UP and PP comprised sera from cattle which were found to be parasitaemic on at least one occasion. Group UN, Group PN and Group PP sera were tested by ELISA. In

addition a pool was prepared from the Group UN sera which was used in a test of parallelism with the standards used in the ELISA. Sera were available for testing from ten cattle in Group PN and from six cattle in Group PP. Group UP sera were not tested.

Enzyme-linked Immunosorbent Assay

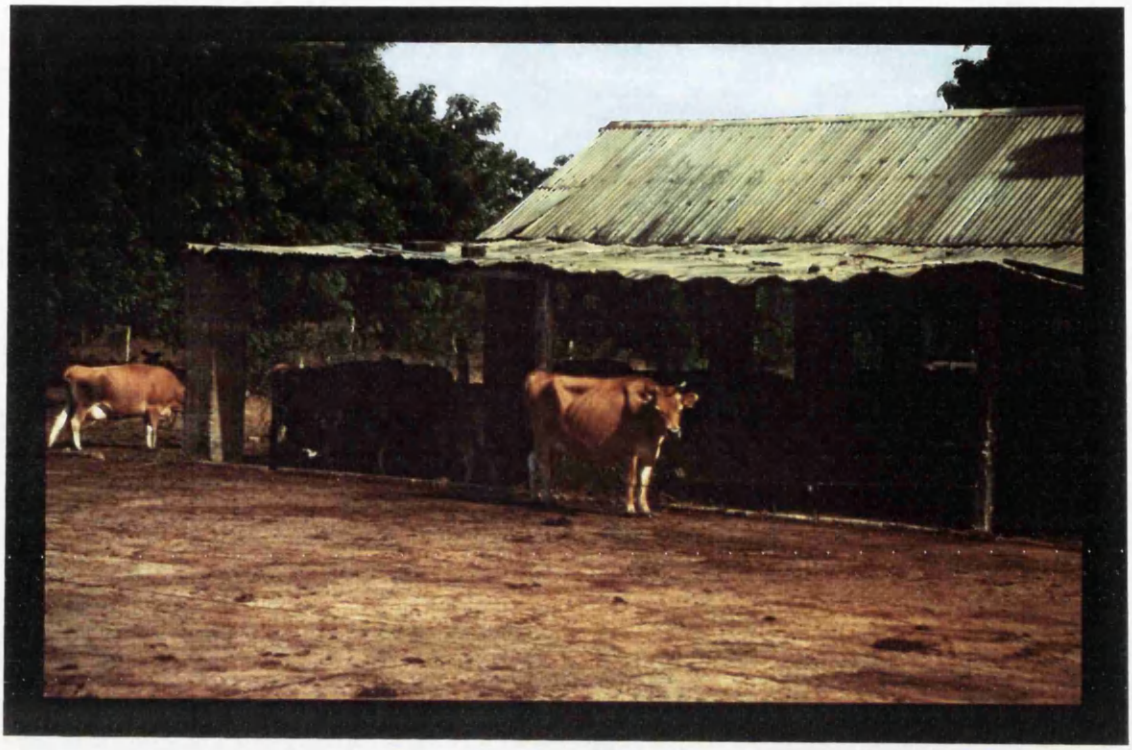
Sera collected following isometamidium treatment were tested using the indirect competition ELISA for isometamidium (Chapter 3). Briefly, microtitre plates (Immulon 4, Dynatech) were coated by overnight incubation of 100 µl per well of a 1/2000 dilution of isometamidium-ovalbumin conjugate in carbonate-bicarbonate buffer (pH 9.2). Plates were washed 5 times using a 1/5 dilution of phosphate buffered saline (PBS, pH 7.2) in distilled water containing 0.05% Tween 20 (Sigma), and blotted dry. Test sera, standards, and quality control sera were pre-diluted 1/10 in a 1/2400 dilution of biotinylated sheep anti-isometamidium IgG in PBS containing 0.05% Tween 20 (PBST), and vortex mixed. Pre-dilutions were added to duplicate wells of microtitre plates (100 µl per well) which were then incubated with shaking at 37°C for 1 hour. Plates were washed 5 times as described above, and 100 µl of a 1/3000 dilution of streptavidin:horseradish peroxidase conjugate (Sigma) in PBST was added to every well. After incubation with shaking at 37°C for 45 minutes, plates were washed 5 times as described above, and 100 µl per well of substrate/chromogen solution added. This solution contained 120 µl of 42 mM tetramethylbenzidine (Sigma) in dimethyl sulphoxide (Sigma), 12 ml of 0.1 M sodium acetate/citric acid buffer (pH 6.0), and hydrogen peroxide to a final concentration of 1.3 mM. The colour reaction was quenched ten minutes after adding this solution with 100µl per well of 2 M sulphuric acid.

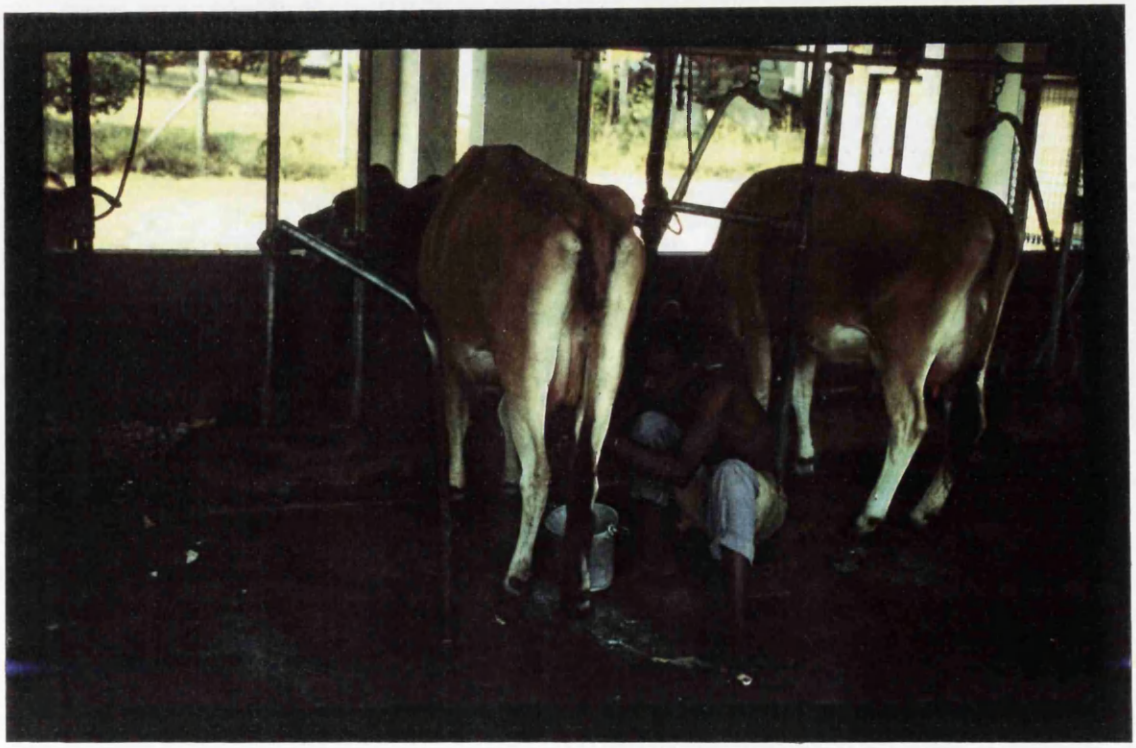
Assay standards were prepared from pooled, untreated Boran cattle serum. Absorbances were read using a multichannel spectrophotometer (Multiskan MCC Mk II, Labsystems), connected to a personal computer (386SX, AST).

Data Analysis

Four-parameter logistic calibration-curve fitting, precision profiling, and calculation of drug concentrations of unknown samples, were performed using spreadsheet software (Lotus 1-2-3 R3.1) specifically programmed for this purpose (M.C.E., unpublished).

Plate 8. Jersey dairy cattle at the Regional Research Centre of the Kenya Agricultural Research Institute, at Mtwapa, in the coconut-cassava agro-ecological zone of the high rainfall coastal strip of the Coast Province of Kenya.





Results

Untreated Controls, Parallelism and Assay Standards

Sera from Jersey cattle which had not been treated with trypanocidal drugs (Group UN) were tested using the isometamidium ELISA, and gave negative results. A pool prepared from these sera showed parallelism with the assay standards. Hence these standards were appropriate for assay calibration in the testing of sera from isometamidium-treated Jersey cattle in the same population (Group PN and PP sera).

Isometamidium-Treated Cattle

Sequential serum samples were available for ELISA testing from ten isometamidium-treated cattle which became infected with trypanosomes (Group PP sera), and from six isometamidium-treated cattle which remained uninfected over the six prophylactic periods (Group PN sera). No serum samples were taken before the last third of the first prophylactic period. Each sample was tested in at least two assays. Mean isometamidium concentrations for Group PN and PP sera determined by ELISA for each sampling date are shown in Figure 8.

Data from all six prophylactic periods were pooled by calculating the mean of all determinations made during the first 30 days of a prophylactic period, the mean of all determinations made between days 30 to 60 of a prophylactic period, and the mean of all determinations made after day 60 of a prophylactic period. Pooled data were calculated for sera from cattle undergoing at least one break-through trypanosome infection (Group PP sera; n = 10), for sera from cattle never detected parasitaemic (Group PN sera; n = 6), and for sera from both Groups. The results are shown in Figure 9.

In general, mean isometamidium concentrations increased following prophylactic treatments, and decreased thereafter. Drug levels varied between lower than the limit of detection of the ELISA, and approximately 2 $ngml^{-1}$. Most observations were below 1 $ngml^{-1}$, and frequently concentrations were below 0.5 $ngml^{-1}$, or were undetectable.

Figure 8. Isometamidium concentrations determined by ELISA in sera from Jersey cattle under a regular prophylactic treatment regimen. Solid line: mean concentration in cattle undergoing at least one break-through trypanosome infection (n = 10). Dashed line: mean concentration in cattle never detected parasitaemic (n = 6). Arrows indicate dates of isometamidium treatments.

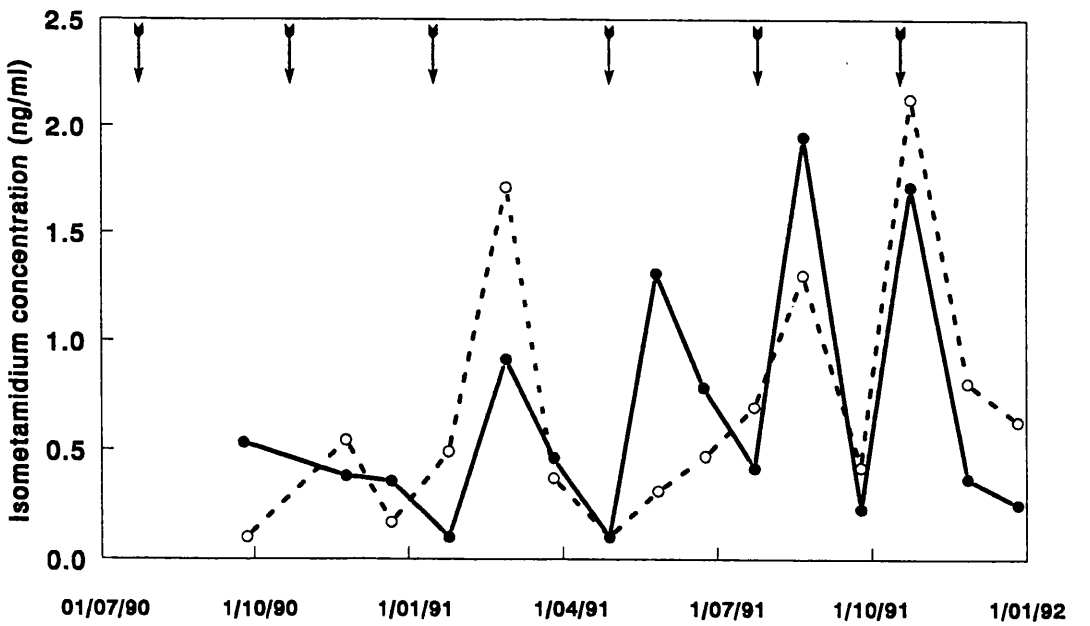
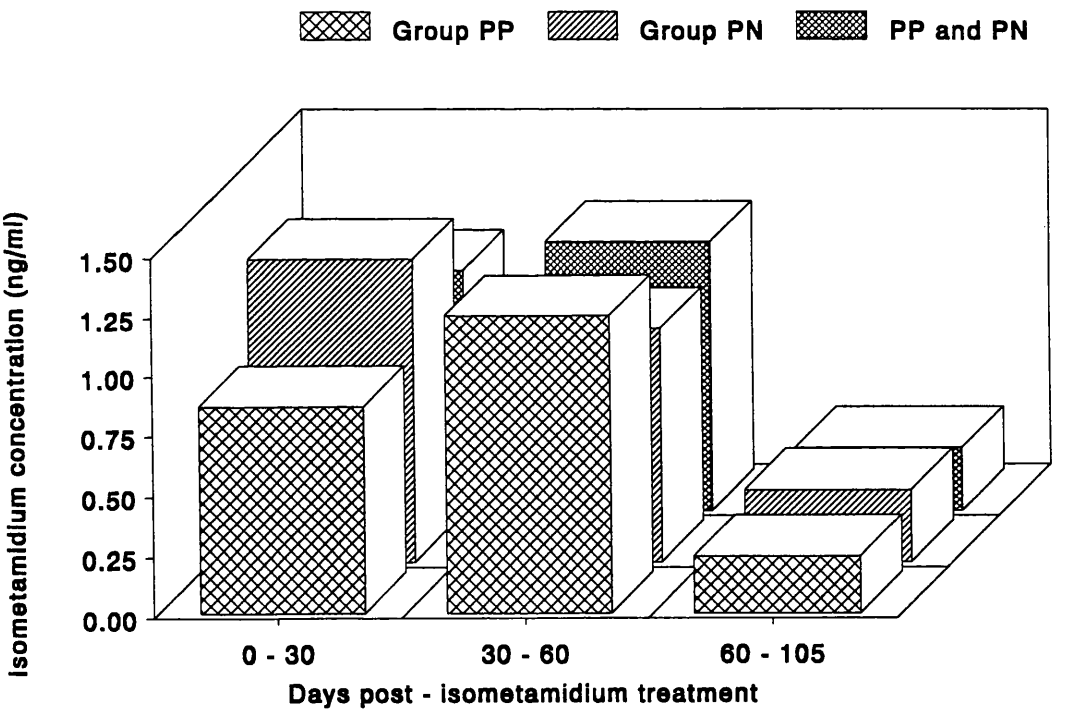


Figure 9. Isometamidium concentrations in sera from treated Jersey cattle pooled from six prophylactic periods (i.e. inter-treatment intervals) determined by ELISA. Data were pooled by calculating the mean of all determinations made during the first 30 days of a prophylactic period, the mean of all determinations made between days 30 to 60 of a prophylactic period, and the mean of all determinations made after day 60 of a prophylactic period. Pooled data were calculated for sera from cattle undergoing at least one break-through trypanosome infection (Group PP sera; n = 10), for sera from cattle never detected parasitaemic (Group PN sera; n = 6), and for sera from both Groups.



Discussion

In this study, for the first time, levels of circulating isometamidium have been quantified in cattle maintained under a chemoprophylactic regime in the face of virulent natural trypanosome challenge, using the isometamidium ELISA. The concentrations measured were similar in magnitude to concentrations measured by the ELISA in cattle under experimental conditions (Chapters 3 and 4). This assay has therefore been shown to be useful for the investigation and rationalisation of chemoprophylactic strategies in the field.

The concentrations of isometamidium determined in the sera of treated Jersey cattle (Groups PN and PP) were within the range of values previously determined in Boran cattle treated at the same isometamidium dose rate (0.5 $mgkg^{-1}$ body weight by intramuscular injection) under controlled laboratory conditions (Chapters 3 and 4). Together with the demonstration of parallelism between pooled untreated Jersey (*Bos taurus*) serum and the assay standards prepared using pooled untreated Boran (*Bos indicus*) serum, this suggested that the genotype of the cattle under test had no effect on the ELISA.

The level of cross-reactivity (10^{-3}) of diminazene aceturate in the isometamidium ELISA is relatively low (M.C.E., unpublished data). Hence the smallest concentration of diminazene which might interfere with the isometamidium ELISA would be 1000 times greater than the minimum detectable concentration of isometamidium. Studies on the pharmacokinetics of diminazene (Aliu, Mamman and Peregrine, 1993) show that levels present more than 5 days after intramuscular treatment of Boran cattle at a dose rate of 3.5 $mgkg^{-1}$ body weight would not interfere with the isometamidium ELISA. Assuming that the absorption and disposition kinetics are similar in Jersey cattle administered diminazene at 7.0 $mgkg^{-1}$ body weight, interference due to diminazene would not have occurred in this study more than 10 days after treatment. Cross-reactivity was not likely to have occurred, since in fact no sample was tested within two weeks of diminazene treatment.

All trypanosome infections detected in isometamidium-treated and untreated cattle using the buffy-coat technique were found to be caused by *T. vivax*, and some were characterised by a haemorrhagic syndrome (Maloo, 1993).

In general, isometamidium concentrations followed a similar pattern in both uninfected cattle (Group PN sera) and in cattle in which infections were

detected (Group PP sera). The occurrence of infections (Group PP sera) did not appear to be associated with lower drug concentrations than were measured in uninfected cattle (Group PN sera). Hence, factors other than differences in isometamidium concentrations were probably more significant in determining whether individual animals became infected.

The lowest isometamidium concentrations in isometamidium-treated cattle occurred, as would be expected, towards the end of each prophylactic period. It was also generally in the latter stages of prophylactic periods that new infections were detected in the animals investigated by ELISA: all but one of the infections in isometamidium-treated cattle occurred in the last third of a prophylactic period, the remaining one occurring in a middle third. This distribution of new infections over time was probably related more to the level of challenge than to the drug concentrations, because the distribution of new infections over the course of each prophylactic period was similar in the prophylaxis group and in the non-prophylaxis group (Maloo, 1993). The concentrations measured in the last third of each prophylactic period were less than the minimum concentration shown to be protective against a drug-sensitive stock of *T. congolense* (0.4 $ngml^{-1}$, Chapter 5). Although data on protective values are not yet available for *T. vivax*, the work of Peregrine *et al.* (1987, 1991) suggests that these may need to be higher for this species of pathogenic trypanosome, which appears to be less sensitive to the prophylactic activity of isometamidium than it is to the therapeutic use of the drug. As all parasitaemic cattle at Mtwapa were treated with diminazene, regardless of whether they were receiving isometamidium prophylaxis, it was not possible to assess whether the isometamidium dosage regimen would have been beneficial as a result of the therapeutic effect of the drug.

On the basis of the isometamidium ELISA results, there was no evidence of drug resistance at Mtwapa over the period for which serum samples were available; most trypanosome challenge occurred during the last third of an inter-treatment interval, when isometamidium concentrations in cattle under prophylaxis were relatively low. Over this period, the apparent failure of isometamidium chemoprophylaxis to prevent virulent trypanosome infections could be attributed, in part, to low levels of circulating drug, which suggests that the dosage regimen used in this study was inappropriate for the local *T. vivax* populations. Either the dose of Samorin® administered (0.5 $mgkg^{-1}$ body weight), the lower of two doses recommended by the manufacturer, was

inadequate, or the inter-treatment interval was too long. It would therefore be appropriate to test the effectiveness of isometamidium prophylaxis at a dose rate of 1.0 $mgkg^{-1}$ body weight in dairy cattle exposed to natural tsetse challenge in coastal East Africa.

It was unfortunate that serum samples from the first and middle thirds of the initial prophylactic period were not available for the isometamidium-ELISA, as the distribution of challenge over time was noticeably different during this period (Maloo, 1993): the majority of infections which occurred over this period were during the first and middle thirds. If serum isometamidium concentrations during the first prophylactic period were similar to those in subsequent prophylactic periods, then indirect evidence for drug-resistance would have been obtained, as infections occurred when drug concentrations would be expected to be protective against drug-sensitive trypanosomes (Chapters 4 and 5).

This work has shown that the isometamidium ELISA is appropriate for field studies, and can provide novel information in treated cattle. In the present study, drug levels at the time of maximal trypanosome challenge were found to be lower than might be expected to provide protection against Kenyan *T. vivax*, even in the absence of a particular drug-resistance problem. The result of these ELISA investigations is concordant with the conclusions of a complex statistical evaluation of epidemiological data from same study (Maloo, 1993) which similarly suggest that the chemoprophylactic regime in use was not effective in protecting against tsetse challenge. This indicates that the ELISA can provide useful information in other African situations in which detailed epidemiological information is not normally available.

Chapter 7:
A Simple Competitive Enzyme Immunoassay for the Detection of the Trypanocidal Drug Isometamidium

Introduction

Isometamidium chloride is one of only a few drugs widely available for use in the control of African bovine tsetse-transmitted trypanosomiasis (Holmes and Torr 1988), and the only one of these proven to have useful chemoprophylactic activity (Trail *et al.*, 1985; Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988; and Chapter 4). However the lack of analytical methods sufficiently sensitive to quantify levels of drug in treated cattle has limited efforts to improve prophylactic drug regimens, and to confirm that apparent drug-resistance is not due to inadequate dosage, or variation in pharmacokinetics between individual animals.

An enzyme-linked immunosorbent assay (ELISA) capable of measuring the concentration of isometamidium in the sera of treated cattle has been described in Chapter 3, and evaluated using isometamidium-treated cattle under experimental (Chapter 4) and natural (Chapter 6) tsetse challenge. This ELISA was capable of detecting isometamidium for at least three months following treatment, and had a mid-point of detection of approximately 1.5 $ngml^{-1}$. However, it was considered possible to improve on a number of aspects of the assay. Firstly, every run of the assay involves four incubation stages; an overnight coating incubation, two subsequent hour long incubations, and a final, short, colour development incubation (Chapter 3). Clearly, the more steps the assay involves, the more susceptible it is to the possibility of technical errors, and the more time and materials are wasted if the assay needs to be repeated because of these errors. Secondly, coated plates could not be stored without deterioration in the quality of the results obtained, and thus a new batch of coated plates was used for every run of the assay. This in turn may have been a contributing factor to the day to day variation in the B_0 absorbance values (Chapter 3), which was a further feature of the ELISA regarding which improvement was considered possible.

This chapter describes a new competitive enzyme immunoassay (CEIA) for isometamidium, which was developed as an alternative to the indirect competition ELISA (ICE) described in Chapter one. The CEIA offers distinct advantages over the ICE in terms of reagent preparation, assay procedure, and day to day variability in absorbances.

Materials and methods

Normal bovine sera

Sera were obtained from normal healthy cattle of mixed breeds (*Bos taurus*), age and sex from both Scotland and Northern Ireland. None of these cattle had ever received treatment with isometamidium.

Isometamidium-spiked bovine sera

Assay standards were prepared as described for use in the indirect competition ELISA (Chapters 3). Briefly, 50 mg isometamidium chloride (Samorin®) were dissolved in 100 ml distilled water and stored in a dark glass bottle at room temperature (approximately 20 - 22°C). The solution was further diluted 1/10 in distilled water, and immediately diluted again in normal bovine serum (NBS) to a final concentration of 1.5 $\mu g ml^{-1}$. From this dilution a threefold serial dilution was performed in pooled NBS, to produce ten spiked "standards" ranging in concentration from 500 to 0.025 $ng ml^{-1}$, which were stored at 4°C for up to 7 days.

Isometamidium chloride treatment

Isometamidium chloride (Samorin®) was administered to cattle by injection of a 1% aqueous solution into the jugular vein, following the method of Dowler *et al.*, (1989), or by deep intramuscular injection of a 2% aqueous solution into the middle third of the neck, except in animals in which the musculature of the neck was poorly developed, in which case the gluteal muscles were used. Injections were given using a 1½ inch 19 gauge needle at a dose rate of 1.0 $mg kg^{-1}$ body weight.

Cross-reactivity with other trypanocidal compounds

Cross-reactivities of diminazene aceturate (Berenil®, Hoechst Veterinär Gmbh, Frankfurt am Main, Germany) and homidium bromide (Ethidium®, Camco) were determined by spiking pooled NBS with a range of concentrations of these compounds. Cross-reactivity was assessed by comparing the concentration of each drug which resulted in 50% inhibition of the optical density obtained in the absence of competing drug. Cross-reactivity was expressed as the concentration of isometamidium divided by the concentration of cross-reacting drug x 100%.

Hyperimmune sheep anti-isometamidium serum

Four Scottish Blackface wethers were immunised using an isometamidium-porcine thyroglobulin conjugate as described previously (Whitelaw *et al.*, 1991). Briefly, isometamidium chloride (Samorin®) was conjugated to porcine thyroglobulin by a diazotization reaction, and primary immunisation was by sub-cutaneous injection of conjugate emulsified in Freund's complete adjuvant. Five subsequent booster immunisations of the same conjugate emulsified in Freund's incomplete adjuvant were given by sub-cutaneous injection at intervals over the following six months. Blood was collected into plain Vacutainers (Beckton-Dickinson), and serum separated by centrifugation, and stored at -20° C until use.

Isometamidium : horseradish peroxidase conjugate

One-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) dissolved in distilled water (40 $mgml^{-1}$) was added, with constant mixing, in 50 µl aliquots to 0.5 ml of a 38 mM solution of isometamidium chloride in 50% (v/v) pyridine (Sigma) in distilled water. The resulting solution was added in 200 µl aliquots over 15 minutes with constant mixing, to 10 mg of horseradish peroxidase (HRP; Boehringer Mannheim) dissolved in 1 ml distilled water, and the resulting mixture was incubated at 37°C for 20 hours. The mixture was then dialysed against phosphate buffered saline (PBS), pH 7.2, and passed down a G-25 Sephadex (Sigma) column (bed volume 30 ml), and the coloured portion of the void volume collected. Finally the conjugate was mixed for 15 minutes with 0.5 ml of a 0.25% (w/v) suspension of activated charcoal (Sigma) containing 0.025% (w/v) dextran T70 (Pharmacia, Milton Keynes, UK) in distilled water, then centrifuged (Eppendorf) at 14,000 rpm, and the supernatant removed and stored in aliquots at -20°C after mixing well with an equal volume of glycerol (Sigma).

Microtitre plate coating and storage

Ninety-six well microtitre plates (Immulon 4, Dynatech) were coated overnight at 4°C with 100 µl per well of hyperimmune sheep anti-isometamidium serum diluted optimally (see below) in 0.1M carbonate/bicarbonate buffer, pH 9.2. Plates were then frozen with the coating solution *in situ* and stored at -20° C for up to two months.

Competitive enzyme immunoassay

Test sera, standards and quality controls were prediluted 1/10 in an optimal dilution (see below) of isometamidium-HRP conjugate in PBS containing 0.05% Tween 20 (Sigma), in 8 x 12 racks of polypropylene tubes (Micronics systems, Muratech Scientific, Aylesbury). Coated plates were allowed to thaw and equilibrate to room temperature before washing five times in a 1/5 dilution of PBS in distilled water containing 0.05% Tween 20. Racked predilutions were vortex mixed and transferred to duplicate wells of microtitre plates (100 µl per well). Plates were shaken for ten minutes at room temperature, and then incubated overnight at 4°C. Plates were allowed to equilibrate to room temperature before washing five times as described previously. Enzyme levels were determined using 100 µl per well of a two-component horseradish peroxidase substrate containing 3,3,5,5 tetramethylbenzidine (Cambridge Veterinary Sciences, Littleport, UK) pre-warmed to 37°C. After 10 minutes incubation at 37°C with orbital shaking (Varishaker, Dynatech), the colour reaction was quenched by the addition of 100 µl per well of 2M sulphuric acid. Absorbances were read at 450 nm using a multichannel spectrophotometer (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

For the testing of unknown samples, 8 standards were included in duplicate wells of every microtitre plate, at the following concentrations ($ngml^{-1}$): 500, 18.5, 6.17, 2.06, 0.686, 0.229, 0.762, 0.0254. Three quality control samples were each included twice (in duplicate) on every microtitre plate, once at the beginning of the series of unknown samples, and again at the end. The quality controls comprised one normal (untreated) bovine serum, and two dilutions, 1/2 and 1/5, in normal bovine serum, of serum from a particular steer treated intramuscularly with isometamidium, as described above, at a dose rate of 0.5 $mgkg^{-1}$.

The principle of the CEIA is illustrated in Plate 9.

Reagent titrations

Optimal dilutions for hyperimmune sheep anti-isometamidium serum and isometamidium-HRP conjugate were determined by checkerboard titration of the two reagents using the CEIA as described above, using pooled isometamidium-free NBS, or pooled NBS spiked with 0.69 $ngml^{-1}$ isometamidium. The optimal combination of reagent dilutions was considered to be that resulting in a B_0 absorbance (i.e. the absorbance obtained with isometamidium-free NBS) of approximately 1.0 optical density units, and with the highest percentage

competition (i.e. absorbance obtained with isometamidium-spiked NBS divided by B_0 absorbance, x 100%).

Data Analysis

Data were downloaded from the multichannel spectrophotometer via the RS232C serial connection to an IBM-compatible microcomputer. Calculation of the mean and coefficient of variation of absorbances of duplicate wells, four-parameter logistic curve-fitting of calibration standards, and calculation of concentrations of unknowns were obtained using an executable program ("Quikfit", M.C.E., unpublished) written in C++ (Turbo C++, Boorland International), and additional statistical calculations and precision profiling (see below) were performed using macro-language programs (M.C.E., unpublished) for three-dimensional spreadsheets (1-2-3 for Windows, Lotus Development Ltd, Staines, UK).

Precision profiling

The mean CEIA responses (absorbances) of duplicate standards, quality control samples and unknowns were allocated into eight "bins" of equal width (i.e. local segments of the graph) within the range between the highest and lowest values, and the median standard deviation of duplicates within each bin calculated (Rodbard, 1981). The response-error relationship was modelled using simple linear regression of the median standard deviation against the mean response within each bin. Precision profiles were calculated using the method of Ekins ((1983)), from the response-error relationship of individual assays, and from pooled responses in a series of 28 assays.

Response variance calculations

Fifty-seven normal bovine sera were tested using the CEIA to assess the variability of the mean response at zero-dose (variance of sample means, σ^2_x). Each serum was tested in accordance with the test protocol, except that from each serum two predilutions were prepared (each of which was pipetted into duplicate wells of a microtitre plate), on four different days. This allowed partitioning of the overall variance into a variability component associated with the use of duplicate wells (intra-predilution variance, σ^2_{wd}), a variability component associated with the predilution of sera (inter-predilution variance, σ^2_{bd}), and a component associated with true inter-sample variance (σ^2_{bs}). Variances were calculated according to the model:

$$\sigma^2_x = \sigma^2_{bs} + \sigma^2_{bd}/r_1 + \sigma^2_{wd}/r_2$$

where r_1 is the number of predilutions prepared from each serum, and r_2 is the number of replicate wells filled from each predilution.

Additionally, the inter-assay response variability was assessed by calculating the mean variance of the mean responses for each sample on each day. Outlying data points were rejected using Cochran's one-sided outlier test (Anon. 1987). Analysis of variance was performed using Genstat 5 Release 2.2 (Numerical Algorithms Group Ltd., Oxford).

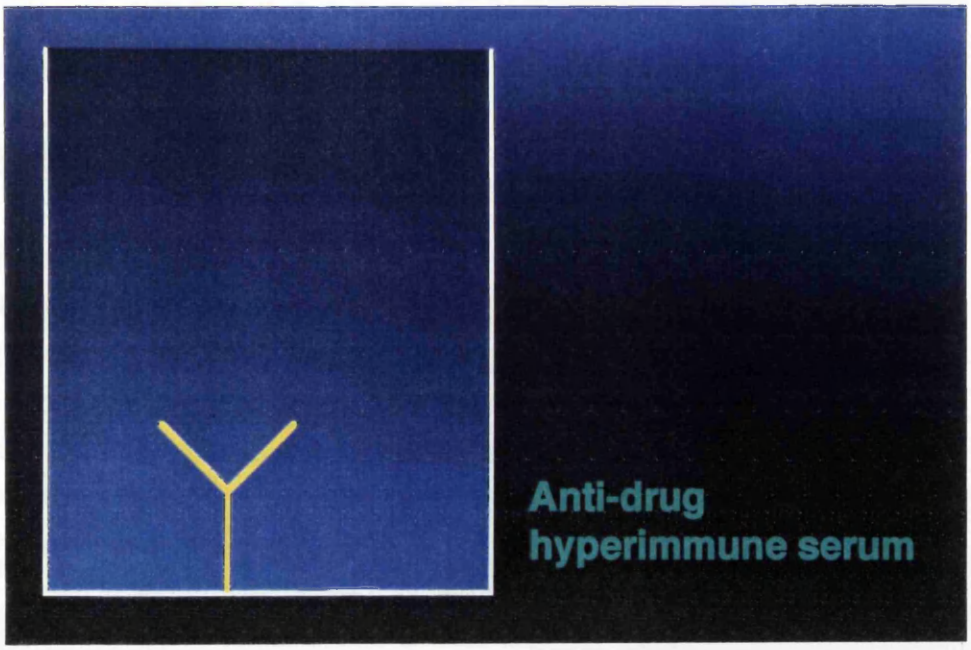
Plate 9. The three stages of the competitive enzyme immunoassay for the detection of isometamidium. Between each stage, unbound reagents are removed by washing.

a) The wells of a 96-well polystyrene microtitre ELISA plate are coated with specific antibody by passive adsorption of hyperimmune sheep anti-isometamidium serum.

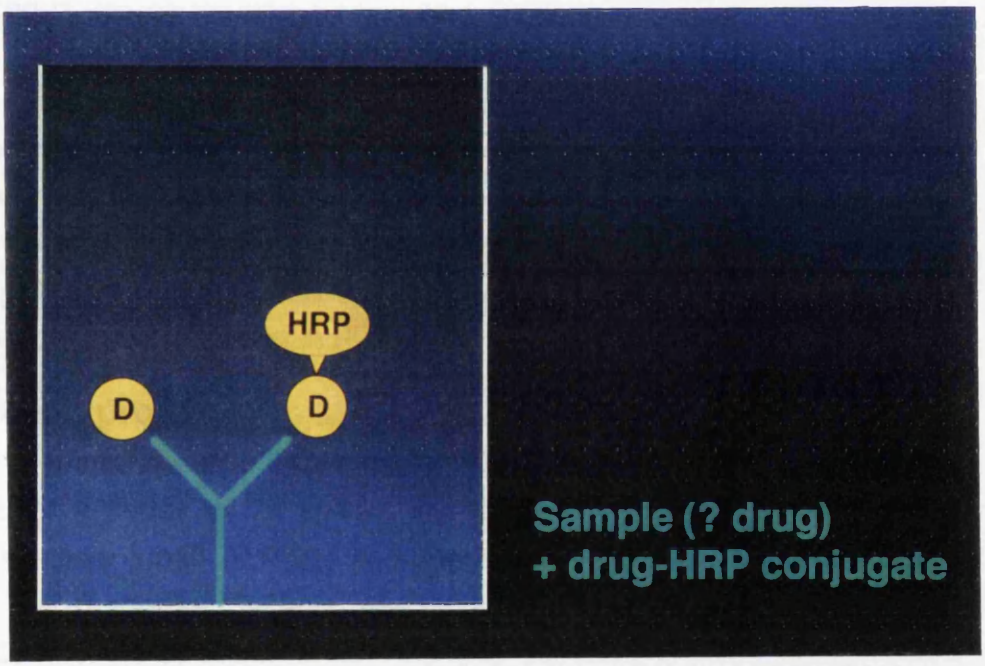
b) The sample, containing an unknown quantity of the drug, is incubated in the ELISA plate wells together with a fixed quantity of drug-horseradish peroxidase conjugate. Sample drug competes for immobilised specific antibody, reducing the amount available to bind to drug-horseradish peroxidase.

c) In the presence of drug-horseradish peroxidase and hydrogen peroxide, colourless tetramethylbenzidine is converted into a coloured product.

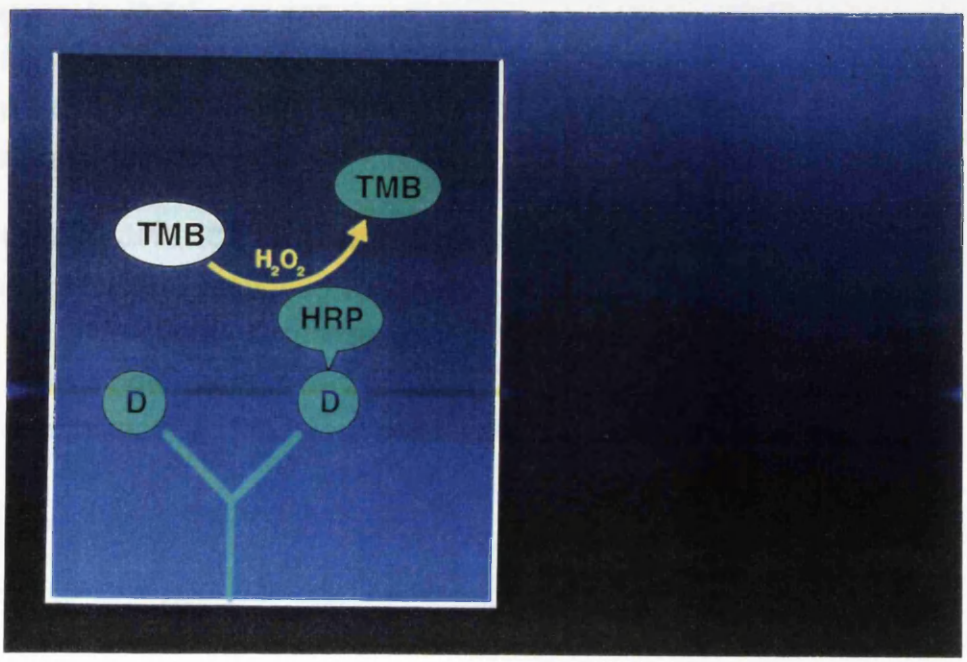
a


Anti-drug
hyperimmune serum

b


HRP
D
D
Sample (? drug)
+ drug-HRP conjugate

c


TMB
TMB
H_2O_2
HRP
D
D

Results

Reagent titrations

All four Blackface wethers responded well to the isometamidium-porcine thyroglobulin conjugate immunisation. Anti-isometamidium hyperimmune serum from the wether which had the highest titre was used in subsequent assays. Optimal dilutions determined by checkerboard titration were 1/12000 for anti-isometamidium hyperimmune serum, and 1/2000 for isometamidium : horseradish peroxidase conjugate.

Calibration curves

Calibration curves fitted well to the four-parameter logistic model, with adjusted r^2 values usually greater than 0.98, and outlying data points occurred rarely. There was relatively little day to day variation in the four parameters, which indicated that the B_0 and background absorbances, the drug concentration resulting in 50% competition, and the dynamic range of the assay were consistent. Mean B_0 and background responses, the four parameters (A, B, C, D), and concentrations corresponding to 10%, 50% and 90% B_0 values obtained over a series of 28 assays are shown in Table 16. The corresponding calibration curve is shown in Figure 10.

Precision profiling

The response-error relationship and resultant precision profile for pooled data over a series of 28 assays are shown in Figure 11. As the regression constant was small (0.002), and the response-error relationship linear (r^2 = 0.97), the response CV was relatively consistent across the response range, with CV = 3.4% at absorbance 1.00 and CV = 5.1% at absorbance 0.10. This resulted in a precision profile giving dose CV less than 25% between approximately 0.5 and 100ngml^{-1}, with the most precise dose estimates occurring between 5 and 10 ngml^{-1} (dose CV approximately 5%). Similar response-error relationships and precision profiles were derived from individual assays (data not shown).

Response variance of untreated cattle

The partitioned response variances of 57 normal bovine sera are shown in Table 17. The overall response variance (i.e. variance of sample means, σ^2_x), based on a single predilution from each serum, was low, with a corresponding CV of approximately 10%. True inter-sample variability accounted for 76.9% of

this overall variance, giving a corresponding CV of 8.7%. Inter-predilution variability accounted for an additional 9.6% of overall variance, with corresponding CV of 3.1%. The remaining 13.5% of the overall variance was accounted for by intra-predilution variability, with a corresponding CV of 5.2% for duplicate wells prepared from a single predilution.

The variability of responses between identical assays conducted on different days was also low, giving a CV of 10.9%.

Similar results were obtained with other normal bovine sera, tested using the normal assay procedure (i.e. only one predilution per serum). For example the CV of the sample means of 34 normal bovine sera was 9.0% (results not shown). It was not possible to partition the variance for these sera.

Isometamidium-treated cattle

Isometamidium could be detected in the sera of cattle treated with isometamidium by the intramuscular route for up to ten weeks following administration of the drug. Concentrations were similar to those obtained using the indirect competition ELISA (Chapters 3, 4 and 5). One hundred and seventy one samples from isometamidium treated cattle were analysed using both the ICE and the CEIA; the results of these determinations are shown in Figure 12. Linear regression of log concentration determined by the CEIA on log concentration determined using the ICE resulted in an adjusted r^2 value of 0.96 indicating close agreement between the two methods.

Determinations performed on two separate days on 108 individual samples from isometamidium-treated cattle were analysed by linear regression as a measure of the reproducibility of the method. The results of these determinations are shown in Figure 13. An adjusted r^2 value of 0.94 was obtained, signifying a high level of reproducibility.

Cross-reactivity with other trypanocidal compounds

The CEIA showed only very low levels of cross-reactivity with diminazene aceturate, so that concentrations of diminazene required to reduce B/B_0 to 50% were approximately 1000 times greater than those for isometamidium. Calibration curves obtained with homidium chloride were non-sigmoidal, which made it difficult to calculate a cross-reactivity factor. However, significant cross reactivity with homidium chloride did occur in these assays. All four immunised sheep showed similar patterns of cross reactivity.

Table 16. Mean B_0 and background responses, the four parameters of logistic curve-fitting (A, B, C, D), and concentrations corresponding to 10%, 50% and 90% B_0 values obtained over a series of 28 assays.

Parameter:	Value:
A	0.933
B	0.393
C	1.19
D	0.035
B_0	0.968
Signal to noise ratio (B_0 / background)	27.5
10% B_0 ($ngml^{-1}$)	13.90
50% B_0 ($ngml^{-1}$)	2.19
90% B_0 ($ngml^{-1}$)	0.35

B_0 = absorbance at zero dose

Table 17. Response variance of untreated cattle

Day	σ^2_x		σ^2_{bs}		σ^2_{bd}		σ^2_{wd}	
	Variance component	CV	Variance component	CV	Variance component	CV	Variance[1] component	CV
1	100%	10.3%	73.2%	8.8%	8.0%	2.9%	18.8%	6.3%
2	100%	10.0%	70.5%	8.4%	20.9%	4.6%	8.6%	4.1%
3	100%	10.4%	87.7%	9.7%	1.0%	1.1%	11.2%	4.9%
4	100%	8.9%	72.9%	7.6%	8.5%	2.6%	18.5%	5.4%
ALL	100%	9.9%	76.9%	8.7%	9.6%	3.1%	13.5%	5.2%

σ^2_x = variance of sample means (based on single predilutions)
σ^2_{bs} = true inter-sample variance
σ^2_{bd} = inter-predilution variance
σ^2_{wd} = intra-predilution variance ([1]based on two replicate wells per predilution)

Figure 10. Calibration curve for the isometamidium CEIA. Mean responses (absorbances at 450 nm) for isometamidium-spiked standards prepared using normal bovine serum were fitted to the four-parameter logistic equation using "Quikfit". The parameters for the curve are shown in Table 16.

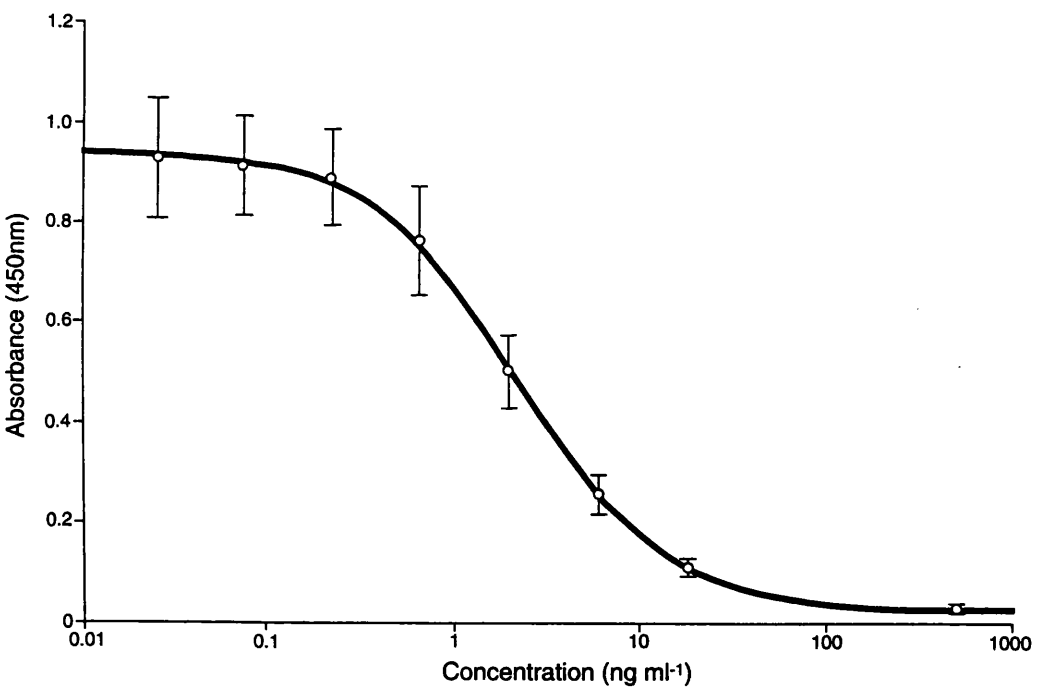


Figure 11(a). Intra-assay response-error relationship constructed using pooled data from 28 assays. Mean responses of duplicate wells were divided into eight evenly spaced categories between the maximum and minimum values. Within each category, the median standard deviation (S.D.) of duplicates was plotted against the mean response (X). The line represents that of best fit obtained by linear regression (S.D. = 0.032X + 0.002, adjusted $r^2 = 0.96$). Figure 11(b): see overleaf.

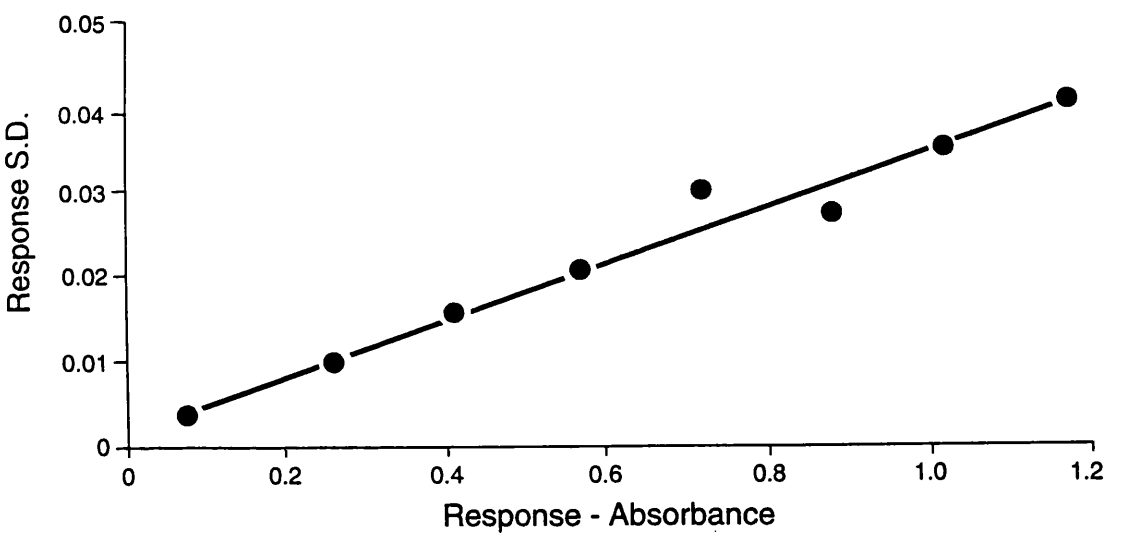


Figure 11(b). Precision profile obtained using the response-error relationship shown in Figure 11(a), and the calibration curve shown in Figure 10. The y-axis shows the predicted coefficient of variation (%) of concentrations determined in the isometamidium CEIA, obtained by dividing the standard deviation predicted at each concentration by that concentration, and multiplying by 100. Standard deviation of measured concentration, as a function of concentration (H), was predicted by dividing the response-error relationship function by the gradient (Γ) of the calibration curve calculated from the equation:-

$$\Gamma = (ABCH^{1/C})/(1+2BH^{C}+B^{2}H^{2C})$$

where A, B, C are three of the four parameters of the calibration curve.

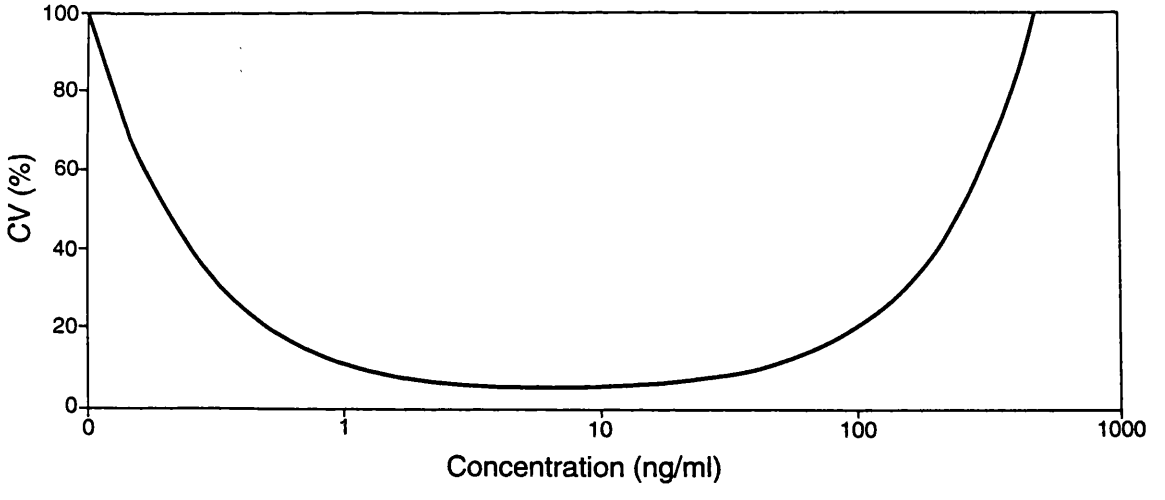


Figure 12. Results in $ngml^{-1}$ of determinations on 171 serum samples from isometamidium-treated cattle using the indirect competition ELISA (Chapter 3) (x-axis) and the competitive enzyme immunoassay (y-axis). The line shows best fit by linear regression of log concentrations; adjusted $r^2 = 0.96$.

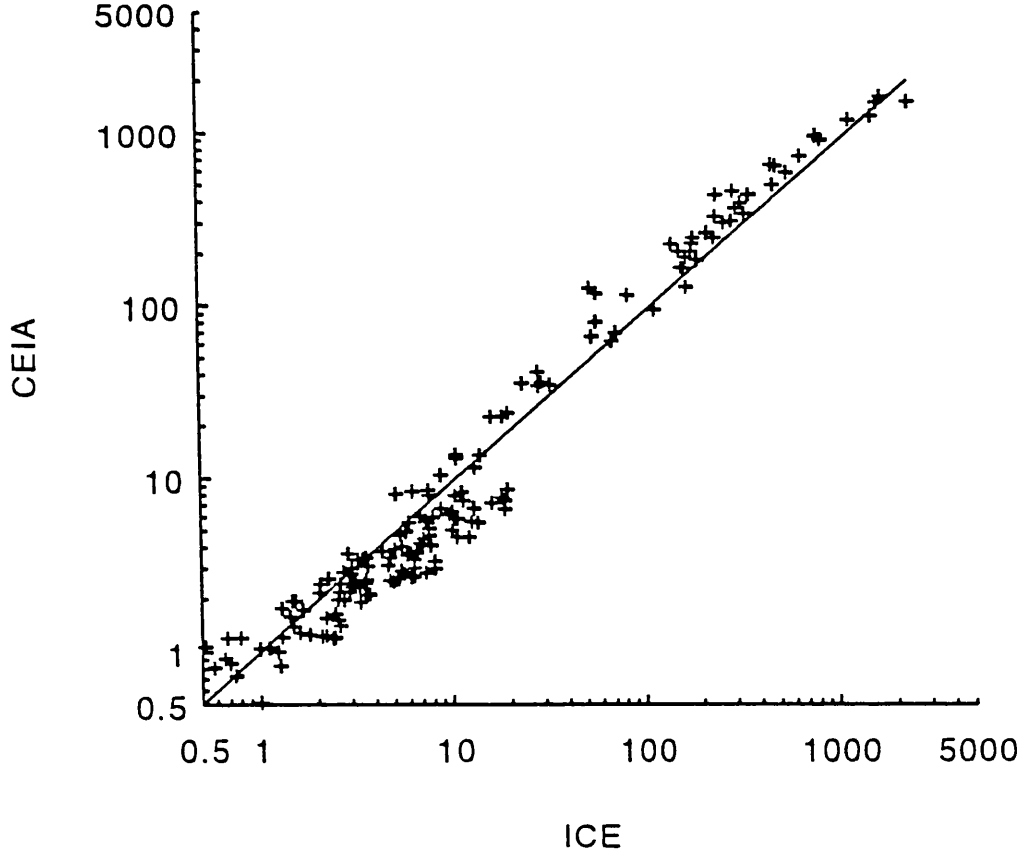
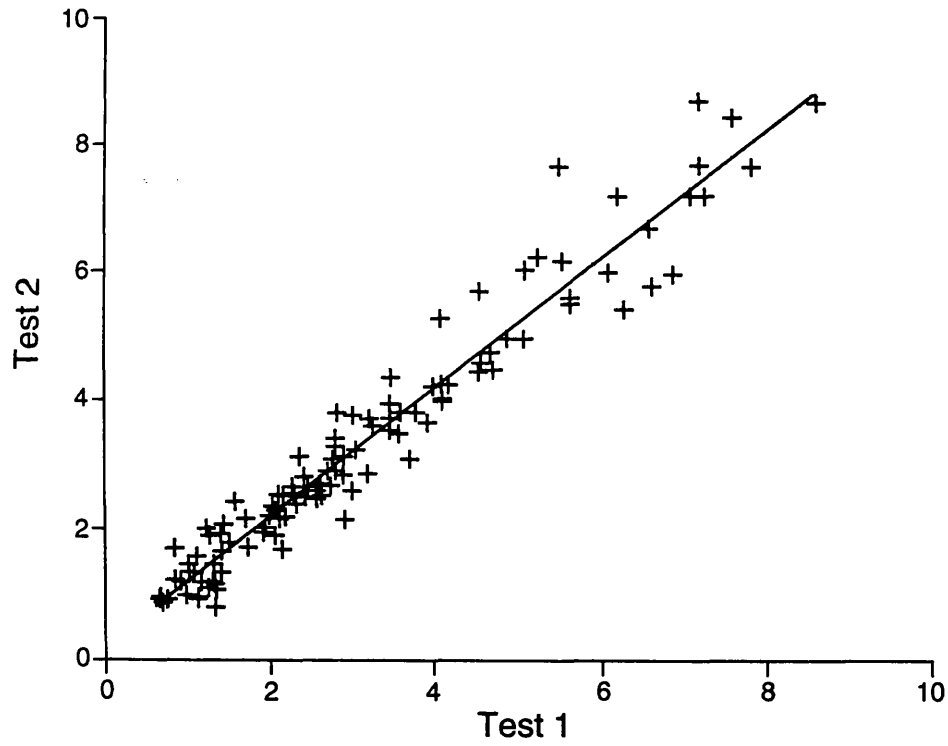


Figure 13. Results in $ngml^{-1}$ of two determinations in separate assays on different days on 108 serum samples from isometamidium-treated cattle. The line shows best fit by linear regression, adjusted $r^2 = 0.94$.



Discussion

The competitive enzyme immunoassay (CEIA) for isometamidium has been shown to be a useful new analytical method for the detection and quantification of the drug in bovine serum.

The CEIA differs from the indirect competition ELISA (ICE) for isometamidium (Chapter 3 and Plate 7) in the strategy adopted to quantify the level of analyte. Whereas in the ICE there is competition between solid phase-bound isometamidium conjugate and liquid phase isometamidium for specific antibody in the liquid phase, in the CEIA, competition for solid phase-bound specific antibody is between conjugated and unconjugated isometamidium both in the liquid phase (see Plate 9). Whole hyperimmune serum, suitably diluted in carbonate/bicarbonate buffer is passively adsorbed onto the solid phase, namely high quality, 96-well polystyrene microtitre plates. In the competition reaction, test samples containing unknown quantities of isometamidium are diluted in buffer containing a fixed amount of enzyme-conjugated isometamidium and incubated overnight in the wells of coated microtitre plates. Finally, after washing to remove unbound reagents, the substrate-chromogen solution is added.

The CEIA has a number of advantages over the ICE in terms of reagent preparation, repeatability, the number of assay steps, and in the "waiting time" during incubations. Firstly, the use of hyperimmune sheep anti-isometamidium serum in the CEIA, rather than the anti-isometamidium biotinylated IgG used in the ICE, obviates the need for immunoglobulin purification and biotinylation. Secondly, whereas in the ICE plates needed to be coated each day for use the following day, in the CEIA plates may be coated as a large batch using a single aliquot of coating solution. This reduces the day to day variability in optical density seen in the ICE. As coating is no longer part of the assay protocol *per se*, the CEIA effectively consists of only two stages, firstly the incubation of samples with isometamidium-HRP conjugate, and secondly the colour development step. In comparison the ICE has four separate stages for each assay. Finally the CEIA is less time consuming as the first stage has in effect zero "waiting time", being an overnight incubation, and the second stage, colour development, takes only ten minutes. By contrast the ICE has an overnight incubation, and three subsequent incubation stages, a total of almost two hours "waiting time".

The inter-sample response variation (σ^2_x) of isometamidium-naïve normal bovine sera in the CEIA was found to be low, with a corresponding CV of approximately 10%. Partitioning of variance showed the within-predilution variability (σ^2_{wd}), attributable to the CEIA method *per se* (excluding variability associated with preparation of predilutions) to be relatively low, with a corresponding CV of approximately 5%, and using duplicate wells, accounting for approximately 13.5% ($\sigma^2_{wd} / 2.\sigma^2_x \times 100\%$) of overall variability. Predilution of samples did not introduce much additional variability as inter-predilution variance (σ^2_{bd}) accounted for only 9.5% of overall variance, and the overall within sample variability ($\sigma^2_{bd} + \sigma^2_{wd} / 2$) accounted for only 23% of overall variance.

The main contribution to overall inter-sample variability was attributable to the isolated, true inter-sample variability (σ^2_{bs}), or sample matrix effect (Chard, 1990). This is a result of the inclusion of a relatively high concentration (10%) of serum sample in the reaction mixture. Investigations at other sample concentrations (unpublished data) suggest that the sample inclusion rate of 1/10 is the best compromise between reducing inter-sample variability by diluting out the sample matrix, and reducing detectability by diluting out the analyte. At the chosen sample dilution (1/10), sample matrix effect occurs but is acceptable as it results in only slight inter-sample variability, which still permits a relatively low limit of detection.

Rodbard (1978) discussed the use of a one-tailed Student's t statistic for the estimation of the minimum detectable concentration or sensitivity of radioligand assays. However, the approach taken did not allow for inter-sample variability, σ^2_{bs}. It may not, therefore, be appropriate for the CEIA for isometamidium, in which the response variance of unknown samples at or around zero-dose which does include σ^2_{bs} as a component, may be greater than the variance of the estimate of B_0, which does not. We suggest 0.5 $ngml^{-1}$ as an approximation of the limit of detection, obtained using $B_0 - 1.6 \times \sigma^2_x$ as an estimate of the greatest response significantly different to zero.

The CEIA was found to give results in close agreement with those obtained in the ICE (Figure 12). The new immunoassay technique was also shown to have a high level of reproducibility (Figure 13).

One potential disadvantage of the CEIA compared to the ICE for isometamidium is the greater sensitivity of the latter method (approximately 0.1 $ngml^{-1}$, Chapter 3). However it should be possible to increase the sensitivity of

the CEIA should this be found necessary. Studies underway in this laboratory indicate that high titre antisera against isometamidium may be raised in rabbits, and that these antisera may produce assays with sensitivities similar to that of the indirect competition ELISA. Furthermore, if it is considered necessary to detect smaller (sub-nannogramme) quantities of isometamidium, an extraction and concentration step could be employed (Kinabo and Bogan, 1988). Such a step was not used in the work described here because of the importance of keeping the procedure as simple as possible for use in African laboratories. Finally, investigations into the relationship between isometamidium concentrations circulating in treated cattle and prophylaxis against tsetse-transmitted trypanosomiasis (Chapter 4) suggest the CEIA has adequate sensitivity to detect concentrations in the range of interest in field situations.

In these studies, the CEIA for the detection of isometamidium in bovine sera has been shown to be a simple and reliable method, both in terms of the *in vitro* characteristics of the assay using sera from isometamidium-naïve European cattle, and in terms of the repeatability of the determinations of the drug in the sera of treated European cattle. Other studies have shown it to be a useful and reliable method for use in the investigation of the pharmacokinetics of isometamidium, and in the determination of concentrations of the drug in African cattle under field situations. The results of these studies are presented in subsequent chapters.

Chapter 8:
Pharmacokinetics of the Chemoprophylactic and Chemotherapeutic Trypanocidal Drug Isometamidium Chloride in Cattle

Introduction

Of the few drugs available for use in African bovine trypanosomiasis, isometamidium chloride (Samorin®, Trypamidium®) is the only one widely used for its prophylactic and therapeutic activity. The drug has been widely used under field conditions for over thirty years, and its effectiveness in a commercial situation has been demonstrated (Trail *et al.*, 1985). In controlled laboratory experiments in cattle, up to six months prophylaxis against tsetse challenge with *Trypanosoma congolense* has been demonstrated following a single intramuscular dose (Whitelaw *et al.*, 1986; Peregrine et al, 1988; and Chapter 3).

In spite of a long history of widespread use, the approach to dosage regimens with isometamidium has remained empirical, as until recently analytical techniques capable of determining drug concentrations in the circulation of treated cattle were not available and meaningful pharmacokinetic analyses were not possible. Philips *et al.* (1967), used a spectrophotometric method of analysis which was not sufficiently sensitive to determine concentrations less than 0.7 μgml^{-1} in plasma. Perschke and Vollner (1985) developed a high performance liquid chromatography (HPLC) method in which isometamidium was converted to homidium before separation and detection, and thus the method was not specific. Using this method, concentrations as low as 20 $ngml^{-1}$ could be detected, but this required as much as 5 ml of serum per sample. Kinabo and Bogan (1988a) developed a more sensitive and specific ion-pair reverse-phase HPLC method for isometamidium, which could detect as little as $10ngml^{-1}$, but even with this improved level of sensitivity the drug could only be detected in the serum of treated cattle for two hours following intramuscular doses of 0.5 $mgkg^{-1}$. Similarly a radioimmunoassay for isometamidium was relatively insensitive because of the low specific activity of the ^{14}C-labelled tracer, and was unable to detect the drug in serum of calves treated at the same dose rate for more than four hours (Kinabo and Bogan, 1988d).

More recently two ELISA methods for isometamidium (Chapters 3 and 7) have been shown to be capable of detecting sub-nannogramme quantities of isometamidium in the sera of treated cattle for several weeks following intramuscular treatment at 0.5 or 1.0 $mgkg^{-1}$; one of these ELISAs has been used to investigate the relationship between circulating concentrations of isometamidium following the administration of prophylactic injections, and

protection against experimental tsetse challenge with *Trypanosoma congolense* (Chapter 4). In this study another ELISA technique for isometamidium, the competitive enzyme immunoassay (CEIA) described in Chapter 7, has been used to determine the disposition kinetics of isometamidium following intramuscular and intravenous injection.

Materials and methods

Cattle

Three to six month old Friesian (*Bos taurus*) steers weighing 92 - 122 kg were maintained on hay, and a commercial concentrate ration, and had access to water *ad libitum*. These cattle were raised and maintained in a country without tsetse (UK), and had never previously been exposed to trypanocidal drugs.

Isometamidium chloride treatment

Isometamidium chloride (Samorin®) was administered to cattle by injection of a 1% aqueous solution into the jugular vein, following the method of Dowler *et al.* (1989), or by deep intramuscular injection of a 2% aqueous solution into the middle third of the neck, except in animals in which the musculature of the neck was poorly developed, in which case the gluteal muscles were used. Injections were given using a 1½ inch 19 gauge needle at a dose rate of 1.0 $mgkg^{-1}$ body weight.

Serum collection

Blood samples were collected using indwelling intravenous jugular cannulae for the first forty-eight hours following isometamidium treatment. Thereafter blood samples were collected using plain Vacutainers (Becton-Dickinson). Blood samples were incubated for approximately four hours at 37°C, and then overnight at 4°C, before separation of serum by centrifugation. Serum samples were stored at -25°C until required for testing.

Isometamidium determinations

Serum isometamidium concentrations were determined using the competitive enzyme immunoassay (CEIA) described in Chapter 7.

Briefly, sera were prediluted 1/10 in a 1/2000 dilution of isometamidium-HRP conjugate in phosphate buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (Sigma). Ninety-six well microtitre plates (Immulon 4, Dynatech) which had previously been coated overnight with 100 µl per well of an appropriate dilution of hyperimmune sheep or rabbit anti-isometamidium serum in carbonate/bicarbonate buffer, pH 9.2, and then frozen and stored at -20°C with the coating solution *in situ*, were allowed to thaw and equilibrate to room temperature. Plates were then washed five times in a 1/5 dilution of PBS in distilled water containing 0.05% Tween 20. Serum predilutions were vortex

mixed and transferred to duplicate wells of microtitre plates (100 µl per well). After shaking for ten minutes at room temperature, plates were incubated overnight at 4°C, and then allowed to equilibrate to room temperature before five washes as described previously. Enzyme levels were determined using 100 µl per well of a two-component horseradish peroxidase substrate containing 3,3,5,5 tetramethylbenzidine (Cambridge Veterinary Sciences, Littleport, UK) pre-warmed to 37°C. After 10 minutes incubation at 37°C with orbital shaking (Varishaker, Dynatech), the colour reaction was quenched by the addition of 100 µl per well of 2M sulphuric acid. Absorbances were read at 450 nm using a multichannel spectrophotometer (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

A series of eight standards, ranging in concentration from 0.025 to 500 $ngml^{-1}$, were prepared using commercially available normal bovine serum from a single batch (Equine and Ovine Blood Products Ltd., Bonnybridge, Stirlingshire) which had been shown to give a response in the CEIA close to the mean of the responses obtained with sera from the experimental cattle prior to any trypanocidal drug treatment, and were included in duplicate wells of every microtitre plate. Three quality control samples, comprising one normal (untreated) bovine serum, and two dilutions, in normal bovine serum, of serum collected from a single steer 48 hours after intramuscular treatment with 0.5 $mgkg^{-1}$ isometamidium chloride, were each tested twice (and in duplicate) on every microtitre plate. Samples were tested in duplicate, and concentrations of unknowns and quality control samples were derived by four-parameter logistic curve-fitting of calibration standards on the same ELISA plate. Results from individual ELISA plates were only included in the final data analysis if the standards and quality controls gave the results expected.

Data Analysis

Data were downloaded from the multichannel spectrophotometer via the RS232C serial connection to an IBM-compatible microcomputer. Calculation of the mean and coefficient of variation of absorbances of duplicate wells, four-parameter logistic curve-fitting of calibration standards (Rodbard and Hutt, 1974), and calculation of concentrations of unknowns were obtained using the program "Quikfit" (M.C.E. unpublished) and additional statistical calculations and precision profiling were performed using macro-language programs (M.C.E. unpublished) for three-dimensional spreadsheets (1-2-3 for Windows, Lotus Development Ltd, Staines, UK).

Pharmacokinetic analysis

The serum isometamidium concentration data obtained using the CEIA was analysed using the exponential curve stripping program JANA (SCI Software), and the nonlinear regression/pharmacokinetic modelling program PCNONLIN (SCI Software). Data from cattle treated intravenously was analysed using a three compartment open model (Kinabo and McKellar, 1989). The suitability of the model was assessed using the Akaike (Yamaoka, Nakagawa and Uno, 1978) and Swartz (1978) information criteria. Data from cattle treated intramuscularly was analysed using a non-compartmental model (Kinabo and McKellar, 1989). Pharmacokinetic parameters are calculated by the software using standard methods (Gibaldi and Perrier, 1982) and are expressed using the preferred symbols (Rowland and Tucker, 1980). Data were weighted using weights equal to the square of the reciprocal of concentration.

Arithmetic means were calculated for pharmacokinetic parameters obtained for cattle treated by the same route of administration, except in the case of half-lives, for which geometric means were calculated. Differences between pharmacokinetic parameters calculated for two individual cattle were considered to be statistically significant, when greater than twice the mean of their respective standard errors.

Experimental design

Five cattle were treated with isometamidium chloride by the intramuscular route. Blood samples were collected from these cattle at 20, 40, 60, 80, and 100 minutes after injection, then at 2, 3, 6, 12, and 24 hours after injection, then at 12 hourly intervals until 7 days after injection, then daily until 18 days, then biweekly until 50 days after injection, and thereafter weekly until isometamidium could no longer be detected in the serum.

Another similar five cattle were treated with isometamidium chloride by the intravenous route. Blood samples were collected from these cattle at similar times after injection to the intramuscularly treated cattle, with an additional sampling at 10 minutes after injection.

Results

Intravenously Treated Cattle

Isometamidium could be detected using the competitive enzyme immunoassay (CEIA) for over 14 days in all five intravenously treated cattle. Mean isometamidium concentrations determined in the sera of intravenously treated cattle are shown in Figure 14.

Pharmacokinetic parameters obtained using a three compartment open model of isometamidium concentrations determined with the CEIA in the sera of cattle treated with isometamidium chloride by the intravenous route are shown in Table 18. Only data from serum samples collected up to the time that the measured concentration fell below twice the limit of detection of the CEIA (1.0 $ngml^{-1}$) were used in the pharmacokinetic analysis. Hence the analysis was conducted for the first 14 days (2 cattle, nos. 111 and 112), 15 days (no. 113), 42 days (no. 114), and 21 days (no. 115) following treatment.

Of the five cattle to which isometamidium had been administered by the intravenous route, most of the pharmacokinetic parameters calculated for one animal (no. 114) were statistically significantly different from those of the other four animals. This animal was one in which difficulty had been experienced in administering the intravenous injection and at least part of the injection may have had been extravascular. The pharmacokinetic parameters for this animal were therefore excluded from the calculation of the mean values.

The volume of the central compartment (V_C) was large (mean 0.70, range 0.59 - 0.95 lkg^{-1}). The mean area under the curve (AUC) was 143 $ng.h.ml^{-1}$ (range 129 - 170), and the total body clearance (Cl) of isometamidium was 4.9 ml/min/kg (range 4.1 - 5.4 $ml.min^{-1}kg^{-1}$). The mean residence time (MRT) was long at 3.4 days (range 2.6 - 5.2 days) and the volume of distribution at steady state (V_{SS}) was very large (mean 24.5, range 18.6 - 39.3 lkg^{-1}). The half-life of the elimination phase was 5.8 days (range 5.1 - 6.9 days).

Intramuscularly Treated Cattle

Isometamidium could be detected by the CEIA in intramuscularly treated cattle for up to 64 days after treatment. Mean isometamidium concentrations determined in the sera of intramuscularly treated cattle are shown in Figure 15.

Pharmacokinetic parameters obtained by non-compartmental modelling of drug concentrations determined using the isometamidium-CEIA in the sera of

cattle treated with isometamidium chloride by the intramuscular route are shown in Table 19. Only data from serum samples collected up to day 30 following treatment were used in the pharmacokinetic analysis, as beyond this time concentrations fell below 1.0 $ngml^{-1}$ (twice the limit of detection of the CEIA).

The maximum isometamidium concentrations (C_{max}) were attained rapidly, mean time to maximum observed concentration (T_{max}) was 36 minutes (range 20 - 60 minutes). Values of C_{max} varied considerably, with values in two cattle (nos. 5 and 6; C_{max} = 197$ngml^{-1}$ and 191$ngml^{-1}$ respectively) considerably higher than in the other three (nos. 7, 8 and 9; C_{max} = 53 $ngml^{-1}$, 77 $ngml^{-1}$ and 37 $ngml^{-1}$ respectively). Thereafter concentrations fell rapidly, and by 24 hours were not greater than 10% of C_{max}. The mean concentration fell by a further 50% over the next 24 hours, but thereafter the rate of drug disappearance diminished so that a further 6 days were required for another 50% reduction in concentration.

The apparent bioavailability (F) of isometamidium following intramuscular injection was 63%, and the mean absorption time (MAT) was 7.8 days.

Table 18. Pharmacokinetic parameters obtained from cattle treated with isometamidium chloride by intravenous injection at a dose rate of 1.0 $mgkg^{-1}$.

Parameter	Units	Mean	Range		
C_{zero}	$ngml^{-1}$	1496	1049	-	1708
V_{CC}	lkg^{-1}	0.695	0.586	-	0.953
K21	-	5.96	3.21	-	8.89
K31	-	0.202	0.181	-	0.221
K10	-	10.5	8.0	-	13.2
K12	-	12.6	6.8	-	17.8
K13	-	6.31	5.23	-	7.54
K10-$t_{½}$*	h	1.61	1.26	-	2.08
Alpha-$t_{½}$*	h	0.528	0.408	-	0.673
Beta-$t_{½}$*	h	5.56	4.35	-	8.40
Gamma-$t_{½}$*	h	136	123	-	165
AUC	$ng.d.ml^{-1}$	143	129	-	170
Cl	$ml.min^{-1}.kg^{-1}$	4.90	4.07	-	5.37
AUMC	$ng.d^2.ml^{-1}$	490	363	-	679
MRT	h	82.75	63.42	-	123.91
V_{SS}	lkg^{-1}	24.5	18.5	-	39.3

*Means of half-lives are geometric means

Table 19. Pharmacokinetic parameters obtained from cattle treated with isometamidium chloride by deep intramuscular injection at a dose rate of 1.0 $mgkg^{-1}$.

Parameter	Units	Mean	Range		
t_{max}	h	0.605	0.336	-	1.01
C_{max}	$ngml^{-1}$	111	37	-	197
Beta	-	0.103	0.054	-	0.144
$t_{½}$ Beta*	d	7.22	4.81	-	12.8
AUC_{last}	$ng.d.ml^{-1}$	84.6	43.3	-	131
AUC_{INF} (observed)	$ng.d.ml^{-1}$	97.6	64.0	-	140
AUC_{INF} (predicted)	$ng.d.ml^{-1}$	90.4	57.0	-	133
$AUMC_{last}$	$ng.d^2.ml^{-1}$	645	392	-	808
$AUMC_{INF}$ (observed)	$ng.d^2.ml^{-1}$	1186	982	-	1387
$AUMC_{INF}$ (predicted)	$ng.d^2.ml^{-1}$	890	775	-	991
MRT_{last}	d	8.26	6.16	-	11.1
MRT_{INF} (observed)	d	13.6	8.2	-	20.2
MRT_{INF} (predicted)	d	11.2	6.6	-	17.4
Bioavailability	%	63.0		NA	
MAT	d	7.78		NA	

* Mean half-life is geometric mean

Figure 14. Mean isometamidium concentrations ($ngml^{-1}$) in the sera of 4 cattle treated with 1.0 $mgkg^{-1}$ by the intravenous route, determined using the CEIA. Error bars: standard deviation.

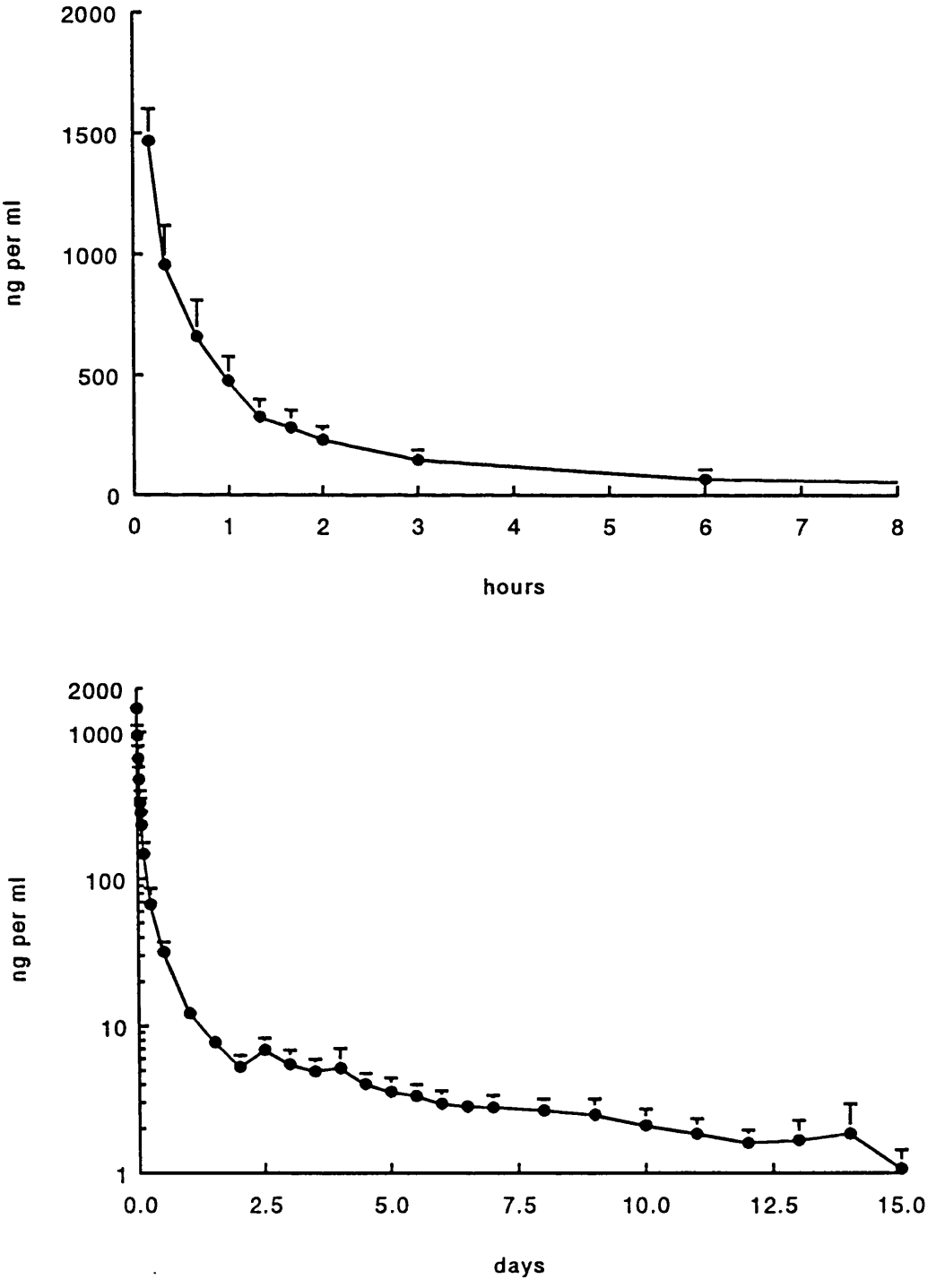
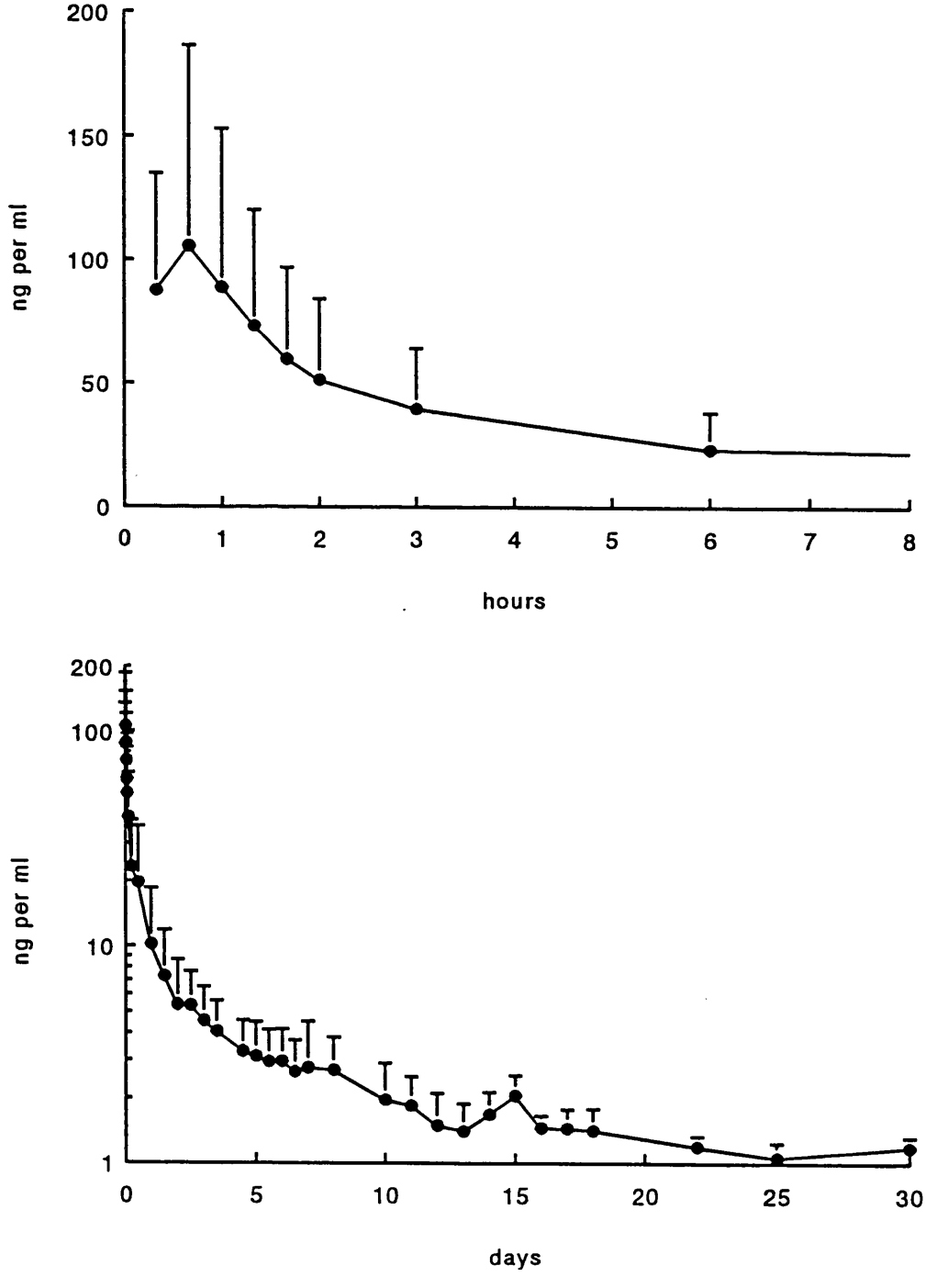


Figure 15. Mean isometamidium concentrations ($ngml^{-1}$) in the sera of 5 cattle treated with 1.0 $mgkg^{-1}$ by the intramuscular route, determined using the CEIA. Error bars: standard deviation.



Discussion

The isometamidium CEIA has permitted, for the first time, a comprehensive pharmacokinetic analysis of the disposition and kinetics of the drug in treated cattle.

Although a cross-over experimental design is usually considered desirable in pharmacokinetic studies, two different groups of cattle were used for the intramuscular and intravenous studies. In view of the extremely long period of biological activity following a single dose of isometamidium chloride (Whitelaw *et al.*, 1986), the length of the washout period required between repeat treatments of the same animals by different routes would have been impractical. Overall mean values for bioavailability (F) and mean absorption time (MAT), could nevertheless be calculated, although the experimental design precluded estimation of individual values, and hence variability, of these parameters.

Weighting of data using weights equal to the square of the reciprocal of concentration was considered necessary because of the wide disparity (over 1000-fold) between the highest and lowest concentrations measured, resulting in a tendency for the non-linear least squares method to fit the earlier data points (the highest concentrations) better than the later data points (the lowest concentrations) for which the residual values (i.e. the difference between the concentrations observed and those predicted by the model) were far smaller. This weighting scheme is justifiable on mathematical grounds because of the error structure of the determination of concentrations, as predicted by the precision profile of the assay (Chapter 7). Because the coefficients of variation of the concentrations are relatively uniform over the dynamic range of the assay in which they are determined, the corresponding standard deviation tends to be related to concentration linearly, and hence the variance tends to be linearly related to the square of the concentration. Weighting on the basis of the reciprocal of the square of the concentration is thus proportional to weighting on the basis of the reciprocal of the variance (in practice weights are "normalised" by multiplying by a factor such that the sum of the weights equals the number of observations).

The large volume of the central compartment (mean 0.70 lkg^{-1}) exceeded both the plasma volume (0.028 - 0.037 lkg^{-1}) and the extracellular fluid volume (0.26 - 0.37 lkg^{-1}) of cattle (Tasker, 1980). The extremely large volume of distribution at steady state (mean 24.5 lkg^{-1}, range 18.6 - 39.3) was greater

than the total body water (0.502 - 0.645 lkg^{-1}) of cattle (Tasker, 1980), and probably reflected extensive uptake of the drug into specific tissues. The prolonged mean residence time (83 h) and terminal phase half-life (136 h) following intravenous injection were consistent with the formation of secondary drug depots. In rats, 41% of a 1.0 $mgkg^{-1}$ dose of isometamidium administered by the intravenous route was present in the liver within 1 minute of injection, and by 10 minutes, 62% of the dose was present in the same organ (Philips *et al.*, 1967). In addition, approximately 5% of the dose administered was detected in kidney as early as 1 minute after injection. Kinabo and Bogan (1988b) using HPLC analysis, confirmed the presence of secondary isometamidium depots in liver, kidney and spleen, one, three and six weeks following intramuscular treatment of cattle. Uptake of isometamidium into these tissues may be due to binding of the drug to proteins, to acid phospholipids and to DNA (Kinabo and Bogan, 1987), or to an isometamidium-specific active transport mechanism as was proposed by Philips *et al.* (1967); such a mechanism has been demonstrated in *T. congolense* (Sutherland *et al.*, 1992; Zilberstein *et al.*, 1993).

The absolute bioavailability (F) of isometamidium following intramuscular injection was found to be approximately 60%. In experiments on the use of isometamidium in pigs, the bioavailability could not be calculated because animals which received the drug via the intravenous route died (Kinabo *et al.*, 1991). In goats, the mean absolute bioavailability of intramuscularly injected isometamidium was 27% (Kinabo and McKellar, 1990), approximately half that obtained in cattle in the study described here. The value of t_{max} following intramuscular injection of cattle (0.6 h) was considerably less than that in goats (6 h) implying absorption of isometamidium was slower in the latter species, at least in the initial phase following injection. In spite of the relatively short time to maximum concentration in cattle, the mean absorption time (MAT) in cattle was prolonged (7.8 d), and considerably longer than that observed in goats (13 h). The low F value and slow absorption rate in goats were considered to be due to binding of the drug to tissue macromolecules and ground matrix components at the injection site (Kinabo and McKellar, 1990), and the same probably holds true, possibly to a lesser extent, in cattle. One possible explanation of the large differences observed between cattle and goats in MAT and F values might be the high sensitivity of the CEIA (detection limit 0.5 $ngml^{-1}$) which enabled pharmacokinetic parameters to be determined using concentration-time data up to 30 days after intramuscular treatment and 15

days after intravenous treatment. The goat study used the less sensitive HPLC method (detection limit 5 $ngml^{-1}$), which was unable to detect isometamidium in plasma samples more than 24 hours after intravenous treatment, and 48 hours after intramuscular treatment. This might have led to underestimation of the value of the terminal phase rate constant (β) and consequently inaccuracy in the parameters derived from it, including AUC, MAT and F.

The relative importance of the injection site and other secondary drug depots in the prophylaxis of isometamidium afforded to rats was investigated by Hill and McFadzean (1963). Following amputation of the injection site in the tail, the period of prophylaxis afforded was reduced from 16 to 2 weeks. Although the tail of a rat may not be a particularly good model of an intramuscular injection in the bovine, that experiment clearly indicated the importance of the primary depot, while also demonstrating a not insignificant period of prophylaxis following its removal.

In the present work the time required for drug concentrations to fall to 1.0 $ngml^{-1}$ following intravenous treatment (mean 16 days) was approximately half, when compared with intramuscular treatment (mean 30 days). Isometamidium has been shown to possess prophylactic activity for up to 48 days following intravenous administration to cattle (Touré, 1973).

In this study C_{max} was over ten times greater following intravenous administration (mean C_0 [= C_{max}] 1.3 μgml^{-1}) than intramuscular administration (mean C_{max} 110 $ngml^{-1}$) of a 1.0 $mgkg^{-1}$ dose. Dowler *et al.* (1989) considered that achievement of initial high blood levels of isometamidium may be important in the elimination of cryptic infections. Intravenous administration of isometamidium also has the advantage of avoiding the severe inflammatory and subsequently fibrotic tissue reaction associated with intramuscular use of the drug (Kinabo and Bogan, 1988b), which may result in loss of value of the carcass (Dowler *et al.*, 1989). The intravenous route may be preferable in slaughter cattle for this reason (Eyidi, 1971; Dowler *et al.*, 1989).

The pharmacokinetic results obtained here with isometamidium are not dissimilar to those obtained with the related trypanocide diminazene aceturate (Aliu *et al.*, 1993) in female Boran cattle, in that following intravenous injection, drug concentrations fell rapidly during a biphasic period of drug distribution, and more slowly during a prolonged elimination phase. The elimination half-life of intravenous isometamidium (137 hours) was, however, far longer than that of

diminazene (31.7 hours), a finding consistent with the prolonged period of biological activity observed with the former drug.

Kinabo and McKellar (1989) reviewed the models available for veterinary pharmacokinetic studies, and proposed a physiological pharmacokinetic model for isometamidium. One of the reasons for the development of this model was that of the very low concentrations present in serum, below the limit of detection of the available methods of determination (Kinabo and Bogan, 1988a, 1988b), which precluded a compartmental analysis. The improved sensitivity of detection afforded by the ELISA (CEIA) has allowed this constraint to be overcome, and has allowed the major pharmacokinetic parameters to be derived for isometamidium. The studies reported here were conducted in Friesian cattle (*Bos taurus*). Although further work will be required to investigate whether there are any significant differences in the pharmacokinetics of isometamidium in *Bos indicus* cattle, earlier studies failed to reveal any evidence of differences between the two species of cattle in the rates of disappearance of the drug from the sera following treatment via the conventional intramuscular route (Chapter 3 and 4).

Chapter 9:
Isometamidium Concentrations in the Sera of Cattle Under a Chemoprophylactic Regime in a Tsetse-Infested Area of Zimbabwe

Introduction

At present, the use of trypanocidal drugs is the mainstay of control of bovine trypanosomiasis in much of sub-Saharan Africa (Jordan, 1992), and is likely to remain so for the foreseeable future.

Isometamidium chloride (Samorin®, Trypamidium®) is the most widely used drug for chemoprophylaxis of bovine trypanosomiasis in Africa. The use of isometamidium until the present has been largely empirical, because of the lack of a suitable analytical method for the determination of its concentration in the circulation of cattle. However, recently developed ELISA techniques for the determination of isometamidium concentrations in bovine serum (Chapters 3 and 6) should provide opportunities to rationalise and improve strategies for the use of this drug by allowing drug levels to be quantified following treatment. This is of particular importance in view of the increasing number of reports of drug-resistant trypanosomes (Kupper and Wolters, 1983; Pinder and Authié, 1984; Ainanshe *et al.*, 1992; Codjia *et al.*, 1993), which may arise as a result of inappropriate use of trypanocidal drugs (MacLennan, 1970; Leach and Roberts, 1981).

The indirect competition ELISA for isometamidium (Chapter 3) was used to measure concentrations of the drug in the sera of cattle that were treated and maintained under controlled laboratory conditions (Chapters 3, 4 and 5). In such animals the assay could detect the drug for up to four months after treatment (Chapter 5). Furthermore, the relationship between serum concentrations of isometamidium and the degree of protection against tsetse challenge with various populations of *Trypanosoma congolense* was investigated (Chapters 4 and 5). This ELISA was also shown to be capable of measuring concentrations of isometamidium in the sera of Jersey dairy cattle under a chemoprophylactic regimen in the field (Chapter 6).

A simpler, more robust, competitive enzyme immunoassay (CEIA) for isometamidium was described in Chapter 7. The CEIA was shown to have a high level of repeatability and reproducibility, and gave results which corresponded closely with those obtained in the indirect competition ELISA for isometamidium (Chapter 7).

In the work described in this Chapter, the CEIA for isometamidium was further evaluated in an experiment carried out under field conditions to determine the concentrations of isometamidium present in the sera of cattle

maintained under an isometamidium chemoprophylactic regimen in the face of natural tsetse challenge.

Materials and methods

Study site

The experiment was carried out at the Rekomitjie Research Station; a tsetse-infested area of the Zambezi valley, Zimbabwe. *Glossina pallidipes* is the predominant tsetse fly species in this area, with smaller numbers of *Glossina morsitans morsitans.* The area supports abundant populations of game animals and a few cattle kept for research purposes (Woolhouse, Hargrove and McNamara, 1993).

Cattle

Twenty-four clinically normal adult cattle were obtained from the tsetse-free area of Makuti, situated on the Zambezi escarpment. A further eighteen cattle, maintained permanently at Rekomitjie, were used as challenge controls during the experiment.

Serum collection

Every two weeks, jugular venous blood was collected into plain Vacutainers (Becton Dickinson), and the serum separated and stored at -20°C until analysis by the competitive enzyme immunoassay for isometamidium.

Trypanocidal drug treatments

Isometamidium chloride (Samorin®) was administered as a 2% (w/v) aqueous solution by deep intramuscular injection into the gluteal muscle mass at a dose of 1.0 $mgkg^{-1}$ body weight (body weight). Diminazene aceturate (Berenil®, Hoechst AG) was administered as a 7% (w/v) aqueous solution by deep intramuscular injection at a dose of 7.0 $mgkg^{-1}$ body weight

Parasitological monitoring

Every two weeks blood samples were collected from an ear vein of each animal into potassium ethylenediamine tetra-acetate. Thereafter, the samples were examined for trypanosomes using the haematocrit centrifugation - buffy coat technique (Murray *et al.*, 1977). Identification of the species of trypanosome was confirmed using Giemsa-stained thin-blood films.

Isometamidium determinations

Serum isometamidium concentrations were determined using the competitive enzyme immunoassay described in Chapter 7. Briefly, sera were prediluted 1/10 in a 1/2000 dilution of isometamidium-HRP conjugate in

phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma). Ninety-six well microtitre plates (Immulon 4, Dynatech) which had previously been coated overnight with 100 µl per well of a 1/12000 dilution of hyperimmune sheep anti-isometamidium serum in carbonate/bicarbonate buffer, pH 9.2, then frozen and stored at -20°C with the coating solution *in situ*, were allowed to thaw and equilibrate to room temperature. Plates were then washed five times in a 1/5 dilution of PBS, pH 7.2, in distilled water containing 0.05% Tween 20. Serum predilutions were vortex mixed and transferred to duplicate wells of the microtitre plates (100 µl per well). After orbital shaking (Varishaker, Dynatech) for ten minutes at room temperature, plates were incubated overnight at 4°C, then allowed to equilibrate to room temperature before being washed five times as described previously. Enzyme levels remaining in each well were then determined by adding 100 µl per well of a two-component horseradish peroxidase substrate containing 3,3,5,5 tetramethylbenzidine (Cambridge Veterinary Sciences, Littleport, UK) pre-warmed to 37°C. After 10 minutes incubation with orbital shaking at 37°C, the colour reaction was quenched by the addition of 100 µl per well of 2M sulphuric acid. Absorbances were then read at 450 nm using a multichannel spectrophotometer (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

Eight calibration standards containing a range of isometamidium concentrations from 500 to 0.025 $ngml^{-1}$ were prepared using a serum pool prepared from an equal volume of serum that was collected from each of the 24 cattle prior to administration of any trypanocidal drug. Thereafter, they were included in duplicate wells on every microtitre plate. Three quality control samples, comprising one normal (untreated) bovine serum sample, and two dilutions, in normal bovine serum, of serum from a steer treated with isometamidium chloride under laboratory conditions, were each tested twice (and in duplicate) on every microtitre plate. Samples were all tested in duplicate, and concentrations were derived by four-parameter logistic curve-fitting of calibration standards on the same ELISA plate. Results from individual ELISA plates were only included in the final data analysis if the standards and quality controls gave the expected results.

Data Analysis

Four-parameter logistic curve-fitting and calculation of concentrations were performed using in-house software (M.C.E., unpublished). Data collation and

statistical analyses were performed using spreadsheet software (Lotus 1-2-3 Release 4 for Windows) on an IBM-compatible personal computer.

The disappearance of isometamidium from the sera of individual cattle, over the first twelve weeks following treatment, was modelled using linear regression of log concentration against time. The regression coefficients were converted into apparent half-lives ($t_{½}$) using the formula:

$t_{½}$ = - log 2 / coefficient.

Experimental design

On 2nd February 1993, 22 cattle in the tsetse-free area of Makuti were examined for the presence of trypanosomes using the method of Murray *et al.* (1977); only one animal was detected parasitaemic. Thereafter, diminazene aceturate was administered to all the cattle. Two weeks later, the cattle were moved to the tsetse-infested area of Rekomitjie, and isometamidium chloride was administered to each animal as described. The cattle were then maintained at Rekomitjie for the following six months. As mentioned, parasitological monitoring and serum collection were carried out fortnightly throughout the experiment. During the study, any animal found to be parasitaemic was treated intravenously with isometamidium chloride at a dose of 0.4 $mgkg^{-1}$ body weight, using a 1% (w/v) solution, and removed from the experiment.

In order to determine the level of trypanosome challenge, eighteen challenge control cattle were maintained together with the drug-treated cattle at Rekomitjie. These animals were not treated with isometamidium chloride, but were monitored on a fortnightly basis throughout the experiment period for the presence of trypanosomes.

Results

Parasitological findings

At the initial screening of the 22 cattle in the tsetse-free area of Makuti, only one trypanosome infection was detected; *T. vivax* in animal number P243. No parasites were detected in the blood of the animal two weeks later, or at any of eleven subsequent fortnightly parasitological screenings.

Table 20 lists the monthly incidence of trypanosome infections in the isometamidium-treated cattle and challenge control cattle at Rekomitjie. No trypanosomes were detected in isometamidium-treated cattle for 126 days following treatment. Thereafter, trypanosomes were detected in only three animals; *T. vivax* was diagnosed in one animal (P225) on day 140; on day 154, mixed infections were detected in two animals, *T. brucei/T. congolense* in J437 and *T. brucei/T. vivax* in P239. One isometamidium-treated animal (P229) died from anaplasmosis, between four and six weeks following arrival at the tsetse-infested area.

By contrast, over the same period, the monthly incidence of trypanosomiasis in the eighteen challenge control cattle, that were not under isometamidium prophylaxis, was as follows: February, nil; March , two cases; April, one case; May, four cases; June , two cases; July, nil (see Table 20).

Isometamidium concentrations

The mean isometamidium concentrations determined using the CEIA in the sera of the 24 drug-treated cattle, over the twelve-week period following intramuscular treatment, are shown in Table 20 and Figure 16. Isometamidium could be detected using the CEIA in the sera of all but one of the 24 cattle following treatment. In one animal (T478), isometamidium, if present, was at concentrations below the limit of detection of the assay (0.5 $ngml^{-1}$). Two weeks after treatment, the concentration of isometamidium in sera of the 23 cattle in which the drug could be detected ranged between 2.4 and 9.1 $ngml^{-1}$. Excluding the animal which died (P229), and the animal in which isometamidium was not detected (T478), the drug could be detected in all the remaining 22 cattle for eight weeks following treatment, and in most (20 cattle) for 10 weeks following treatment. In 12 cattle, the drug could still be detected 12 weeks following treatment. Finally, in five cattle it could be detected after 14 weeks following treatment.

Linear regression of log isometamidium concentrations against time for the first 12 weeks following treatment accounted for between 78% and 99% (median 92%) of the total variance in log concentrations. The range in apparent half-lives calculated from the regression coefficients in 22 individual cattle (regressions could not be calculated for the animal which died, or the animal in which isometamidium was not detectable) was 15.7 to 58.3 days, and the geometric mean half-life was 23.2 days. Among these 22 cattle, the half-life of isometamidium in the majority (20 cattle) were between 15.7 and 27.1 days, while the half-lives in the other two cattle were somewhat longer at 37.8 and 58.3 days.

In all three isometamidium-treated cattle in which trypanosome infections were detected (J437, P225 and P239), isometamidium concentrations had fallen below the limit of detection of the CEIA (0.5 $ngml^{-1}$) by the time trypanosomes were detected. In animal number P225, the last serum sample in which isometamidium could be detected was collected 16 weeks after treatment; the trypanosome infection in this animal was not diagnosed until four weeks later (week 20). In animal number J437, isometamidium was last detected eight weeks following treatment, whereas the trypanosome infection was not diagnosed until 14 weeks later (week 22). This animal was the one in which isometamidium could be detected for the least length of time and was therefore the one in which isometamidium had the shortest apparent half-life (15.7 days). Finally, in animal number P239, isometamidium could be detected for 12 weeks following treatment, whereas the trypanosome infection was not detected until ten weeks later (week 22).

Discussion

In this study, concentrations of isometamidium were determined using the competitive enzyme immunoassay (CEIA) in the sera of 24 cattle that were treated with a prophylactic dose of isometamidium chloride (1.0 $mgkg^{-1}$ body weight) and exposed to natural tsetse challenge at Rekomitjie, Zimbabwe. Trypanosome infections were not detected in any of the drug-treated cattle for 18 weeks following treatment; in 20 of the cattle infections were not detected for at least 22 weeks. By contrast, 9 trypanosome infections were detected in 18 untreated control cattle during the same period, indicating a low to medium tsetse challenge (Whiteside, 1962b).

Isometamidium could be detected in the sera of all but one of the 24 treated cattle. Failure to detect isometamidium in one animal may have resulted from failure to administer the drug, or failure to inject it into the centre of a muscle mass. Intramuscular injections may inadvertently be made into the facial planes between individual muscle groups, which would be likely to result in relatively poor absorption from the injection site. Folkers (1966) attributed the failure of isometamidium to cure a trypanosome infected steer to be due to poor absorption of the drug due to an inflammatory reaction at the injection site. However injections of isometamidium into the subcutaneous connective tissue of the dewlap are apparently effective in prophylaxis against bovine trypanosomiasis (Williamson, 1970). An alternative possibility, that of inadvertent intravascular injection, would not readily explain failure to detect the drug, as the CEIA has been shown to be capable of detecting the drug for over three weeks following intravenous injection of cattle at the same dose rate (Chapter 8).

The isometamidium concentrations measured using the CEIA in the sera of treated cattle showed a drug disappearance rate that was similar to that observed in sera of cattle treated with isometamidium chloride at the same dose rate (1.0 $mgkg^{-1}$ body weight) under laboratory conditions; the mean half-life of elimination, 23 days, was similar to the value of 23.9 days measured in non-infected cattle (Chapters 3 and 4). The initial peak in drug concentration could not be detected because the first occasion serum was sampled was not until two weeks after isometamidium treatment; the initial peak occurs within the first hour after intramuscular treatment (Kinabo and Bogan, 1988; and Chapter 8).

In contrast to drug disappearance rates, the isometamidium concentrations determined in this study were slightly lower than those previously observed in cattle treated under laboratory conditions with the same dose of isometamidium chloride (Chapters 3 and 4). For example, 28 days after treatment, the mean concentration in this study (2.75 $ngml^{-1}$) was significantly lower ($p < 0.001$) than the mean concentration of 6.1 $ngml^{-1}$ measured in the experiment described in Chapter 4. One explanation for this difference might be the fact that the work described here used the CEIA (Chapter 7), whereas the previous work had used the indirect competition ELISA (Chapter 3). However, it was shown in the work described in Chapter 7 that there was a close correlation (r^2 value of 0.96) between the results obtained using the two methods (see Figure 12), and therefore it is unlikely that the slightly lower values for the serum levels in the present study were in part due to the methodology used.

It is also possible that the lower concentrations observed in this study were due to differences between the two experimental situations. Firstly, the cattle used in this study were of a breed different to the pure-bred Boran cattle used in previous laboratory experiments (Chapters 4 and 5); although previous studies have failed to reveal significant differences between isometamidium concentrations in different breeds of cattle (Chapters 4 and 6) there is as yet insufficient data to rule out entirely the possibility of breed effects. Secondly, the cattle in this study were maintained under field conditions and potentially exposed to environmental stresses such as ectoparasite and endoparasite challenge, and poor nutrition. Their full history, particularly in terms of both management and disease, was not known, and at least one animal (P243) was found to be harbouring a trypanosome infection at the start of the experiment. In contrast, the cattle used in the experiments described in Chapters 4 and 5 were kept under laboratory conditions where environmental stresses were intentionally kept to a minimum, and nutrition was adequate. These stresses can have marked effects on body weight and body condition, and are also likely to affect body composition; the distribution of the relatively lipid insoluble drug isometamidium could for example be susceptible to changes in the overall proportion of body fat.

Treatment of the cattle at Rekomitjie with isometamidium chloride at a dose of 1.0 $mgkg^{-1}$ body weight prevented development of trypanosome infections in the majority of cattle for the entire six-month period of the experiment, in spite of a moderate trypanosome challenge. Since the ELISA could no longer detect

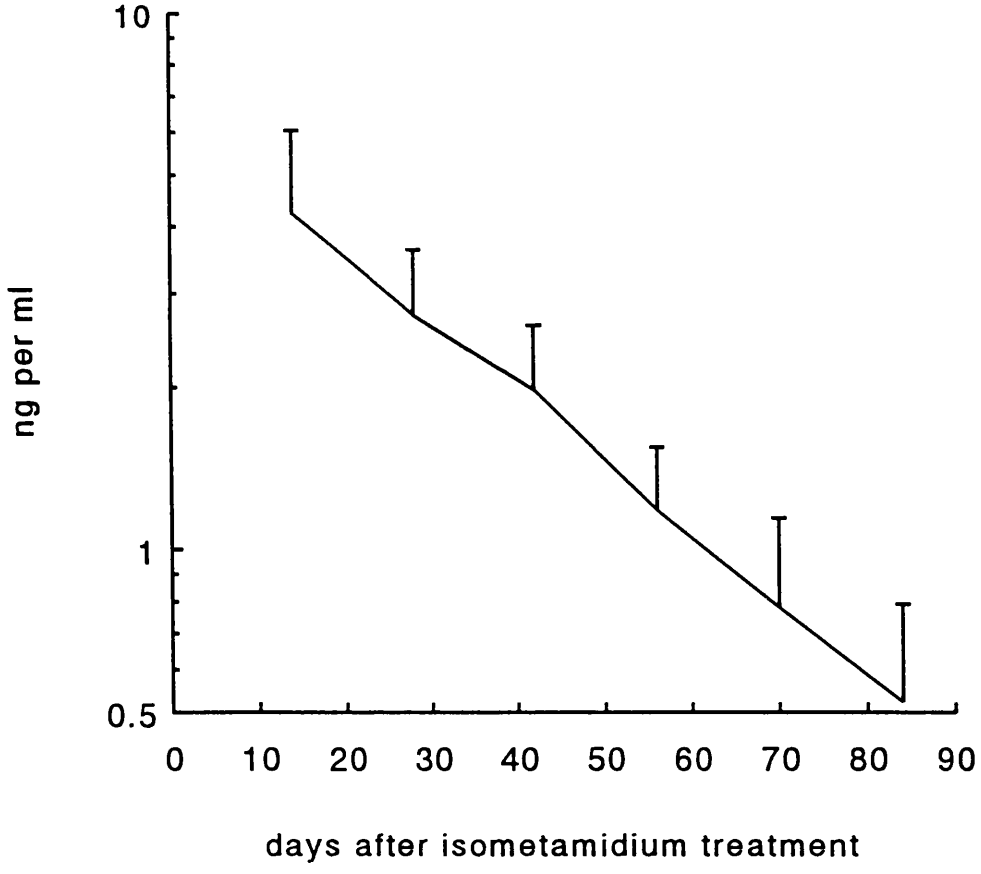
isometamidium in the sera of the majority of cattle by 16 weeks following treatment, it was not possible to correlate drug levels with the degree of protection obtained. However, it was possible to make the observation that 10 to 12 weeks after a single treatment with isometamidium chloride at the aforementioned dosage, concentrations of the drug of 0.5 $ngml^{-1}$, or greater, were sufficient to protect cattle against potentially virulent trypanosome challenge. This result was therefore in agreement with laboratory experiments (Chapters 4 and 5) in which it was shown that four months after isometamidium treatment, concentrations in the sera of cattle as low as 0.4 $ngml^{-1}$ were protective against experimental tsetse challenge with a drug-sensitive population of *T. congolense*. There was therefore no evidence of drug-resistant trypanosomes at Rekomitjie; perhaps not a surprising observation, as the area supports abundant populations of game animals, but only a few cattle (Woolhouse *et al.*, 1993).

This study has demonstrated the competitive enzyme immunoassay for isometamidium to be a useful technique for the demonstration of prophylactic isometamidium concentrations in cattle under natural tsetse challenge in the field. It has also demonstrated that protective levels of isometamidium may not be present in the circulation of all cattle following a treatment protocol carried out under field conditions; isometamidium could not be detected in one of the treated animals (T478). Although the reason for this was not discovered, in many situations in the field it might be the result of failure to administer the drug, of failure to calculate the dose correctly, or it might follow the use of adulterated or counterfeit drug; all of which are recognised as problems affecting the use of trypanocidal drugs in sub-Saharan Africa (Jordan, 1986). In view of widespread concern in tsetse-infested areas about the possibility of increasing numbers of drug-resistant trypanosomes, it is important to distinguish whether trypanosome infections occurring in spite of isometamidium prophylaxis are due to genuine drug-resistance, or whether they are due to other factors resulting in inadequate circulating drug concentrations. The results obtained here suggest that the CEIA for isometamidium would be capable of making this distinction, since circulating drug levels believed to be protective could be detected in most but not all of the treated cattle. Further studies are required to investigate isometamidium concentrations in sera of cattle in areas where drug-resistant trypanosomes are believed to occur.

Table 20. Mean and standard deviation of isometamidium concentrations in the sera of cattle treated intramuscularly with isometamidium chloride at a dose of 1.0 mgkg^{-1} body weight and exposed to natural tsetse challenge at Rekomitjie.

Weeks after treatment	Mean isometamidium concentration (ngml^{-1})	Standard deviation
2	4.25	1.79
4	2.75	0.88
6	1.99	0.63
8	1.19	0.36
10	0.78	0.36
12	0.52	0.27

Figure 16. Mean isometamidium concentrations ($ngml^{-1}$) in the sera of 24 cattle exposed to natural tsetse challenge at Rekomitjie over a twelve week period following intramuscular treatment with isometamidium chloride at a dose rate of 1.0 $mgkg^{-1}$ body weight, determined using the CEIA. Error bars: standard deviation.



Chapter 10:
Concentrations of the Trypanocidal Drug Isometamidium Chloride in the Sera of Maasai Zebu Cattle Following Frequent Repeated Intramuscular Treatments

Introduction

Control of African bovine trypanosomiasis continues to rely heavily on the use of a small number of therapeutic and prophylactic drugs. Of these, isometamidium chloride is the most widely used chemoprophylactic agent, and has been used successfully in many situations (Bourn and Scott, 1978; Leach and Roberts, 1981; Trail *et al.*, 1985). Recently, however, reports of less than satisfactory isometamidium prophylaxis have been attributed in part to the development of drug resistant trypanosomes (Kupper and Wolters 1983; Pinder and Authié, 1984; Schönefeld *et al.*, 1987; Ainanshe *et al.*, 1992), and an indirect competition ELISA for the detection and quantification of isometamidium in bovine serum has been developed (Chapter 3) to elucidate the relationship between circulating concentrations of the drug, and susceptibility to tsetse challenge with pathogenic trypanosomes (Chapters 4 and 7).

Increased frequency of administration of isometamidium has been used as a means of countering the problem of drug resistance (Fox *et al.*, 1991), but has under some circumstances been associated with a wasting syndrome with high mortality (Stevenson, Dolan, Okech, Heath and Njogu, 1990; Dolan *et al.*, 1992). In the study described in this Chapter, the competitive enzyme immunoassay (CEIA) for isometamidium described in Chapter 7 was used to investigate a possible role for the drug in the pathogenesis of a similar syndrome induced experimentally in cattle.

Materials and methods

The experiment was carried out in the Nguruman area of Kajiado District in South West Kenya.

Cattle

Maasai Zebu cattle were purchased from Maasai pastoralists at the foot of the Nguruman escarpment and maintained on natural grazing in the same area for the duration of the experiment. As the area had suffered a period of drought for two years, grazing was poor at the start of the experiment (23/8/91).

Trypanocidal drug treatments

Isometamidium chloride (Samorin®) was administered as a 2% aqueous solution by deep intramuscular injection into the middle third of the neck at a dose rate of 1.0 $mgkg^{-1}$ body weight. Diminazene aceturate (Berenil®, Hoechst AG) was administered as a 7% aqueous solution by deep intramuscular injection at a dose rate of 7.0 $mgkg^{-1}$ body weight.

Prior to the study, cattle had been maintained on an isometamidium prophylactic regimen. However, no isometamidium treatments had been administered within 5½ months of the start of the study. During this 5½ month period, trypanosome infections had been treated with diminazene aceturate. No diminazene treatments were administered within eight weeks of the start of the study.

Parasitological monitoring

Blood samples collected from an ear vein were examined weekly for trypanosome infections using the haematocrit centrifugation - buffy coat technique (Murray *et al.*, 1977). During the study, any animals found to be parasitaemic were treated with diminazene aceturate.

Serum collection

Jugular venous blood was collected into plain Vacutainers (Becton Dickinson), and the serum separated and stored at -20°C until testing.

Isometamidium determinations

Serum isometamidium concentrations were determined using the competitive enzyme immunoassay (CEIA) described in Chapter 7.

Briefly, sera were prediluted 1/10 in an optimal dilution of isometamidium-HRP conjugate in phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma). Ninety-six well microtitre plates (Immulon 4, Dynatech) which had previously been coated overnight with 100 µl per well of an optimal dilution of hyperimmune sheep anti-isometamidium serum in carbonate/bicarbonate buffer pH 9.2, frozen and stored at -20°C with the coating solution *in situ*, were allowed to thaw and equilibrate to room temperature. Pates were then washed five times in a 1/5 dilution of phosphate buffered saline (PBS, pH 7.2), in distilled water containing 0.05% Tween 20. Serum predilutions were vortex mixed and transferred to duplicate wells of microtitre plates (100 µl per well). Plates were shaken for ten minutes at room temperature, and then incubated overnight at 4° C. Plates were allowed to equilibrate to room temperature before washing five times as described previously. Enzyme levels were determined using 100 µl per well of a two-component horseradish peroxidase substrate containing 3,3,5,5 tetramethylbenzidine (Cambridge Veterinary Sciences, Littleport, UK) pre-warmed to 37°C. After 10 minutes incubation at 37°C with orbital shaking (Varishaker, Dynatech), the colour reaction was quenched by the addition of 100 µl per well of 2M sulphuric acid. Absorbances were read at 450 nm using a multichannel spectrophotometer (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

Eight standards prepared using pooled serum from isometamidium-naive Kenyan Boran cattle were included in duplicate wells of every microtitre plate. Three quality control samples, comprising one normal (untreated) bovine serum, and two dilutions, in normal bovine serum, of serum from an isometamidium steer, were each tested twice (in duplicate) on every microtitre plate. Samples were tested in duplicate, and provided the standards and quality controls had given the results expected, concentrations were derived by four-parameter logistic curve-fitting of calibration standards on the same ELISA plate.

Glutamate dehydrogenase concentrations

Serum glutamate dehydrogenase concentrations were determined as an index of hepatocellular damage (Mullen, 1976), using a test kit (GLDH Test Kit, Boehringer Mannheim GmbH Diagnostica).

Experimental design

At the start of the experiment (23/8/91), 40 cattle were allocated into five groups of eight cattle. Group 2 comprised cattle in better body condition at the start of the experiment than Groups 1, 3, 4 and 5, and were maintained in an area of better grazing for the duration of the study (4 months). Groups 1, 2 and 3 received treatment with isometamidium chloride at the start of the experiment, and monthly thereafter for four consecutive months. In addition, Groups 1 and 2 received treatment with diminazene aceturate, three days after the third and fourth isometamidium treatments. Group 4 cattle were treated with diminazene but not isometamidium at the same times as Groups 1 and 2. Group 5 cattle served as untreated controls. The drug administration to the different groups is summarised in Table 21.

Serum isometamidium concentrations and GLDH levels were measured approximately two weeks following the second, third and fourth isometamidium treatments. The precise dates were 16 days (8/10/91) following the second isometamidium treatment, 14 days (4/11/91) following the third isometamidium treatment, and 12 days (3/12/91) following the third isometamidium treatment. Body weights were determined on these occasions, and on others such as the dates of isometamidium administration.

Statistical analysis

For the purposes of analysis the experiment could be considered in two parts. Firstly, Groups 1, 3, 4, and 5 comprised a factorial experimental design, with isometamidium treatments being applied to Groups 1 and 3, diminazene treatments applied to Groups 1 and 4, and Group 5 as untreated controls (Table 21). Secondly, the performance of two groups of animals (Groups 1 and 2), which were differentiated on the basis of starting body condition and quality of grazing, but which received identical drug treatments (isometamidium and diminazene), were compared. The effects of isometamidium treatment, diminazene treatment and their interaction (using data from Groups 1, 3, 4 and 5), and the effects of body condition and nutrition (using data from Groups 1 and 2) on serum isometamidium concentrations, GLDH levels, and body weight losses were investigated by analysis of variance, using a matrix of orthogonal contrasts.

The experiment was divided into four periods (1 to 4), each following an isometamidium treatment. Percentage daily body weight changes were calculated for periods 1 to 3 using the formula:

(body weight at end of period - body weight at start of period) ÷ (weight at start of experiment × number of days in period) × 100.

In the case of four animals which died during the second half of period 3, and all animals surviving in period 4, percentage daily body weight change was calculated over the first two weeks of the period only. Serum isometamidium concentrations and GLDH levels measured approximately two weeks following the second, third and fourth isometamidium treatments were compared with percentage daily body weight changes over the corresponding periods, using linear regression. Body weights were modelled as dependent variables, isometamidium concentrations, and GLDH concentrations were analysed as independent variables, and treatment groups and periods as fixed effects. The significance of differences between results for particular groups on particular dates was determined by t-tests. Statistical analyses were performed using Genstat 5 Release 2.2 (Numerical Algorithms Group Ltd., Oxford).

Results

Trypanosome infections

Four animals were found to be infected on the first day of the study (23/8/91), one in Group 1 (*T. vivax*), two in Group 2 (both *T. brucei* / *T. congolense* mixed infections), and one in Group 3 (*T. brucei*). Thereafter, only two trypanosome infections were detected, both on 16/10/91, both *T. vivax*, and both in Group 4 cattle. These infections were treated with diminazene aceturate.

Body weight changes

During the first treatment period percentage daily body weight changes were small (mean = 0.021 % per diem), and were not significantly different ($p > 0.1$) between groups, nor did isometamidium treatment or body condition/nutrition have any significant effect on body weight changes.

During the second treatment period, percentage daily body weight changes were significantly different between groups ($p < 0.001$), and body weight losses were significantly greater ($p < 0.001$) in isometamidium treated cattle (Group 1 and 3 means respectively: -0.49 and -0.55 % per diem) than in cattle which had not received isometamidium (Group 4 and 5 means respectively: -0.34 and 0.39 % per diem). As no diminazene treatments had yet been given, treatments for Groups 1 and 3, and Groups 4 and 5 were identical at this stage in the experiment. Isometamidium treated cattle in better body condition at the start of the experiment, and obtaining better nutrition, (Group 2) suffered significantly smaller ($p < 0.001$) weight losses (mean -0.19 % per diem) than either the remaining isometamidium treated cattle (Groups 1 and 3), or untreated cattle on a lower plane of nutrition (Groups 4 and 5).

During the third treatment period, the increased weight loss associated with isometamidium treatment remained highly significant ($p < 0.001$): cattle given isometamidium and diminazene (Group 1) had higher weight losses (mean: -0.58 % per diem) than those given only diminazene (Group 4; mean: -0.16 % per diem), and cattle given only isometamidium (Group 3) had higher weight losses (mean: -0.38 % per diem) than those which received neither drug (Group 5; mean: -0.13 % per diem). Although mean weight losses were higher in cattle given diminazene (Group 1 compared with Group 3, and Group 4 compared with Group 5), these differences were of marginal significance ($p = 0.065$), and the interaction between the two drugs was not significant ($p = 0.19$). There was no effect of better nutrition, and better body condition at the start of the

experiment, on cattle treated with both trypanocidal drugs (Group 2, mean -0.59 % per diem, compared with Group 1, mean: -0.58 % per diem) during this period ($p > 0.89$).

During the fourth treatment period, body weight increases (overall mean 0.62 % per diem), rather than decreases were observed in all Groups. The main effect of isometamidium on body weight change ceased to be significant ($p = 0.68$) during this period, whereas the main effect of diminazene became significant ($p < 0.01$), as did the interaction between the two trypanocidal drugs ($p < 0.001$). Cattle given isometamidium and diminazene (Group 1) had smaller weight gains (mean 0.36 % per diem) than cattle given only diminazene (Group 4; mean: 0.67 % per diem), whereas cattle given only isometamidium (Group 3) had higher weight gains (mean: 0.86 % per diem) than cattle given neither drug (Group 5; mean: 0.61 % per diem). These results must be interpreted taking into consideration that four Group 1 cattle, including three (B16, B19 and B59) which had the highest drug concentrations during the third treatment period, died during the second half of that period (i.e. prior to the fourth drug administration). Finally, cattle in better body condition at the start of the experiment and with better nutrition (Group 2), had significantly smaller weight gains (mean: 0.07 % per diem) than all the other treatment groups ($p < 0.01$), except Group 1 in which too few animals ($n = 4$) had survived for a meaningful comparison.

Isometamidium concentrations

Isometamidium concentrations in sera collected from each of the treated groups (Groups 1, 2 and 3) during the second, third and fourth treatment periods were determined using the CEIA. Comparisons of concentrations between treatment groups were conducted using analysis of variance and t-tests, and comparisons over time of concentrations within treatment groups were made using paired t-tests. Mean and standard deviation isometamidium concentrations are shown in Table 22 and Figure 17.

Isometamidium concentrations were not determined following the first drug administration as sera were not collected.

During the second treatment period (see Table 22), the mean isometamidium concentration found in the serum of Group 1 cattle (5.3 $ngml^{-1}$) did not differ significantly ($p > 0.1$) from those of the other two treated groups (Group 2 mean: 3.0 $ngml^{-1}$; Group 3 mean: 7.8 $ngml^{-1}$).

During the third treatment period, the mean isometamidium concentrations in Group 1, 2 and 3 (respectively 8.8, 7.1 and 10.4 $ngml^{-1}$) did not differ significantly from each other ($p > 0.05$), but were all higher than the previous determinations for the same groups, significantly so in the case of Groups 2 and 3 ($p < 0.05$).

During the fourth treatment period, the mean isometamidium concentration in Group 2 (11.8 $ngml^{-1}$) was significantly higher ($p < 0.05$) than that of Group 3 (5.6 $ngml^{-1}$). The mean of Group 1 (8.5 $ngml^{-1}$) fell between and was not significantly different ($p > 0.1$) from the means of either Groups 2 and 3. Only in Group 2 was the mean drug concentration in period 4 significantly greater ($p < 0.05$) than that of period 3, in spite of the deaths of two cattle (B6 and B61) during the fourth treatment period (on 26/11/91 and 30/11/91) which had the highest drug concentrations during the third treatment period. In Groups 1 and 3 the mean concentrations in period 4 were actually lower than their previous values. The deaths of four cattle in Group 1, including three (B16, B19 and B59) which had the highest drug concentrations during the previous period, should be taken into account in interpreting the data: excluding the cattle which died, there was a rise in the mean concentration in the 4 surviving cattle from 5.8 to 8.5 $ngml^{-1}$. There were no deaths in Group 3 during the period in question, and the fall in mean drug concentration could not be attributed to the effect of two animals (B26 and B68) in which no isometamidium was detectable following the fourth administration: the fall in concentration was significant ($p < 0.05$) whether or not these two animals were included in the analysis.

There was no effect ($p > 0.1$) of diminazene treatment on concentrations of isometamidium ($p > 0.1$) during any of the treatment periods during which it was determined (periods 2, 3 and 4).

Finally, cattle (Group 2) in better body condition at the start of the experiment and with better nutrition than Group 1 cattle, were not significantly different ($p > 0.1$) to them in terms of serum isometamidium concentrations, in any of the three relevant treatment periods, although by the fourth period too few animals ($n = 4$) in Group 1 had survived for a meaningful comparison.

Isometamidium concentration - body weight relationship

The relationship between isometamidium concentration and percent daily body weight change was analysed by linear regression. Weight was modelled as the response variable using the main effects and interactions of treatment

group and isometamidium concentration as explanatory variables. This analysis was conducted for each of the three periods during which isometamidium concentrations were determined (periods 2, 3 and 4). Animals not treated with isometamidium (Groups 4 and 5) were excluded from the analysis.

During the second treatment period, the relationship between isometamidium concentration and body weight change was not significant ($p > 0.1$). However during the third and fourth treatment periods the relationship was significant, and body weight change was best described by the model

constant + isometamidium concentration.Group

The model accounted for 54% and 68% of the overall variance in isometamidium concentrations during the third and fourth periods respectively. The regression coefficients, standard errors and associated probabilities are shown in Table 23. During the third and fourth treatment periods, the regression coefficients were significantly different to zero in Groups 1 and 2 ($p < 0.01$), but not in Group 3 (period 3: $p > 0.08$; period 4: $p > 0.8$). The regression coefficients for Groups 1 and 2 did not differ significantly in either period. All the significant regression coefficients ($p < 0.05$) were negative, implying an inverse relationship between isometamidium concentration and body weight change.

GLDH concentrations

All serum GLDH levels measured at the start of the experiment fell into the normal range (up to 13 ul^{-1}, Rushton 1981), except for one animal in Group 8, in which the level was marginally higher (13.8 ul^{-1}). There were no significant differences ($p > 0.1$) between the treatment groups at that time. GLDH levels in Groups 4 and 5 were never significantly elevated ($p > 0.5$) above their initial values during the experiment, nor did they differ significantly ($p > 0.5$) from each other.

During the second treatment period, GLDH levels were not significantly elevated ($p > 0.1$) above their starting values in any of Groups 1 to 5 (overall mean 6.1ul^{-1}), nor were there any significant differences ($p > 0.1$) between the groups.

During the third treatment period, elevations in GLDH levels became apparent in Groups 1, 2 and 3 (means respectively: 15.9, 21.1and 19.9 ul^{-1}). These were significantly higher than the GLDH concentrations in Groups 4 and

5 (means respectively 6.5 and 5.0 ul^{-1}; $p < 0.05$), and their initial levels ($p < 0.01$). The differences in GLDH levels between Groups 1, 2 and 3 were not significant ($p > 0.1$) during this period.

During the fourth treatment period, GLDH levels in Group 2 increased further (mean 38.4 ul^{-1}) to become significantly greater ($p < 0.001$) than their previous values, and the levels in all the other groups. The levels in Groups 1 and 3 actually decreased slightly (means respectively 11.9 and 15.8 ul^{-1}), but not significantly so ($p > 0.1$), and, of these, only those in Group 3 remained significantly elevated ($p < 0.05$) above their starting values. Again, the deaths of four cattle in Group 1, and two cattle in Group 2 need to be taken into consideration in the interpretation of the results for this period.

GLDH level - body weight relationship

The relationship between GLDH level and percent daily body weight change was analysed by linear regression in a similar way to that used for isometamidium concentrations. Weight was modelled as the response variable using the main effects and interactions of treatment group and GLDH level as explanatory variables. This analysis was conducted for the same three periods used in the analysis of isometamidium concentrations (periods 2, 3 and 4). Animals not treated with isometamidium (Groups 4 and 5) were excluded from the analysis.

During the second and third treatment periods, the relationship between GLDH level and body weight change was not significant ($p > 0.1$). During the fourth treatment period GLDH level was best described by the model:

Group constant + GLDH.Group

This model accounted for 74% of the total variance in GLDH levels during the fourth period.

The regression constants, standard errors and associated probabilities are shown in Table 24. Only in Group 1 was the GLDH regression constant significantly different to zero ($p < 0.001$). The sign of the regression constant was negative, indicating an inverse relationship between GLDH levels and body weight changes.

Isometamidium concentration - GLDH concentration relationship

The relationship between serum isometamidium concentration and GLDH levels was examined using linear regression. The response variable in the

model was GLDH level, while the terms of the model examined as explanatory variables were the main effects and interactions of isometamidium concentration, group, and treatment period. The best fitting model accounted for 37% of overall variance, and included the terms:

constant + isometamidium concentration.group + isometamidium concentration.treatment period.

The regression constants, standard errors and associated probabilities are shown in Table 25. Overall there was a significant positive relationship between isometamidium concentration and GLDH level ($p < 0.01$). No effect of isometamidium was seen during the second treatment period ($p > 0.9$), but significant effects became apparent during the third and fourth treatment periods ($p < 0.05$). The effect in the fourth treatment period was greater than that in the third, although this difference was not significant ($p > 0.4$). Finally, the effect of isometamidium concentration on GLDH levels was significantly greater ($p < 0.01$) in Group 2, from that observed in either Group 1 or Group 3.

Mortality

Overall, nine cattle died during the experiment, and a further six cattle were considered unlikely to survive because of general weakness, emaciation, collapse or recumbency, and were therefore slaughtered after 3/12/91. Four of the deaths occurred in Group 1 cattle during the second half of the third treatment period, within a period of four days (11/11/91 to 14/11/91). Four deaths occurred in Group 2, two during the first two weeks of the fourth treatment period (26/11/91 and 30/11/91), and two five to ten days later (5/12/91 and 10/12/91). The remaining death occurred ten days later still (20/12/91), in an animal in Group 3. Of the cattle which were slaughtered, two were in Group 1, and there was one in each of Groups 2, 3, 4 and 5.

The relationship between mortality, and body weight changes, isometamidium concentrations, and GLDH levels was investigated in isometamidium treated cattle (Groups 1, 2, and 3). Body weight changes over the first, second, third, and fourth treatment periods, and isometamidium concentrations and GLDH levels during the second, third, and fourth treatment periods in cattle which died were compared with those in cattle which survived. Of these comparisons, only body weight changes over the third treatment period showed a significant difference ($p < 0.001$). The mean body weight change during this period was -0.73 % per diem in cattle which died, whereas in the survivors it was -0.39 % per diem. If the four cattle in Groups 1, 2 and 3 which

were slaughtered were included in the analysis, GLDH levels during the fourth treatment period were also significantly greater ($p < 0.05$) in animals which died or were slaughtered (mean 38.3 ul^{-1}), than those which survived (mean 12.4 ul^{-1}).

Table 21. Experimental design: drug treatments

	Isometamidium:	
	+	-
Diminazene:		
+	Groups 1 & 2	Group 4
-	Group 3	Group 5

Table 22. Isometamidium concentrations in cattle following repeated treatments.

Treatment period	2	2	3	4
Date of isometamidium treatment	23/8/91	22/9/91	21/10/91	21/11/91
Date of serum collection	Not done	8/10/91	4/11/91	3/12/91
Days after treatment	-	16	14	12
Isometamidium concentrations: mean & (standard deviation)		$ngml^{-1}$	$ngml^{-1}$	$ngml^{-1}$
Group 1	-	5.3 (4.0)	8.8 (5.1)	8.5 (3.5)
Group 2	-	3.0 (1.9)	7.1 (2.7)	11.8 (4.8)
Group 3	-	7.8 (5.6)	10.4 (4.1)	5.6 (4.3)

Table 23. Regression coefficients, standard errors and associated probabilities for the linear regression of isometamidium concentrations on body weight changes

Treatment Period	Term	regression coefficient	standard error	probability
2	Group 1	-0.043	0.0090	< 0.001
	Group 2	-0.051	0.0125	< 0.001
	Group 3	-0.016	0.00851	0.083
	constant	-0.217	0.0814	0.015
3	Group 1	-0.060	0.0198	0.009
	Group 2	-0.061	0.0129	< 0.001
	Group 3	0.0041	0.0204	0.84
	constant	0.827	0.131	< 0.001

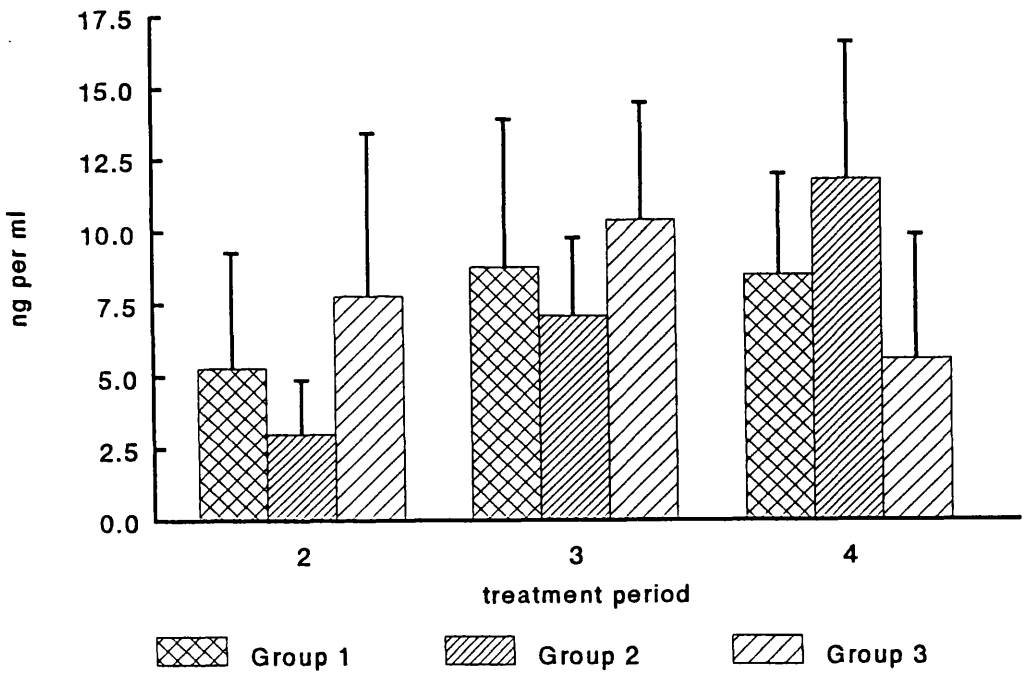
Table 24. Regression coefficients, standard errors and associated probabilities for the linear regression of GLDH levels on body weight changes during treatment period 4.

Term	regression coefficient	standard error	probability
GLDH.Group I	-0.0488	0.0141	0.005
GLDH.Group II	-0.0011	0.0033	0.742
GLDH.Group III	0.0103	0.0126	0.427
Group I constant	0.935	0.203	<0.001
Group II constant	0.111	0.157	0.491
Group III constant	0.693	0.215	0.007

Table 25. Regression coefficients, standard errors and associated probabilities for the linear regression of isometamidium concentrations on GLDH levels.

Term	regression coefficient	standard error	probability
concentration.period 2	-0.009	0.456	0.985
concentration.period 3	0.768	0.382	0.049
concentration.period 4	1.096	0.446	0.017
concentration.Group I	-0.172	0.385	0.657
concentration.Group II	1.287	0.422	0.003
concentration.Group III	NA	NA	NA
Constant	8.61	2.67	0.002

Figure 17. Mean isometamidium concentrations in sera of repeatedly treated cattle (treatment Groups 1, 2 and 3) during the second, third and fourth treatment periods. For explanation of treatment groups, see text. Error bars: standard deviation.



Discussion

In this study the use of a regimen of frequently repeated intramuscular treatments with the trypanocidal drug isometamidium chloride, with and without concurrent administration of a second trypanocide, diminazene aceturate, was investigated in Maasai Zebu cattle. Parameters measured were body weight change, levels of the hepatocellular leakage enzyme GLDH, and circulating isometamidium concentrations. The effect of body condition and nutrition on the treatment regimen involving both trypanocides was also investigated. A recently developed competitive enzyme immunoassay (CEIA) for the determination of isometamidium concentrations in bovine serum (Chapter 7) enabled circulating concentrations of the drug to be measured for the first time in the context of a possible overdose problem.

The experiment was primarily designed in an attempt to recreate a syndrome believed to be associated with repeated isometamidium and diminazene treatments in cattle under poor nutritional conditions (Dolan and Stevenson, 1992). As the experiment was apparently successful in this respect (Stevenson, *et al.*, in preparation), the relationships between the parameters listed above were investigated, in spite of some deficiencies in the data available. To repeat the experiment in order to restore these deficiencies would be difficult to justify because of considerations of animal welfare and expense.

It should be emphasised that the experiment was deliberately conducted in a location where, and during a period when, poor grazing would result in weight loss even in the absence of *heavy* trypanocidal treatment. The results, therefore, do not necessarily indicate what would happen in cattle on a more adequate plane of nutrition and in positive energy balance.

In Maasai Zebu cattle under conditions of poor grazing and negative energy balance, while a single intramuscular treatment with isometamidium chloride at a dose rate of 1.0 $mgkg^{-1}$ had no significant effect on body weight, two or more such treatments at monthly intervals caused significant increases in weight loss. The additional weight loss in isometamidium treated cattle (Groups 1 and 3) over the month following two treatments, compared to that of untreated cattle (Groups 4 and 5) over the same period was over 0.25 kg per diem. The effect of isometamidium on body weight changes was maintained following a third isometamidium treatment, whether diminazene was administered 2 days after that isometamidium treatment (Group 3) or not (Group 1). However, whether

cattle had previously received three isometamidium treatments (Group 1) or none (Group 3), diminazene itself had no significant effect on body weight.

Cattle in better body condition at the start of the experiment, with access to better grazing, and treated similarly with two doses of isometamidium (Group 2), had smaller body weight losses than either treated cattle (Groups 1 and 3) or untreated cattle (Groups 4 and 5) on poorer nutrition. The sparing effect on body weight losses afforded by better initial body condition and better nutrition was no longer apparent following three monthly isometamidium treatments and one diminazene treatment. Weight losses during the third treatment period were greater under this treatment regimen (Group 2) than in untreated controls on poorer nutrition (Group 5). By the end of the this treatment period, the mean percentage of body weight lost since the start of the experiment in Group 2 cattle (31.2%) had surpassed that of cattle (Group 1) under an identical drug regimen but with poorer nutrition and initial body condition (mean 26.5%).

During the fourth and final treatment period, body weights increased in all treatment groups, reflecting improved grazing following the onset of the rains. The comparison of the drug treatment regimens in this period was additionally complicated by the deaths of six animals, four in Group 1, and two in Group 2. The effect of four successive isometamidium treatments appeared to depend on whether diminazene had also been administered. Cattle which received only isometamidium (Group 3) had significantly higher weight gains than untreated controls (Group 5), whereas cattle given both trypanocides (Group 1) had lower weight gains than either untreated controls, or those receiving only diminazene (Group 4). The apparent growth promoting effect of isometamidium on Group 3 cattle may have been a "rebound effect" resulting from the greater weight losses experienced during the previous treatment periods when grazing was scarce.

Although there was no evidence that one or two monthly treatments with diminazene alone had any effect on body weight change, there was some evidence that two diminazene treatments following the third and fourth of four isometamidium treatments had a negative effect on body weight. Cattle under this treatment regimen, which had been in better body condition at the start of the experiment, and which had access to better grazing for the entire duration of the experiment (Group 2), had significantly lower weight gains than all other treatment groups and controls (Groups 1, 3, 4,and 5) during the fourth treatment period. The adverse effects of four monthly treatments with isometamidium, and two of diminazene, may have been more than an improvement in nutritional

status at the onset of the rains could compensate. The high levels of GLDH in this group of cattle during this period, indicating hepatocellular damage, appear to support this explanation.

Isometamidium concentrations were measured to investigate differences in concentrations between the different treatment regimens, to investigate whether there was evidence of drug accumulation, and to determine the relationship between drug concentrations and weight loss, liver damage, and survival. Differences in serum isometamidium concentrations between the various treatment groups were not observed until all four monthly administrations of the drug had been given. Isometamidium concentrations following up to three doses of the drug at monthly intervals were not influenced by initial body condition and nutritional status, nor by the additional administration of diminazene following the third of these monthly doses.

Isometamidium concentrations following four monthly doses of the drug were influenced by either initial body condition and nutritional status, or by the additional administration of diminazene shortly after the third and fourth doses of isometamidium. During the fourth treatment period, isometamidium concentrations in Group 2 cattle were found to be significantly higher than those in Group 3. The difference might have been related to the additional administration of diminazene to cattle in Group 2, or the difference in the nutritional status of these two treatment groups. Unfortunately, the comparison of isometamidium concentrations in similarly nourished cattle with and without diminazene (Group 1 and Group 3 cattle, respectively), and the comparison of cattle treated with both trypanocides with poorer or better initial body condition and nutrition (Group 1 and Group 2 cattle, respectively) were biased by the deaths of four cattle in Group 1 and two cattle in Group 2. The reduction in the sizes of the treatment groups reduced the sensitivity of statistical tests, and the results were further confounded by the non-random nature of the experimental drop-out: five of the six cattle that died had concentrations above average for their treatment groups in both previous determinations. Hence it seems reasonable to conjecture that had more cattle in Group 1 survived, the mean isometamidium concentration in that group would have been higher, and similar to that in Group 2, and therefore the difference between Groups 2 and 3 was more likely to be related to diminazene than nutritional status.

There was evidence of accumulation of isometamidium following intramuscular treatments repeated at monthly intervals. Following the third such

treatment, the mean concentrations in all treated cattle (Groups 1, 2 and 3) were higher than those following the second treatment (significant in Groups 2 and 3). In cattle treated with both isometamidium and diminazene (Groups 1 and 2), still further increases in isometamidium concentrations (significant in Group 2) still were observed in those which survived following the fourth isometamidium treatment until the date of sampling. However, in cattle treated with isometamidium alone (Group 3), there was a significant decrease in the mean isometamidium concentration over the same period, which was paralleled by a decrease in GLDH level. This was consistent with the explanation that improved nutritional status during this period permitted recovery of hepatic function which may have been part cause, and part effect, of increased elimination of the additional drug burden.

Although there was no evidence that better initial body condition or better grazing had any effect on isometamidium concentrations (Group 2 compared with Group 1), there was, however, evidence of a significant relationship between isometamidium concentrations and body weight changes during the third and fourth treatment periods, in cattle treated with both isometamidium and diminazene (Groups 1 and 2). There was no significant differences in this relationship between these groups, implying that it was not influenced by the differences in initial body condition and nutritional status.

The hepatocellular enzyme GLDH was measured as an indicator of liver damage (Mullen, 1976). Evidence of liver dysfunction was obtained in isometamidium treated cattle following three monthly doses of the drug. In all three groups of cattle given this trypanocide, GLDH levels increased significantly during the third treatment period, and, like isometamidium concentrations, GLDH levels in Group 2 cattle continued to rise during the fourth period. Diminazene administration had no effect on GLDH levels during any treatment period. A relationship between GLDH levels and body weight was less apparent than that between isometamidium and body weight. Evidence found for such a relationship was found only during the fourth treatment period, and only in one treatment group (Group 1), by which time comprised only four cattle. There was however a significant positive relationship between isometamidium concentrations and GLDH levels during the third and fourth treatment periods. The relationship was significant in all three isometamidium treated groups of cattle, and significantly greater in Group 2 than in Groups 1 or 3.

Altogether nine cattle died during the experiment, of which all had been treated with isometamidium, and eight also with diminazene. Unsurprisingly, cattle which died following the fourth isometamidium treatment had lost more weight over the previous (third) treatment period than those which survived. The same number of cattle (four) died in each of the two groups treated with both trypanocides (Groups 1 and 2), a mortality rate of 50%. Nutritional status and initial body condition did not, therefore, appear to influence the mortality rate, although deaths occurred slightly later (26/11/91 to 10/12/91) in cattle with better initial body condition and nutritional status (Group 2) than those without (Group 1; 11/11/91 to 14/11/91). Neither isometamidium concentrations nor GLDH levels during the third treatment period were significantly different in cattle which subsequently died to those which survived. When animals which were slaughtered because of general weakness, emaciation, collapse or recumbency were included in the analysis with those which died, GLDH levels, but not isometamidium concentrations, during the fourth treatment period were significantly greater in cattle which died or were slaughtered than in the survivors.

Weight loss and mortality was observed in improved Boran steers at Galana Ranch, Kenya treated with three doses (1.0 $mgkg^{-1}$ body weight), and between 1 and 3 doses of diminazene (7.0 $mgkg^{-1}$ body weight) over a five month period of poor grazing (Dolan *et al.*, 1992). Although the diminazene treatments were administered on the basis of diagnosis of trypanosome infections in individual steers, other infected steers treated with diminazene, which were not on isometamidium prophylaxis, did not loose weight, and suffered significantly lower mortality. The study described here has shown that trypanosome infections are not an essential factor for weight losses or death to occur in cattle under nutritional stress treated frequently with these trypanocides.

Elevated GLDH levels were regarded as evidence of hepatotoxicity as a mechanism for the weight loss and mortality associated with these frequent treatments. Other phenathridinium derivatives (homidium, prothidium and metamidium) have been shown capable of capable of causing liver damage at doses higher than the therapeutic level (Williamson 1970). The liver has been shown to be one of the main sites of accumulation of isometamidium in cattle (Kinabo and Bogan 1988a). While there was no evidence of increases in either γ-glutamyl transferase (γ-GT) or aspartate aminotransferase (AST) in cattle

following intravenous administration of isometamidium (Schillinger *et al.*, 1985), significant increases in γ-GT and alanine aminotransferase were found in pigs following large toxic intramuscular doses of the drug (Kinabo *et al.*, 1991). In the present study, in spite of elevations in GLDH, there was no evidence of elevation in γ-GT levels (data not shown), findings consistent with hepatocellular rather than hepatobiliary pathology.

The ELISA techniques for the determination of isometamidium concentrations in bovine serum described in Chapters 3 and 6 have been used to investigate the relationship between circulating concentrations and protection against tsetse transmitted trypanosomiasis challenge (Chapter 4 and 7). The mean concentration of the drug found in the circulation of Boran cattle under laboratory conditions one month after intramuscular administration of a dose of 1.0 $mgkg^{-1}$ was 6 $ngml^{-1}$ (range 3 to 12 $ngml^{-1}$). In the present study concentrations were determined to investigate concentrations of the drug in Maasai Zebu cattle under conditions of poor grazing, following frequent repeated intramuscular treatments at the same dose rate. Although samples were not collected for isometamidium determination after a single dose of the drug, two weeks after two, three or four similar doses, the concentrations found were not dissimilar to those observed in the laboratory experiment at the same interval after treatment. Although there were no obviously marked elevations in concentration which might have allowed the use of the CEIA as a predictor of a potential toxicity problem, the small but significant increases in concentration which occurred with successive treatments did suggested drug accumulation, and should be considered in the context of the low therapeutic index of isometamidium (Kinabo and Bogan 1988b).

In this study the CEIA for isometamidium has been successfully used to investigate the relationship between circulating concentrations of the drug, and a syndrome associated with repeated trypanocidal treatments. As few as two isometamidium treatments one month apart were shown to be associated with significant weight loss in cattle under conditions of poor nutrition. Cattle treated with diminazene aceturate after three or four isometamidium treatments suffered a 50% mortality rate. Isometamidium concentrations in cattle treated repeatedly at monthly intervals increased significantly with the number of treatments administered, and were significantly higher in cattle to which diminazene had also been administered. In cattle treated with both trypanocides, body weight losses and GLDH levels were correlated with isometamidium concentrations.

These observations support the hypothesis that the syndrome observed was related to the use of repeated doses of isometamidium in conjunction with diminazene aceturate, and the pathogenesis involved a component of hepatic damage. On the basis of these findings it is recommended that cattle, particularly under nutritional stress, are not subjected to repeated treatments with isometamidium at intervals a short as one month, and where frequently repeated treatments with isometamidium are considered unavoidable, the concurrent use of diminazene should be avoided.

Chapter 11:
General Discussion

Summary of achievements

In the work described in this thesis two new enzyme-linked immunosorbent assay (ELISA) procedures for the detection and quantification of isometamidium in bovine serum were developed. The first of these assays was an indirect competition ELISA (ICE) and was capable of detecting isometamidium to approximately 0.1 $ngml^{-1}$. Following the treatment of cattle with isometamidium chloride by intramuscular injection at the recommended prophylactic dose rate of 1.0 $mgkg^{-1}$, the drug could be detected in serum samples for up to four months.

In experiments described in Chapters 4 and 5, the concentrations of isometamidium in the circulation of Boran cattle treated with isometamidium were related to the ability of the cattle to withstand tsetse challenge with populations of *T. congolense* of various degrees of resistance to the drug. These experiments were conducted to enable the interpretation of isometamidium concentration data obtained using serum samples from cattle in the field. If cattle in the field are found to be harbouring *T. congolense* infections in spite of measurable isometamidium concentrations greater than those found to be protective against the drug-sensitive *T. congolense* clone IL1180, these trypanosome infections may be considered to possess a degree of resistance to the drug; the higher the isometamidium concentration, the greater the degree of drug resistance which may be inferred. *T. congolense* which are established in the presence of circulating drug concentrations greater than 6 $ngml^{-1}$ may be considered to be markedly resistant, by comparison to the two highly isometamidium-resistant *T. congolense* populations IL 3893 and IL 3889 which were able to establish break-through infections at this drug concentration (Chapter 4). Similarly *T. congolense* which are able to establish infections at concentrations greater than 2 $ngml^{-1}$ may be considered moderately resistant by comparison with the moderately isometamidium-resistant *T. congolense* population IL 3343 (Chapter 5). *T. congolense* which are able to establish infections in the presence of concentrations greater than 0.4 $ngml^{-1}$ may be considered to possess a low level of drug resistance, since this concentration was protective against challenge with the drug-sensitive clone *T. congolense* IL 1180 (Chapter 5).

The ICE was used to examine serum samples collected on a regular basis from a herd of Jersey cattle in coastal Kenya in which trypanosome infections occurred in spite of an isometamidium chemoprophylactic regimen. The study

provided an opportunity to use the ELISA on sera from cattle under natural tsetse challenge, and to evaluate its potential as a tool for discriminating genuine break-through infections by drug-resistant trypanosomes from failure of prophylaxis due to inadequate drug levels. Isometamidium concentrations could be determined in the samples, and an attempt was made to relate the isometamidium concentrations to the incidence of trypanosome infection over a number of three month prophylactic periods. The distribution of trypanosome challenge over the period of the study was uneven, and it was perhaps unfortunate, both for the cattle and the purposes of this investigation, that almost all challenge (based on the infection rate in a control group of cattle not given isometamidium) occurred at the end of prophylactic periods. Unsurprisingly therefore, at the time of challenge, drug concentrations in the treated group were at their lowest and no inferences could be made about drug resistance in the infecting trypanosomes on the basis of the results obtained using *T. congolense* in the laboratory studies described in Chapters 4 and 5. In fact, all infections diagnosed during in this study were caused by *T. vivax* which is possibly inherently less sensitive to the prophylactic activity of isometamidium (Peregrine *et al.*, 1987, 1991). It should be emphasised that failure to demonstrate drug resistance using the ELISA was not due any failure of the ELISA *per se*; had serum samples been collected during the first prophylactic period, in which trypanosome infections occurred relatively soon after treatment, a rather different result might have been obtained.

Another simpler ELISA procedure for the determination of isometamidium in bovine serum was developed using isometamidium-enzyme conjugates (Chapter 7). This assay, described as a competitive enzyme immunoassay (CEIA) had a number of advantages over the ICE in terms of reagent preparation, assay procedure, and day to day variability in absorbances. The purification of immunoglobulin from whole serum is not required, and there are fewer steps to the assay. The method was shown to be highly repeatable and reproducible, and gave results similar to those obtained with the ICE. It was however slightly less sensitive, with a detection limit of approximately 0.5 $ngml^{-1}$.

The CEIA was used to investigate the pharmacokinetics of isometamidium in Friesian cattle (*Bos taurus*) treated via the intravenous and intramuscular routes at a dose rate of 1.0 $mgkg^{-1}$ body weight (Chapter 8). The major pharmacokinetic parameters were calculated using non-linear curve-fitting of

standard pharmacokinetic equations. The large volume of distribution at steady state (mean 24 lkg^{-1}), the prolonged mean residence time (83 h) and the terminal phase half-life (136 h) following intravenous injection were consistent with extensive uptake of the drug into specific tissues such as liver, kidney and spleen. The relatively low bioavailability (60%), and prolonged mean absorption time (7.8 days) following intramuscular injection was consistent with formation of a primary depot at the site of injection due to binding of isometamidium to tissue macromolecules and ground matrix components.

The ability of the CEIA to quantify isometamidium concentrations in serum samples taken from cattle under field conditions in Africa was evaluated in an experiment conducted at the Rekomitjie Research Station in a tsetse-infested area of the Zambezi valley, Zimbabwe. Twenty-four cattle were treated with a prophylactic dose of isometamidium chloride (1.0 $mgkg^{-1}$ body weight) and exposed to natural tsetse challenge. Isometamidium could be detected in the sera of all but one of the 24 treated cattle for up to 14 weeks following treatment; drug was detectable in 22 cattle for eight weeks, in 20 cattle for 10 weeks, in 12 cattle for 12 weeks and in five cattle for 14 weeks following treatment. The geometric mean apparent half-life of isometamidium in 22 individual cattle was 23.2 days (range 15.7 to 58.3 days). Although trypanosome challenge was judged to be a low to medium on the basis of infection rates in untreated control cattle, few trypanosome infections occurred in the prophylaxis group. Infections were not detected in any of the isometamidium-treated cattle for 18 weeks following treatment, after which three infections were detected. By this time isometamidium concentrations had fallen below the limit of detection of the CEIA (0.5 $ngml^{-1}$), and there was no evidence therefore of drug resistance at this site.

Finally, the CEIA was used to investigate a possible role for the isometamidium in the pathogenesis of a wasting syndrome induced experimentally in Maasai Zebu cattle under a regimen of frequently repeated trypanocidal drug treatments. Other parameters measured were body weight change and the hepatocellular leakage enzyme Glutamate dehydrogenase (GLDH). The effect of body condition and nutrition on a treatment regimen involving two trypanocides was also investigated. As few as two isometamidium treatments one month apart were shown to be associated with significant weight loss in cattle under conditions of poor nutrition, and cattle treated with diminazene aceturate after three or four isometamidium treatments suffered a

50% mortality rate. In cattle treated with both trypanocides, body weight losses and GLDH levels were correlated with isometamidium concentrations. Although there were no obviously marked elevations in isometamidium concentration which might have allowed the use of the ELISA as a predictor of a potential toxicity problem, small but significant increases in concentration occurred with successive treatments. These suggested accumulation of the isometamidium may have occurred; this should be considered in the context of the drug's low therapeutic index. It was therefore recommended that such frequently repeated treatment regimens be avoided wherever possible.

Recommendations for further scientific investigation

Protection against *T. vivax*

There are, as yet, no data available to relate isometamidium concentrations to protection against *T. vivax*. There have been fewer controlled laboratory studies on the prophylaxis afforded by isometamidium to cattle against challenge by *T. vivax*, although the limited evidence available suggests *T. vivax* may be less sensitive to the prophylactic activity than the therapeutic activity of the drug (Peregrine *et al.*, 1987, 1991). Isometamidium prophylaxis experiments in cattle under controlled laboratory conditions, similar to those described in Chapters 4 and 5, with tsetse challenge using isometamidium-sensitive and resistant *T. vivax* populations should therefore be given a high priority among future investigations.

Influence of level of challenge on duration of prophylaxis

It has often been suggested on the basis of field observations, that the duration of prophylaxis afforded by isometamidium is dependant upon the level of tsetse challenge (Fiennes, 1953; Davey,1957; Whiteside, 1962a). However, challenge experiments using intradermal titrated doses of metacyclic forms of *T. congolense* IL1180 failed to demonstrate any relationship between the duration of isometamidium prophylaxis and the level of challenge (Whitelaw *et al.*, 1986; Peregrine, *et al.*,1988). These experiments did not represent the field situation closely, firstly, because experimental challenge with metacyclic trypanosomes took place on a single occasion (Whitelaw *et al.*, 1986) or on a single day each month (Peregrine *et al.*, 1988), and secondly, because a single clone of *T. congolense* was used for challenge, whereas in the field, challenge by a wide variety of strains of trypanosomes of the three species pathogenic to cattle

would be expected. Nevertheless, it is difficult to envisage why the duration of prophylaxis should be related to the level of challenge.

In the work described in Chapters 4 and 5, there was some evidence of a potential interaction between trypanosome infection and the circulating concentrations of isometamidium in cattle which appeared to decline more rapidly in infected animals. However, this phenomenon was only observed after the onset of parasitaemia caused by resistant trypanosomes and therefore it would not appear to explain how a high level of challenge might overwhelm prophylaxis. Moreover, the duration of isometamidium prophylaxis against *T. congolense* IL 1180 observed when the drug was administered to cattle already infected with an unrelated serodeme was the same as observed in similarly challenged cattle which had not previously experience trypanosome infection (Peregrine *et al.*, 1988). In this situation, trypanosomes would have been exposed to the initial peak in concentration following intramuscular isometamidium administration (range approximately 40 - 200 $ngml^{-1}$, Chapter 8), so that the proportion of the overall circulating drug taken up by the trypanosomes might be expected to be significantly less than that taken up during later stages of the prophylactic period, and that isometamidium which was taken up by these trypanosomes would be expected to be released on lysis of the parasites.

There is evidence that effective isometamidium prophylaxis prevents the entry of viable trypanosomes into the circulation (see below). Although trypanosome multiplication has been demonstrated at the site of the tsetse bite (Luckins and Gray, 1978; Akol and Murray, 1982), it is difficult to imagine a biomass of trypanosomes at this location sufficient that it could significantly influence the pharmacodynamics of isometamidium in an animal as large as an ox, and thereby reduce the prophylactic period afforded by the drug.

Drug concentrations in tissue fluid

Trypanosome challenge which is prevented from establishing infection because of isometamidium prophylaxis appears not to progress beyond the location of the tsetse-fly bite in the skin; skin reactions (chancre), enlargement of local drainage lymph nodes, and the development of measurable antibody responses did not occur in cattle protected by isometamidium against tsetse challenge or intradermal metacyclic challenge with *T. congolense* IL 1180 (Whitelaw *et al.*, 1986; Peregrine *et al.*, 1988). In view of the site of prophylactic action of isometamidium being apparently located at the fly bite,

investigations of the relationship between isometamidium concentrations in tissue fluid and those in the circulation would be particularly interesting. Possible methods for such investigations (tissue cages or lymphatic cannulation) were suggested in Chapter 8. Of critical importance would be whether circulating drug concentrations are representative of skin tissue fluid concentrations, and whether the relationship between drug concentrations in these two locations was consistent or variable among animals.

Endogenous drug binding and extraction

The extraction of an analyte from a biological matrix often results in the measurement of only a proportion of the total analyte present in the matrix. This is usually described as the free fraction, to distinguish it from the remainder which is bound to a macromolecular element of the matrix (endogenous binder). The bound and free fractions normally exist in a state of dynamic equilibrium dependant on the dissociation constant of the interaction with the endogenous binder. The concentration of free analyte is frequently of greater significance than the total, as this may be the fraction having biological activity; for example many drugs and hormones can interact with their receptors only in the free state.

Isometamidium is believed to be highly bound to serum macromolecular components such as albumin (Philips *et al.*, 1967; Smith *et al.*, 1991), and this binding probably contributes to the sample matrix effects seen in the indirect competition ELISA (Chapter 3) and the competitive enzyme immunoassay (Chapter 7). When used therapeutically, the principal interaction of isometamidium with trypanosomes probably takes place in the circulation, at least in infections with *T. congolense* and *T. vivax*. It is not certain to what extent the bound fraction of drug is involved in this interaction, or whether the free fraction alone is taken up by trypanosomes. In *T. brucei* infections, and more importantly, in chemoprophylaxis, the interaction of isometamidium with parasites outside the circulation will be important; drug concentrations in these extravascular sites will be likely to be related to the free drug concentrations in the circulation, but will also depend upon the mechanism by which isometamidium exits the capillaries.

The ELISAs for isometamidium were developed with a view to their use in laboratories in African countries, and the inclusion of an extraction step was considered undesirable on the grounds of the additional materials, equipment, technical expertise and expense involved. However, an extraction procedure

could assist in quantifying macromolecular binding of isometamidium in bovine serum, and would remove the analyte from interfering substances in the sample matrix. This might allow the evaluation of effects of endogenous binding and other matrix components on the precision and accuracy of assays without an extraction step. An extraction procedure would also be an important stage in the preparation of tissues samples for estimation of isometamidium residues; although outside the scope of this thesis, the determination of residues in animal products intended for human consumption could prove to be an important use of the isometamidium ELISAs.

Some preliminary attempts to extract isometamidium from bovine sera have already been made. The method of partitioning drug into inorganic solvents immiscible with water, following which, the solvent phase is dried down and reconstituted in a defined, matrix-free, aqueous assay buffer, is widely practised in steroid hormone immunoassay (Chard, 1990). This methodology was found to be unsuitable for use with isometamidium which, being highly polar, is generally insoluble in solvents which are immiscible with water. An alternative technique was investigated, utilising the property of certain water-miscible, polar organic solvents such as methanol, ethanol and acetonitrile to precipitate serum proteins (data not shown). Following addition of such polar solvents samples containing ^{14}C-isometamidium could be centrifuged and the quantity of isometamidium in the supernatants determined measured by liquid scintillation counting (Tri-Carb 1600-TR, Packard, Groningen, the Netherlands).

Recoveries of ^{14}C-isometamidium in supernatants over a fairly wide range of concentrations (10 - 160 ngml^{-1}) were approximately 30% using methanol precipitation, and 70% using acetonitrile precipitation. Consideration of the law of mass action suggests that this plateau in percentage binding would continue at lower drug concentrations (Goldstein, Aranow and Kalman, 1974), although this could not be verified experimentally because of the limited specific activity of the labelled drug. On redissolving methanol precipitates in aqueous buffer, the presence of 60 to 65% of the non-recovered isometamidium could be demonstrated. Unlike methanol precipitates, acetonitrile precipitates could not be redissolved readily in aqueous buffer, and hence the higher recovery of drug (and lower apparent percentage binding) obtained using acetonitrile might be accounted for by denaturing of serum proteins. The methanol precipitation data were thus considered to be the better estimate of reversible drug binding occurring *in vivo*, and were relatively consistent with data obtained by Philips *et*

al. (1967) which showed 66% to 74% of isometamidium was bound in solutions of 0.2% bovine serum albumin.

The relatively high recovery of isometamidium in supernatants obtained following using acetonitrile precipitation suggested that this might be a suitable extraction method use with ELISA for estimation of total drug. Accordingly, these supernatants evaporated to dryness, and reconstituted in suitable buffer. Acetonitrile extracts tested in the ICE for isometamidium gave quantitative responses over a range of concentrations. However, following precipitation of drug-free normal bovine serum and reconstitution of the dried down extract in assay buffer there was attenuation of the absorbance obtained in the ICE, when compared with the absorbance obtained with buffer alone. This implied that during the extraction procedure there was carry over either of matrix components capable of interfering in the ICE, or acetonitrile. Because of the absorbance quenching effect the extraction procedure showed no advantage over the original assay methodology described in Chapter 3. The use of these extracts in the CEIA could be tested but would be expected to have similar drawbacks.

Solid-phase extraction methods are being used increasingly for sample clean up prior to immunoassay and HPLC analyses. The extraction of isometamidium from bovine serum using substituted silica solid-phase cartridges was used successfully in the development of an HPLC system for isometamidium (Kinabo and Bogan, 1988a) and further investigations in the use of this sample preparation technology with the isometamidium ELISAs are clearly warranted.

An alternative strategy to preparation of biological samples for immunoassay by the use of extraction procedures is the so called "direct" approach in which the analyte is assayed within the biological matrix in which it was collected. Such direct assays avoid the inconvenience and expense of an additional sample extraction procedure, and both the bound and free fractions may be measured, although the extent to which the bound fraction is measured in an immunoassay will depend on whether the endogenous binder competes with binding by the specific antibody reagent, and on the relative affinities of the two forms of binding. The two ELISAs for isometamidium described in this thesis fall into this category.

In order to increase the proportion of bound drug measured in a direct, unextracted immunoassay, another ligand with high affinity for the endogenous

binder, but not the specific antibody may be added to displace the analyte from its binding site (Ratcliffe, 1983). In an ELISA for melarsoprol, chlorophenoxyisobutyric acid was used to displace the drug from human serum albumin (Maes *et al.*, 1988). In preliminary experiments neither this agent, nor aspirin, phenylbutazone, danazol or 8-anilino-1-naphthalene sulphonic acid (Ratcliffe, 1983) influenced the position of displacement curves in the isometamidium ELISAs. This suggested that either none of these agents were capable of displacing the drug from the endogenous binder, or that the ELISAs already measure the total drug concentration. The latter explanation is quite plausible in view of the high affinity of the specific antibodies used. Further investigations to confirm this would of course be warranted.

Other supporting work

Further investigations in a number of areas related to the use of the isometamidium ELISAs would support their continued development and application. In many of these areas some preliminary work have already been conducted during the preparation of this thesis.

In the work described in this thesis, ELISAs for isometamidium were developed for the investigation of concentrations of the drug in serum rather than whole blood or plasma. The rationale for this was that samples of serum are easiest to collect under African conditions. Anticoagulant is not required for the collection of serum, whereas for plasma or whole blood samples use of one of various possible anticoagulants is essential. The quality, quantity, type and availability of anticoagulants used are all extra variables over which the laboratory investigator would have little control. In addition, a centrifuge is desirable, but not essential for the preparation of serum, as clots may be removed manually. Centrifugation is essential for the preparation of plasma samples, and must be performed within a relatively short period of sample collection if haemolysis is to be avoided. In this respect, blood samples collected for serum preparation are relatively more robust. The possibility of successfully establishing serum banks in Africa from cattle in the field has been proven under programmes such as the Pan-African Rinderpest Campaign. Finally, the trypanosome antigen ELISA uses serum samples, and it would clearly be useful if the same sample could be analysed both for evidence of trypanosome infection, and for the presence of isometamidium.

The above notwithstanding, investigations to determine whether there are differences between isometamidium concentrations in serum and plasma would

be warranted. Differences in levels between serum and plasma might be due to binding of isometamidium to fibrinogen or other plasma proteins which are involved in the clotting process. If binding of isometamidium to fibrinogen occurs, the increases in fibrinogen levels that have been shown to occur in cattle in response to many inflammatory conditions could have a significant effect on drug determinations. Similarly, disseminated intravascular coagulation which has been proposed as an important pathogenic mechanism in bovine trypanosomiasis (Forsberg, Valli, Gentry and Donworth, 1979) and in which circulating fibrinogen levels are often severely depleted could be influential.

Theoretical considerations suggest that there may be loss of the positively charged quaternary ammonium compound isometamidium during storage in glass containers, through ionic absorption onto negatively charged silanol groups of the glass. Perschke and Vollner (1985) avoided the use of glass containers for storage of isometamidium solutions for this reason. The recovery of ^{14}C-labelled isometamidium following short term storage of solutions of the drug in various media and containers was investigated to enable the validity of various experimental procedures to be determined (data not shown). It was found that the recovery of isometamidium, measured by liquid scintillation counting (Tri-Carb 1600-TR), in phosphate buffered saline (PBS) was less than 50% even after very brief periods (a few hours) of storage in polyethylene flip-top tubes (Eppendorf). The addition of 20% normal bovine serum (NBS), or 0.1% gelatin to the PBS improved recovery to between 95% and 100% and such solutions could be stored for at least 10 days without significant loss in recovery. In addition recovery of isometamidium solutions stored in glass tubes was better than in the plastic Eppendorf tubes. The improvement in recovery obtained with NBS or gelatin is probably in part due to the fact that the drug has greater affinity for these macromolecules than for the walls of the container, and in part due to a blocking effect of these proteins on the binding sites of the glass or plastic.

In accordance with these results, the immunoassay studies described in this thesis were designed to avoid the need to prepare and store isometamidium in aqueous solution without the addition of proteins such as serum or gelatin; if preparation of such solutions was necessary glass rather than plastic containers were be used, and the solutions were used immediately, without storage. Bovine sera containing isometamidium are unlikely to suffer problems of poor recovery because of the presence of serum proteins such as albumin.

Phillips et al. (1967) found that isometamidium bound to, but did not pass through dialysis tubing. Nevertheless dialysis of isometamidium has been used in a number of experimental protocols including preparation of isometamidium-protein conjugates (Chapter 2). A few simple experiments were conducted to investigate whether dialysis of isometamidium is effective under various conditions. These confirmed that dialysis of isometamidium is only partially effective, and is less effective at higher concentrations of the drug. A difference in colour is apparent visually between the dialysate and the starting material; whereas solutions prepared from Samorin® have a characteristic dark reddish-brown colour, the dialysate is purple. This suggests dialysis may favour particular constituent isomers of the product, especially the purple isomer. Other methods, such as gel filtration and use of dextran-charcoal have been shown to be more effective in the removal of free drug from enzyme-conjugates (Chapter 2).

A radioimmunoassay (RIA) method for isometamidium using ^{14}C-isometamidium tracer and charcoal separation of the bound and free fractions was described by Kinabo and Bogan (1988c). For various reasons, particularly the lack of sensitivity of the method, and the lack of availability of ^{14}C-labelled drug it has not found widespread use. However, RIA remains a useful tool in the development of ELISA techniques because it permits the determination of the bound and free fractions of the tracer, with which information Scatchard analysis may be used to determine affinities of antibodies in various hyperimmune sera and immunoglobulin preparations. In contrast, the configuration of ELISA generally precludes the use of these analyses. RIA thus has a potential role as a reference method with which reagents for use in the ELISA may be evaluated quantitatively.

A new RIA for isometamidium has been developed, incorporating several improvements over the earlier version (Kinabo and Bogan, 1988c). These improvements include the use of superior isometamidium conjugates and hyperimmune sera produced during the development of the isometamidium ELISAs, and a superior better method of separating bound and free drug. The new radioimmunoassay method used ^{14}C-isometamidium, hyperimmune sheep anti-isometamidium IgG, and a polyethylene glycol separation step, and was successful in detecting and quantifying isometamidium (data not shown). Scatchard analysis of the data was used to estimate the affinity of antibodies in hyperimmune sheep serum at approximately $10^{8.5}$ $lmol^{-1}$.

The studies on isometamidium described in this thesis were conducted using the commercial product, isometamidium chloride (Samorin®, RMB Animal Health Ltd.) for the treatment of cattle, and the production of reagents (specific antibody, plate-coating conjugates, enzyme conjugates and assay standards) for use in the immunoassay of the drug. Samorin®, however, is a mixture of similar compounds (see Chapter 2); the commercial product contains approximately 70% of a red isomer (i.e. true isometamidium chloride), 20% of a purple isomer, 10% of a bis species, small quantities of a positional isomer, and traces of homidium (J. Wilkes, personal communication). The most abundant, red isomer has also been shown to be the one possessing the greatest trypanocidal effect against *T. congolense* both *in vivo*, in prophylactic and therapeutic trials in mice (Wragg, Washbourne, Brown and Hill, 1958), and *in vitro* (A.S. Peregrine, personal communication), using a photometric growth inhibition assay (Zinsstag, Brun and Gessler, 1991).

The reactivities of the individual constituent isomers of the commercial product isometamidium chloride has been tested (result not shown) in the competitive enzyme immunoassay for isometamidium described in Chapter 7. Individual purified isomers were shown to react to differing degrees in the CEIA on a weight basis. The red isomer, true isometamidium, reacted to the greatest extent, and was shown to be the constituent responsible for the greater part of the response observed with whole Samorin® in the CEIA. Thus, of the compounds present in Samorin®, the CEIA appears to have the greatest sensitivity for the one which is both the most abundant and the most trypanocidal. The less trypanocidal constituents are present in lesser quantities (together representing only approximately 30% of the total), and those amounts which are present are poorly detected by the CEIA. The next best detected constituent, the positional isomer, is approximately five times less reactive (by comparison of drug concentrations resulting in 50% competition), and the purple isomer ten times less reactive than the red isomer on a weight basis. The bis-species shows comparatively little cross-reactivity, approximately 1000 times less than the red isomer.

The use of monoclonal antibodies as primary immunoassay reagents offers certain theoretical advantages in terms of continuity of supply of a reagent of defined specificity. Two murine monoclonal antibody secreting cell-lines were raised against isometamidium-porcine thyroglobulin conjugate (Peregrine, Eisler, Katende, Gault, Flynn, Kinabo, and Holmes, In press). Competition

ELISAs were developed using these antibodies, but were less sensitive than the ELISAs using polyclonal hyperimmune sheep serum. Similarly, no advantage in terms of assay specificity was obtained. In fact good high titre hyperimmune sera are preferred by many investigators in the immunoassay field because of their greater stability, and generally higher affinity. In competitive immunoassays, as opposed to sandwich-type assays (which are not generally applicable to molecules as small as isometamidium), the law of mass action dictates that the sensitivity of the assay will depend upon the affinity of the antibody used. Obtaining a satisfactory monoclonal antibody secreting cell line is to some extent a question of luck as resources normally allow only a finite number of candidate hybridomas to be screened. The production of monoclonal antibody based immunoassays for isometamidium could therefore be considered further, but with no guarantee of success.

Genetic and environmental influences on the efficacy of isometamidium prophylaxis

Isometamidium prophylaxis may be used in cattle over a very large area of Africa, encompassing some 37 countries affected by bovine trypanosomiasis. There are accordingly a wide variety of cattle breeds which may be involved. The effect of the breed of cattle on the disposition kinetics of isometamidium could therefore be an important factor in the efficacy of chemoprophylaxis in different locations. Most obviously, there is the difference at species level, between the *Bos taurus* breeds of trypanotolerant cattle which are reared in much of West and Central Africa, and the Zebu-type *Bos indicus* cattle also found in West and Central Africa and which predominate in the rest of the continent. Although trypanotolerant cattle are so called because of a degree of genetic resistance to the disease, they are nevertheless susceptible to infection and may be severely affected in high challenge situations, unless their genetic resistance to disease is supplemented by the use of trypanocidal drugs (Roelants, 1986; Otesile, Akpokodje and Ekwuruke, 1991). There may also be differences within each of the two species of cattle between the many different breeds. Some differences may be physiological, based on size, weight, and body composition, and other s related to unknown genetic factors. Other differences may be related to the physiological status of individual animals, such as age, sex, growth rate, gestation, lactation and nutritional status, or to the effects of other diseases or parasites. These factors all merit further investigation.

Recommendations for application of the isometamidium ELISAs in Africa

The development of the ELISAs for the determination of isometamidium in bovine serum has provided a valuable new tool for the improvement of the chemoprophylactic control of African bovine trypanosomiasis. Chemoprophylactic drug dosage regimens can now be based on a understanding of the levels of drug achieved in the circulation of treated animals, rather than on the empirical basis used hitherto. Furthermore, the ELISAs appear to be promising as a technique to investigate the appearance of resistance to isometamidium in the field, by allowing treatment failure to be distinguished from true drug resistance as a cause of apparent drug failure. The simultaneous demonstration in the same animal of trypanosome infection and levels of isometamidium which would under normal circumstances be considered protective, would provide strong circumstantial evidence that drug resistance had developed. In such a situation the use of a "sanative" drug, i.e. diminazene aceturate, in an attempt to eliminate isometamidium-resistant parasites, and the integrated deployment of tsetse control techniques, of which the most promising and environmentally sound currently appear to be the use of insecticide impregnated traps and targets and the application of insecticides onto cattle, should be considered.

Extension of the technology through international programmes

The provision of the trypanocidal drug technology to African veterinary laboratories concerned with the diagnosis and control of bovine trypanosomiasis is envisaged through the programmes of international organisations such as FAO and IAEA. Between 1987 and 1992 a Co-ordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture successfully implemented the trypanosomiasis Ag-ELISA in Veterinary laboratories in ten sub-Saharan countries (Ooijen, 1993). The equipment necessary for conducting ELISAs, and training in its use was given to a large number of participating scientists and technicians. This Programme has now entered a second phase, involving an even greater number of countries, and it is hoped that during this phase trypanocidal drug ELISAs will be distributed, in kit form, to suitable laboratories. With the financial assistance of the ODAs Animal Health Programme, this transfer of technology has already commenced in the case of the Kenya Trypanosomiasis Research Institute, and

plans are underway to do likewise in laboratories in Tanzania and Zambia in the near future.

General List of Abbreviations

Ab	Antibody
ADI	Acceptable daily intake
B_0	Absorbance at zero dose
BCT	Phase contrast / buffy coat technique
CEIA	Competitive enzyme immunoassay
CV	Coefficient of variation
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
GLDH	Glutamate dehydrogenase
HCT	Haematocrit centrifugation technique
HPLC	high performance liquid chromatography
ICE	Indirect competition ELISA
Ig	Immunoglobulin
IM	Intramuscular
IV	Intravenous
Mab	Monoclonal antibody
MRL	Maximum residue level
NBS	Normal bovine serum
OD	Optical density or absorbance
PBS	Phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
PTG	Porcine thyroglobulin
SA-HRP	Streptavidin-horseradish peroxidase
TMB	Tetramethylbenzidine

List of Pharmacokinetic Abbreviations

AUC	Area under the concentration-time curve
AUMC	Area under the moment curve
Cl	Clearance
C_{max}	Maximum concentration
C_{zero}	Concentration at time zero
F	Bioavailability
K_{21}	Rate constant for movement of drug from compartment two to compartment one of the three compartment pharmacokinetic model. K_{31}, K_{10}, K_{12} etc. likewise, such that K_{xy} is the rate constant for movement of drug from compartment x to compartment y.
MAT	Mean absorption time
MRT	Mean residence time
RIA	Radioimmunoassay
$T_{½}$	Half-life
T_{max}	Time to maximum concentration
V_{CC}	Volume of central compartment
V_{SS}	Volume of distribution at steady state

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