AFRICAN ANIMAL TRYPANOSOMIASIS : IMMUNOCHEMICAL STUDIES OF THE TRYPANOCIDE, ISOMETAMIDIUM CHLORIDE

by

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DECLARATION

I, Thomas Masselle Mayombo do hereby declare that the work presented in this thesis is original, was carried out by me and has not been presented for an award of a degree in any other University.

Date: 29th MAY 1990

DEDICATION

This work is dedicated to my sons Kurwa and Dotto, my daughter Sizya Gudilla, their mother Y.B. Mauki, and all those whose work and ideas are aimed at controlling and eradicating trypanosomiasis.

SUMMARY

African animal trypanosomiasis affects about half of the habitable land in Africa hampering improvements in livestock production and mixed agriculture. Though the annual economic losses have been difficult to quantify, the disease is a major economic handicap to the continent and denies its ever-growing population much needed food and livestock products.

In this study, chapter one provides a review of African animal trypanosomiasis with regard to its aetiology, hosts, geographical location and economic importance, life cycle and transmission, pathogenesis, clinical signs, diagnosis and control. Of the various methods which are employed in controlling the disease, chemotherapy and chemoprophylaxis remain the most widespread. However, the trypanocidal drugs in use today have been used for over 30 years and problems such as drug resistance appear to be increasingly reported.

The remaining chapters deal with various immunochemical studies of isometamidium chloride, the main trypanocidal drug used in the treatment and control of the disease. In chapter two, details of purifying an anti-isometamidium antibody produced in sheep and its use in the quantification of isometamidium chloride in sera of treated cattle by enzyme linked immunosorbent assay (ELISA) are given. The levels of free and bound isometamidium in sera of treated cattle were assessed by employing trichloroacetic acid precipitation and ultrafiltration methods. It was found that, in cattle treated by an

intramuscular injection of isometamidium chloride, about 95-99% of the circulating drug is bound to serum proteins and very little if any of it is free and unbound.

In chapter three, details of a "Chemiluminescent Western-blot technique" are given. This technique was used in the characterisation of the serum proteins that bind isometamidium. These proteins were shown to be about 122, 140 and 155 kD in cattle and about 300 kD in mice and goat. The major isometamidium binding proteins in cattle appear to be serum albumin. The significance of such bindings in treated animals are discussed.

The possibility of developing and using a new technique, "Chemiluminescent dot-blot" for the measurement of isometamidium in sera of treated animals is examined in chapter four. With improvements this new technique may offer a simple, quick, less expensive and sensitive method for the quantification of isometamidium chloride in body fluids under field conditions.

Chapter five gives the general conclusions drawn from this study and future areas of immunochemical research of isometamidium chloride are suggested.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

TRYPANOSOMIASIS - DEFINITION AND AETIOLOGY

Trypanosomiasis is an acute, subacute or more commonly a chronic disease of man and animals caused by various species of trypanosomes. It is clinically characterised by intermittent relapses of fever, anaemia, chronic wasting and death.

In Africa, the most important trypanosome species are those transmitted by tsetse flies (Glossina spp) particularly, T. vivax, T. congolense and T. brucei in ruminants; T. uniforme, T. suis and T. simiae in pigs; and T. brucei gambiense and T. brucei rhodesiense in man.

Trypanosomes are protozoan parasites of the genus Trypanosoma belonging to the order Kinetoplastida. The blood stream forms are elongated, spindle shaped organisms of 8 - 39 um long. They all possess a flagellum arising at the posterior end from a basal body at the foot of a flagellar pocket, and runs to the anterior end of the body. The flagellum is attached along its entire length to the pellicle to form an undulating membrane; thereafter, it may continue forward as a free flagellum. A centrally placed oval nucleus; and a small dense staining structure, the kinetoplast, which is a DNA of a single mitochondrion adjacent to the flagellar pocket can be seen in a stained specimen (Mulligan, 1970).

Trypanosomes possess a surface coat of about 15 nm thick which uniformly covers the entire plasma membrane. This cell coat is predominently composed of a single glycoprotein molecule

of about 65 kDa, and is only visible at the ultrastructural level (Vickerman and Barry, 1982; Pays, 1986; Barbet et al, 1989).

HOSTS

There are large numbers of different strains of each trypanosome species which show wide varying virulence for the hosts. In domestic animals, susceptibility varies greatly with the different species of trypanosomes.

Table 1

Susceptibility of Domestic Animals to Pathogenic Trypanosomes

(from Mulligan, 1970)

Trypanosomes	Host				
	Cattle	Sheep	Goat	Pig	Horse
T. vivax	+	++	++	-	+++ or +
T. congolense	+++	++	++	±	++
T. brucei	+	+	++	±	+++
T. uniforme	++	++	++	-	++
T. suis	-	-	-	++	-
T. simiae	-	+	++	+++	-

(+++ : Highly Susceptible; ++ : Susceptible;
+ : Low Susceptibility; ± : May or may not be Susceptible;
- : Not Susceptible)

The susceptibility also differs with different breeds within some domestic animal species. In cattle, breeds that have shown resistance or reduced susceptibility (trypanotolerance) to trypanosome infections include, West African Longhorn-Ndama, West African Shorthorn (Murray et al, 1981; Murray and Trail, 1984; Roelants, 1986; Akol et al, 1986; Ikede and Taiwo, 1986; Roelants et al, 1987; Pinder et al, 1988) and East African Orma Boran

(Dolan et al, 1986; Ismael et al, 1986). Indigenous breeds of sheep and goats in East and West Africa have also been shown to be more resistant than exotic breeds (Griffin and Allonby, 1979; Munyua, 1986; Kanyari et al, 1986; Mutayoba et al, 1988).

In most of the wild animal species, despite high infection rates, the pathogenicity of the trypanosomes is generally low. For example, infection rates of about 30 - 50% have been observed in normal eland, bushbuck, giraffe, reedbuck, kudu and waterbuck; whereas in buffalo, roan antelope, hartebeeste, impala, duiker, warthog and bushpig the infection rates are 10 - 15% (Mulligan, 1970).

However, some trypanosome infections are fatal in wild animals, for example, T. brucei in Thomson's gazelle, dikdik, wild foxes, jackals, hyrax and blue forest duiker; and T. vivax in red-fronted gazelle (Henson and Noel, 1979).

Despite T. brucei brucei being morphologically identical in all its phases of life cycle to T.b. rhodesiense and T.b. gambiense the causative agents of the more acute East African and the more chronic West African human sleeping sickness respectively, it does not appear to infect man and is confined to wild and domestic ungulates and carnivores (Vickerman and Barry, 1982; Boothroyd, 1985).

GEOGRAPHICAL LOCATION AND ECONOMIC IMPORTANCE

The incidence of African trypanosomiasis in domestic animals, particularly ruminants, is directly related to the distribution of tsetse flies which is, in turn, influenced by the

climatic conditions. Three main ecological groups are found:-

(a) The fusca group (subgenus Austerina): These are the forest tsetse, which are difficult to find and catch, and are of least economic importance;

(b) The palpalis group (subgenus Nemorhina): These are the riverine tsetse, which are important vectors of human trypanosomiasis. Some are also important vectors of animal trypanosomiasis;

(c) The morsitans groups (subgenus Glossina): These are the savannah tsetse which are very important vectors of animal trypanosomiasis. They are found almost throughout the year, however they retreat to denser woodland and other refuges during the hot, dry season.

In Africa, an area of about 10 million square kilometres between latitudes 14^{o}N and 29^{o}S is tsetse infested. This is 37% of the continent, and about half of the habitable land affecting 37 countries (Urquhart and Holmes, 1987; Trail et al, 1985). The extent of infestation ranges from one country to another, in Tanzania for example, 70% of its land is tsetse infested and trypanosomiasis was responsible for about 74,818 cattle deaths between 1982 and 1986; this being the second major disease after East Coast Fever (Mbwambo et al, 1988).

Hendy et al (1986) reported mortalities due to trypanosomiasis in Southern Somalia of 30%, 10% and 8% in cattle, sheep and goats respectively. In cattle, it amounted to 12 - 14% of the herd per year in areas of moderate to high tsetse fly infestation.

Trail et al (1985) argued that 7 million square kilometres of the tsetse infested area could be suitable for livestock and mixed agriculture. It could support a further 140 million cattle and equivalent numbers of sheep and goats.

The annual loss in Africa due to tsetse and trypanosomiasis in meat and milk production, draught power and manure in mixed agriculture, and biogas is difficult to quantify. However, if all these factors are taken into consideration, it is estimated that the development of livestock and agriculture in tsetse infested areas could generate a further US dollars, 50 billion annually (Trail et al, 1985).

Human sleeping sickness in Africa is solely due to T.b. gambiense and T.b. rhodesiense infections. Man is refractory to T. vivax, T. congolense, T. uniforme, T. simiae and T. suis (tsetse transmitted trypanosomes), and there are strong indications that he is highly resistant to infection by T. brucei brucei organisms (Stephen, 1986). However, there has been some indications of changes in resistance to human serum occurring in some animal isolates of T. brucei brucei, and this poses a high likelihood of a zoonosis (The Merck Veterinary, 1986).

LIFE CYCLE AND TRANSMISSION

The African pathogenic trypanosomes undergo a cycle of development only in an insect host, the tsetse fly, following the fly's ingestion of blood from a trypanosome infected animal before they are transmitted to a new host by a fly bite. This is referred to as "cyclical transmission". In the fly, the

trypanosomes lose their surface coat after being ingested; they then multiply and develop to trypomastigotes, epimastigotes and finally the metatrypanosomes (metacyclic forms) which re-acquires a surface coat and becomes infective for another mammalian host (Mulligan, 1970).

The development sites for the three stages of trypanosomes vary in each species and may be used as a criterion for identification. T. vivax has an invertebrate life cycle of 7 - 13 days multiplying and developing in the fly mouth parts only. T. congolense develop in the midgut as long trypomastigotes, then migrate to the proboscis where they assume the epimastigote form; all forms lacking a free flagellum. They multiply for some time still being attached to the proboscis wall before they pass into the hypopharynx where they develop into trypomastigotes. This cycle lasts for 15 to 20 days (Levine, 1985).

The T. brucei life cycle in the tsetse fly takes 15 to 35 days. Following ingestion, localisation occurs in the posterior part of the mid-gut, where they develop and multiply in the trypomastigote form for about ten days. These are at first relatively broad; later, slender forms appear which migrate to the proventriculus between days 12 and 20. Further migration occurs to the oesophagus, pharynx, hypopharynx and finally into the salivary glands. It is in the salivary glands that the trypomastigotes develop into epimastigotes, multiply further, then transform into small and stumpy metatrypanosomes having a short free flagellum (Stephen, 1986; Levine, 1985).

Under natural conditions, the infection rates in tsetse

flies is low, rarely exceeding 20%. In African animal trypanosomiasis, tsetse flies are the primary transmitters, with wild animals as the main reservoirs of infection. However, mechanical transmission has also been reported (Mulligan, 1970; Henson and Noel, 1979; Stephen, 1986; Levine, 1985; Urquhart and Holmes, 1987). Trypanosomes on mouthparts of tsetse flies and other biting flies such as Tabanus, Stomoxys, Chrysops, Haematopata and Pangonia can be passed from host to host during interrupted feeding. T. vivax is commonly transmitted mechanically on the fringe of tsetse areas. Prenatal transmission at parturition, and on surgical instruments and needles, rarely do occur.

Ikede and Lossos (1972) reported intrauterine transmission in pregnant sheep and Ogwu et al (1986) in cattle infected with T. vivax.

PATHOGENESIS, CLINICAL SIGNS AND PATHOLOGICAL FEATURES

PATHOGENESIS

Skin Reaction

Within 5 - 8 days after an animal has been bitten by a tsetse fly, the multiplying metacyclic trypanosomes cause the development at the site of the bite of an inflamed, oedematous, raised cutaneous swelling of several centimetres in diameter, which persists for two to three weeks. This skin lesion is referred to as "chancre". The chancre develops only following a bite by trypanosome infected fly, and trypanosomes are found

extravasculary within the chancre (Morrison et al, 1985).

Generally, T. congolense and T. brucei produce local skin reactions which can measure up to 10 cm in diameter, whereas T. vivax produce a much smaller reaction of 2 - 3 cm diameter at maximum. With some T. vivax strains, no detectable reaction develops, for example, isolated stocks of T. vivax from Kilifi, Kenya, reported by Paling et al (1987).

The cells found within the chancre are polymorphonuclear leukocytes and large numbers of lymphocytes and plasma cells. The appearance of the chancre is followed within a few days by detectable parasitaemia, fever and marked enlargement of the draining lymph nodes.

Lymphoid System Changes

The lymph nodes draining the chancre are first enlarged followed by a generalised lymphadenopathy and splenomegaly which is associated with marked proliferation of lymphoid cells. Numerous large active germinal centres develop, and a marked increase of plasma cells occur in the medullary cord of the lymph nodes and splenic red pulp. There is also an increase in the splenic red pulp of active macrophages, some of which are engaged in erythrophagocytosis. Hepatic Kupffer cells are also increased in number and activity, and a pronounced hypergamma-globulinaemia occurs concurrently with these changes. Despite the pronounced activation of the lymphoid system, immunosuppression has been demonstrated by significant reduction in humoral responses to a variety of infectious agents (antigens) in trypanosome infected animals (Scott et al, 1977; Whitelaw et al, 1979; Rurangirwa

et al, 1983).

In chronic infections, the lymphoid organs may be decreased in size with depletion of lymphoid cells.

Circulatory Changes

(a) Anaemia

Anaemia is a reliable indicator of the progress or severity of trypanosomiasis as demonstrated by packed cell volume (PCV) values, and it can be divided into two phases.

The onset and severity of anaemia in the first phase is directly related to the appearance of the trypanosomes in the circulation, and the level and duration of parasitaemia. This phase may last four to six weeks after infection (Holmes and Jennings, 1976; Dargie et al, 1979; Morrison et al, 1985).

Studies by Jennings et al (1974) and Mamo and Holmes (1975), demonstrated that the principal factor causing anaemia is accelerated red blood cell loss from the circulation. The loss is a result of erythrophagocytosis in the spleen, lungs, haemal nodes, bone marrow, and is particularly marked in the liver. There is evidence that, immunological factors play a significant role in erythrophagocytosis in that, antibodies specific for trypanosomes become complexed with antigen on the erythrocytes (erythrocyte sensitisation) resulting in red cell sequestration and destruction in the reticulo-endothelial system (RES). The erythrocytes are destroyed through phagocytosis via fragment crystallisalde (FC) and complement receptors on the macrophage (Murray and Dexter, 1988).

Fever increases erythrocyte fragility, and the damaged red cell membrane may bind antigen-antibody complexes or complement more readily and therefore, facilitating erythrophagocytosis. Pathogenic trypanosomes produce a haemolytic factor of low molecular weight (about 10 kD) and protein in nature causing direct lysis of erythrocytes (Jenkins and Facer, 1985).

The expanded and active mononuclear system, may also be a contributory cause to increased red cell destruction (Urquhart and Holmes, 1987). In a trypanosome infected animal, the non-specific activation of the RES and pooling of red cells within the enlarged spleen may result in increased removal of normal erythrocytes and injurious effects on red cells with increased osmotic fragility.

Though occasionally, petechiations and ecchymoses are found scattered in animal organs and tissues, widespread haemorrhage is not a feature in animal trypanosomiasis, except with some isolates of T. vivax. Intravascular haemolysis has also not been shown to contribute significantly to the anaemia of this stage. Usually the anaemia is normocytic normochronic, however, macrocytic responses have also been observed (Murray and Dexter, 1988).

Treatment of trypanosome infected animals during the first phase with trypanocidal drugs, usually results in rapid recovery of PCV and other haematological values (Holmes and Jennings, 1976).

The second phase is characterised by a low grade, more chronic anaemia found in animals with chronic trypanosomiasis,

and usually occurs between four to six months after infection in cattle. There is low transient parasitaemia or complete absence of detectable parasites in circulation, the PCV values stabilises at 20 - 25%, and the animal responds poorly or fails to respond haematologically to trypanocidal drug treatment (Murray and Dexter, 1988).

The anaemia in this chronic phase is thought to be due to dyshaemopoesis. There is lack of incorporation of iron into the developing red cell precursors, a decrease in erythrocyte production by the bone marrow, and an intrinsic modest reduction in red cell survival. The principle cause lies in some impairment of bone marrow function; the femoral marrow is often gelatinous and inactive, and there is a large excess of stored iron not used for haemoglobin synthesis (Jenkins and Facer, 1985). Murray and Dexter (1988) speculated that red cell autoantibodies or trypanosome mediated antigen-antibody reactions might contribute to stem cell destruction.

(b) Abnormal Haemostasis

Jenkins and Facer (1985) indicated that the major haemostatic factors which appear to be involved in the pathology of trypanosomiasis are vascular injury, coagulopathy with increased fibrinolysis, and thrombocytopenia.

It has been suggested that release of pharmacologically active substances leads to collection of platelets and circulating leucocytes which causes occlusions and finally the blood vessels disintegrate. The vascular damage is manifested by petechiae and leakage of fluid and protein into extravascular

spaces, and is particularly noticeable in experimental infections with T. brucei.

Disseminated (widespread) intravascular coagulation (DIC) also known as consumption coagulopathy, or defibrination syndrome, is a dynamic pathological process triggered by activation of the clotting cascade, and can be manifested as a wide clinical spectrum. DIC with associated fibrinolysis is responsible for the thrombosis, haemorrhage, tissue necrosis and microangiopathic haemolytic anaemia found in acute trypanosomiasis. Murray and Dexter (1988), suggested that microangiopathy most likely play a significant role in acute infections with T. vivax which are characterised by high parasitaemia. Also the microangiopathic changes that occur are possibly initiated by thrombocytopaenia and could lead to red cell damage and finally to phagocytosis.

Increased serum fibrin/fibrinogen degradation products (FDPs) in trypanosomiasis indicates increased fibrinolysis and DIC (Jenkins and Facer, 1985). "Early" FDP fragments (X and Y) are powerful inhibitors of blood coagulation having antithrombin effects and promoting ADP-induced platelet aggregation. The "late" FDP fragments, protentiate biogenic amines and peptides, increase vascular permeability and have immunosuppressive activity.

In trypanosomiasis, thrombocytopaenia and in vivo platelet clumping are characteristic features (Jenkins and Facer, 1985). Thrombocytopaenia may result from a failure of platelet production, increased platelet destruction, or the dilution

effect of increased plasma volume. A platelet defect in number or function results in abnormal haemostasis.

Tissue Lesions

The nature of the cellular infiltrate and possibly the mechanism involved in cell injury appear to depend on the difference in tissue invasiveness between species of trypanosomes.

During the course of the disease, the host responds to a series of trypanosome antigenic variants *in vivo*, by mounting antibody responses to clear the variants. Immune complexes of trypanosome antigens and parasite-specific IgM or IgG plus Complement (C) are formed and deposited in the vasculature and extravascular spaces of tissues. These complexes range from those with an antigen excess (temporarily) to those with an antibody excess in composition, causing inflammatory changes as those occurring in Arthus lesions, and in acute and chronic immune complex disease (Mansfield, 1981).

The common tissue lesions associated with animal trypanosomiasis are found in the heart, skeletal muscle, liver and kidneys. The lesions are characterised by muscle fibre degeneration, mononuclear cell infiltration and oedema. In the heart there is also fibrinous necrosis of the coronary vessels and the conducting purkinje fibre system is often widely separated from the myocardium by cellular infiltrates and oedema. Invasion of tissues with lymphocytes, plasma cells, macrophages and a few polymorphonuclear leucocytes may be due to attraction by chemotactic factors resulting from complement activation by

antigen-antibody complexes at the site. In the skeletal muscles, the tissue changes may be partly responsible for the emaciation which is a characteristic feature of trypanosomiasis, and muscle pain in dogs. In T. brucei infections, tissue destruction is very marked, and the brain is also involved (Mansfield, 1981; Vickerman and Barry, 1982; Urquhart and Holmes, 1987).

Seed and Hall (1985) concluded that major physiological changes also occur in African animal trypanosomiasis which are important in the pathogenesis of the disease. Formation of toxic catabolites and the depletion of essential nutrients for example, would alter host metabolism and lead to changes in the major metabolic pathways, such as decreased protein synthesis, gluconeogenesis and energy production.

CLINICAL SIGNS

The incubation period in African animal trypanosomiasis is usually one to four weeks with the severity of the clinical disease varying with the species of the animal infected and the trypanosome species involved. Generally, the primary clinical signs observed are intermittent fever, anaemia and weight loss or wasting (Mulligan, 1970); The Merck Veterinary Manual, 1986). The disease syndrome may either be acute or chronic with a poorly defined line of demarcation.

In cattle, intermittent pyrexia is observed during the early stages of infection even before parasitaemia is readily detectable. The superficial lymph nodes are palpably enlarged and the animal has a bright demeanour with occasional signs of

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loss of appetite though it may continue to eat during the course of the disease.

Anaemia is evidenced by the pallor of mucous membranes and PCV which falls to 20% or lower (decreased by about 40 - 50%) over the first four to six weeks. There is a gradual loss of body condition with the severity of anaemia until the animal dies (Morrison et al, 1981). The animals show tachycardia during the course of disease with bradycardia, pulsating jugulars, weak pulse and sometimes subcutaneous oedema terminally.

Affected animals usually trail at the rear of the herd during grazing, they are lethargic with a dull coat and show a "hunched-up" appearance. Terminally, the animal becomes extremely weak and often unable to rise, although there is no paraplegia and reflexes are unimpaired (Mulligan, 1970).

Survivors may, after a variable length of time, show gradual disappearance of parasitaemia and rising of PCV. However, these animals remain unproductive for long periods of time and stunted growth occurs in young animals. In adults, there is decreased fertility and sometimes, pregnant cows abort or give birth to small weak calves (Morrison et al, 1981; Stephen, 1986).

In chronic disease, the animals have a voracious appetite which continues until death, and grass is often found in the mouths of dead cattle. A diseased animal is usually thin and emaciated, the skin is drawn tightly over the ribs and pelvis and lacks the normal healthy looseness. The hair is rough with upstanding appearance, and enlarged subcutaneous supernumerary haemolymph glands are easily palpable, particularly over the ribs

and along the flanks (Mulligan, 1970).

Infection with certain isolates of T. vivax produces an acute haemorrhagic syndrome characterised by fever, sustained high levels of parasitaemia, blood-stained diarrhoea and death occurs within two to four weeks post-infection (Morrison et al, 1981).

Generally, T.b. brucei infection is severe in horses, camels, goats, sheep and dogs. In dogs, there is an addition to the common clinical signs, conjunctivitis, keratitis and blindness (Levine, 1985; Stephen, 1986). Masake et al (1984) reported absence of clinical and histological evidence of cerebral trypanosomiasis in cattle infected with T.b. brucei alone. They further concluded that Berenil (Hoechst) treatment predisposes cattle infected with T.b. brucei to CNS involvement; and suggested an interdependence of T.b. brucei and T. congolense mixed infections in the pathogenesis of cerebral trypanosomiasis.

PATHOLOGICAL FEATURES

The gross and histopathological features of animals dying from trypanosomiasis have been extensively discussed by Mulligan (1970) and Stephen (1986).

Macroscopic Appearance

The common gross lesions observed in ruminants dying from trypanosomiasis depend on the stage of the disease, however the pathological features are not pathognomonic.

There is gelatinous atrophy of body fat particularly around the heart and kidneys, and wasting of skeletal muscle. In chronic cases the blood is pale, watery and clots poorly.

Parenchymatous organs are pale, and often excess serous fluid which may be blood tinged is found in the body cavities, notably the pericardium and sometimes there is gross oedema of the subcutis and viscera.

In early stages of the disease, the spleen is markedly enlarged with blunt edges, and the cut surface is drier showing prominent Malphigian corpuscles. In chronic cases, it is small and atrophic and red marrow in long bones (except in ribs and vertebrae) disappears and becomes gelatinous.

Usually, there is a generalised lymphadenopathy, the lymph nodes are also oedematous and haemorrhagic. Sometimes, the medullary cells contain dark pigment giving a lymph node a blackish appearance. Haemal lymph nodes are markedly enlarged. The lymph nodes are commonly smaller than normal and fibrotic in chronic cases.

The lungs are oedematous and marbling is observed due to dilation of the lymphatic vessels. They do not collapse as normal organs and have a firmer consistency. The liver is often swollen, firm, congested and dark, while the kidneys are nephretic.

The heart is usually pale, hypertrophied, and in advanced cases, the ventricles are dilated giving the heart a globular appearance. Sometimes, irregular streaks of haemorrhages are seen on the epicardium and they may extend to the flabby and oedematous myocardium.

Animals dying of hyperacute T. vivax infection are normally in a good body condition with widespread petechial and ecchymotic

haemorrhages on mucosal and serosal surfaces, and sometimes frank haemorrhages are found into the gastrointestinal tract.

Microscopic Appearance

Myositis is observed in chronic cases, and especially in T.b. brucei infections, wherever trypanosomes are found in the connective tissue, an extensive cellulitis occurs in the dermis and subcutaneous layers, and there is necrotising vasculitis, lymphatic thrombosis, fibrinous exudation and necrosis of mononuclear and polymorphonuclear inflammatory cells.

Proliferation of lymphocyte follicles is evident in the cortex of lymph nodes extending to the paracortical zone along the connective tissue trabeculae. There is an increased number of lymphoblasts, immature plasma cells and macrophages in the enlarged germinal centres, expanded cortex, paracortex, medullary cords and distended sinuses. The medullary reticulum is hypertrophied and the cells often contain dark-brown pigmented granules. In T. vivax infections it is common to observe blood vessels thrombosed with a mixture of fibrin, red blood cells, lymphoid cells and trypanosomes. Phagocytosis and extensive deposits of haemosiderin may be found.

In the spleen, there is hypertrophy of lymphoid cells and of the mononuclear phagocyte system (MPS or RES). Germinal centres are grossly expanded and contain actively proliferating large lymphocytes, macrophages and lymphoblasts. Plasma cells and macrophages are found in great numbers throughout the splenic tissue, even in arteolar sheath regions and red pulp. In peracute and chronic infections, regressive changes evidenced by

focal necroses and fibrosis may be observed.

Obvious heart lesions may be absent in small ruminants, but they are severe in cattle. Haemorrhages are common under epi- and endo-cardium. With advancement of the disease, the cardiac muscles undergo a series of changes from myocarditis to the appearance of thin and atrophic muscle fibres. The degenerating myocardial fibres are widely separated by interstitial and perivascular oedema infiltrated with lymphocytes, macrophages and plasma cells. Trypanosomes are commonly contained within the dilated capillaries and they may also be observed extravascularly. There is usually fibrinous necrosis of coronary vessels. Fragmentation and lysis of Purkinje cells may sometimes be seen. Heart changes are less marked in infections with T. congolense.

Kidneys show mild nephrosis and chronic glomeruritis with mononuclear cell infiltrations which may extend throughout the kidneys. Atrophy of the proximal tubules may occur, and haemosiderin is present in the tubular epithelium. With T. brucei infections, the changes may be more severe.

In the bone marrow, there are infiltrations with islets of lymphoblastic tissue, and the haemopoietic tissue is abundant containing excess of normoblasts in acute stages. The marrow in long bones is practically destroyed in chronic cases. Haemosiderosis may be observed.

There is fatty infiltration of the hepatocytes and varying degrees of central lobular necrosis is observed in the liver parenchyma. Central veins and sinusoids are dilated and the Kupffer cells are excessively enlarged containing haemosiderin

and erythrophagocytosis may be evident. The portal triads are infiltrated with lymphocytes, macrophages, plasma cells and sometimes morula cells.

Some lung alveoli can be grossly dilated and sometimes ruptured. Some may contain fluid, the alveolar cells enlarged and swollen, and the alveolar septa is infiltrated with lymphocytes, macrophages, plasma cells, collagen fibres and is greatly distended with oedema.

Brain lesions have been reported to be found mainly in T. brucei infections, however, Masake et al (1984) demonstrated that a higher incidence of cerebral trypanosomiasis occurs in mixed infections with T. congolense and T. brucei than with either T. brucei or T. congolense alone in cattle. In dogs and cattle (Morrison et al, 1981) the lesions are characterised by cellular infiltrates containing many lymphoblasts and plasma cells. These infiltrates are in the meninges and perivasculary throughout the brain and spinal cord in cattle; and in the choroid plexus and meninges in dogs. Trypanosomes are present in cerebral spinal fluid and there is diffuse meningoencephalitis (Morrison et al, 1985). In T. vivax infections only perivascular cuffing by lymphocytes may be observed.

There is degeneration of seminiferous tubules which are devoid of spermatozoa and spermatids. There is also an increase in connective tissue surrounding epididymal tubules which are also devoid of spermatozoa and spermatids.

Histological lesions reported in the endocrine glands have not been constantly observed (Stephen, 1986). They include

extreme distension of thyroid glands' acini with colloid, and where the acini has disintegrated there is mononuclear cell infiltrations or fibrosis terminally. In the adrenal glands, mononuclear cell infiltrations are observed below the capsule, however in some cases they may extend between the bundles of zona fascicularis. The whole cortex tends to be necrotic and sometimes necrosis and fibrosis extend into the medulla.

IMMUNOLOGY OF AFRICAN ANIMAL TRYPANOSOMIASIS

Trypanosome Antigens

There are two functional groups of antigens in African animal trypanosomes; common (stable) antigens and variable antigens (Mansfield, 1981; Vickerman and Barry, 1982; Urquhart and Holmes, 1987).

The common antigens are components (e.g. structural proteins, enzymes) of membranes and organelles found in all trypanosomes at all stages of infection, in all stocks of the same species, and some in other species. About 30 different common antigens may be detected serologically in trypanosome extracts. Almost all of these antigens are covered by a surface coat and therefore unexposed in the circulating living trypanosomes. They are released only when the trypanosomes are destroyed. In this respect, the common antigens lack the opportunity to contact the relevant immunocytes, and it is expected therefore that they elicit only a weak immune response. However, Shapiro and Murray (1982) showed that animals that are able to recognise certain common antigens control trypanosomiasis

infections more effectively than animals which are unable to recognise them. The induction of immunity by these antigens is not yet very clear.

The variable antigen is a 12 - 15 nm thick surface coat covering the plasma membrane of a trypanosome as seen in an electron micrograph, representing 10% of all trypanosomes' cellular protein. The variable antigens change from one population of trypanosomes to another and are responsible for the differences between serological variants or variable antigen types (VATs) of the trypanosomes. The surface coat of a trypanosome is composed of a matrix of a single glycoprotein with molecular weight of about 65 kD and composed of 600 amino acids and up to 20 carbohydrate residues; the variable surface glycoprotein (VSG). The VSG is the variant specific surface antigen (VSSA) evoking an antibody response in each parasitaemic wave (Mansfield, 1981; Vickerman and Barry, 1982; Urquhart and Holmes, 1987). This coat is lost together with the variable antigen when a trypanosome undergoes a cyclical development in the tsetse fly and is reacquired during the differentiation of the metayclic form.

The trypanosomes in mammalian circulation evade the specific immune response to the VATs by undergoing antigenic variation, that is, switching from expression of one VAT to another. The switching is intrinsic occurring spontaneously in a manner that does not require the presence of antibody as a stimulus (Stephen, 1986).

The number of VATs a single trypanosome can produce is known as the VAT repertoire; cloned trypanosomes are capable of expressing several hundred VATs, however the exact number is still unknown (Vickerman and Barry, 1982; Stephen, 1986). Thirty-nine VATs have so far been defined by Gardiner et al (1986) in T. vivax cloned stock; and 101 in T. equiperdum in rabbit (Capbern et al, 1977). In a natural, tsetse-fly transmitted infection, each parasitaemic wave consists of many subpopulations each expressing a different VAT.

Host Response to Trypanosome Antigens

The mammalian host produces immunoglobulins and develops a cellular response to trypanosome infection which is specific for the antigenic composition of the infecting organism. However, antibodies produced against the common antigens show extensive cross-reactions with trypanosome clones of the same species and other trypanosome species. There is considerable evidence that immunity to trypanosome challenge is mediated by antibody responses against the variable surface glycoprotein (VSG). Antibodies produced against each VSSA will react only with the variable surface antigens of a population of parasites in circulation at the particular time during infection, thus the measure of protection provided to the animal is against antecedent antigenic types. However, due to the ability of the African trypanosomes to change their VSSA, the infection persists. As the immune response to the antigenically predominant population destroys those trypanosomes, the variant parasites expressing different VSSA are spared and subsequently

reproduce to become the next antigenically predominant population of the circulation and tissues. Therefore, parasitaemia is controlled only superficially, the cycle of antigenic variation and immunoselection continuing until the demise of the host (Mansfield, 1981; Vickerman and Barry, 1982; Stephen, 1986; Morrison et al, 1985; Pays, 1986; Seed and Sechelski, 1988) or self cure occurring.

It has been found that, in all infections with any of the pathogenic trypanosomes, there is a marked increase in IgM levels within two weeks of infection with a little change in IgG (Luckins, 1972; Kobayashi and Tizard, 1976; MacKenzie et al, 1979; Nielsen et al, 1978; Musoke et al, 1981). Musoke et al (1981) further showed that, during the first three weeks of infection, the produced IgM and IgG are specific for the variable antigen on the surface of the living trypanosomes and thus have a protective function. Also, IgM antibodies seem to be more efficient in killing trypanosomes than IgG.

In trypanosomiasis, the host response is unique in that following the initial IgM response, IgM continuesto rise and remains at high levels. It appears also that, the switching mechanism from IgM to IgG is uniquely selective for IgG_1 heavy chains. The dysfunction can be due to hypocomplementamia which is a consistent feature in trypanosome infections (Koboyashi and Tizard, 1976; Nielsen et al, 1978; Tabel et al, 1980; Musoke et al, 1981; Vickerman and Barry, 1982).

It should be noted that antibodies which neutralise metacyclic trypanosomes appear in the serum just after the peak

of the local skin reaction and at the time of initial detection of parasitaemia. Thereafter, antibodies are produced to the VATs of each successive wave of parasitaemia, and with the progression of infection, antibodies against the common antigens are also produced (Morrison et al, 1985). Antibody effects removal of circulating trypanosomes in vivo by trypanolysis and opsonisation which are followed by Kupffer cell uptake. Extravascularly, various macrophages and lysis are expected to effect trypanosomal removal.

T-lymphocytes do not appear to play a role in immunity to the African trypanosomes and cell mediated immunity reactions do not appear early in infection, if at all, are not directed against the all-important surface variable antigen, and are not involved in protection (Vickerman and Barry, 1982).

DIAGNOSIS

Diagnosis is a prerequisite in the treatment and control of animal diseases either as individual cases, a herd or over a wide geographical area.

Epizootiological features are important in making a presumptive diagnosis, however, amongst the animal trypanosomiases they may vary considerably in different climatic areas and regions.

The diagnosis based on clinical and pathological features in animal trypanosomiasis poses a considerable problem particularly in the presence of other infections that cause anaemia and weight loss, such as babesiosis, anaplasmosis and theileriosis despite the fact that they can be eliminated by examining stained blood and lymph gland smears.

Diagnostic tests are therefore of paramount importance in the confirmation of animal trypanosomiasis, especially a test that detects a current infection, and can identify and differentiate the trypanosome species involved. These tests can also be employed when conducting surveys in an attempt to determine the overall prevalence and experience of infection.

The diagnosis of animal trypanosomiasis is unsatisfactory as no single test is simple and cheap, yet be sufficiently specific, sensitive and reproducible to warrant its widespread use in the field (Paris et al, 1982; Vickerman and Barry, 1982; Kalu and Lawani, 1986).

Parasitological Tests

These tests make use of tissues removed from the animal body particularly blood and to a lesser extent lymph node biopsies. Giemsa's stained lymph node smears are useful only in examination of T. vivax.

Blood samples collected from ear vein are considered to be more sensitive indicators of parasitaemia than blood collected from jugular vein, especially in cases of T. congolense infections. The usually low and transient parasitaemias in animal trypanosomiasis, renders the difficulty in finding trypanosomes in blood samples with the available parasitological tests (Murray et al, 1979; Stephen, 1986). It is believed that trypanosomes exhibit a phenomenon of diurnal variation, however there are conflicting views as to the best time of the day that blood samples should be taken. Greig et al (1979) indicated that samples taken around midday are more likely to reveal the presence of trypanosome infection than samples taken at other times of the day, whereas Stephen (1986) advocates early morning sampling. Through my personal experience (unpublished), the best times for sampling are early morning and late afternoon in the tropics.

The parasitological methods utilising blood for diagnosis of animal trypanosomiasis include blood film examination, trypanosome concentration techniques, and animal inoculation.

Blood Film Examination

(a) Wet (Unstained Films)

A small drop of blood is placed on a clean, grease free microscope slide and then overlaid with a coverslip such that the blood spreads over the area of the coverslip to form a continuous single red blood cell film layer. Trypanosomes are detected by examining such films with the aid of phase contrast or darkground microscopy techniques. Trypanosome species can be differentiated based on the type of movement they show in the wet smear, however, experience is vital.

This method is relatively inefficient and inferior for the detection and identification of trypanosomes particularly when large numbers are being examined as in herd surveys (Murray et al, 1977; Paris et al, 1982; Ogbunude and Magagi, 1982).

(b) Stained Films

Giema's stain, Romanovsky-type stain, Field's stain and Leishman's stain are commonly used in the staining of blood films. Giemsa's stain gives the best results, however, for rapid examination of large numbers of films, Field's stain is preferred due to its short staining time. In large scale trypanosomiasis surveys, thick and thin stained blood films are cheap and still the commonly used methods.

(i) Thick Stained Blood Films

A few drops of blood, about 5 ul are placed and spread over a small area of a microscope slide (1 - 2 cm diameter) giving a thick film and left to air-dry completely. Using a freshly prepared 10% Giemsa's stain in buffered water pH 7.2, the film is

stained for 30 minutes. To differentiate the stain, the slide is then put in buffered water until the film turns pink. The slide is examined under a microscope for trypanosomes after it has air-dried. However, this method results in distortion and poor preservation of the trypanosome's morphological features.

The morphology of the trypanosomes is much better preserved by employing MacLennan's modification in staining thick smears; the slide with a dried thick blood smear is immersed in 0.5% aqueous methylene blue for one second, rinsed gently and briefly with tap water before being stained with Giemsa's stain (Stephen, 1986).

The use of thick stained blood films is particularly useful in detecting infections with low parasitaemia, whereby the trypanosomes are too scanty to be observed in a thin or wet film (Mulligan, 1970; Paris et al, 1982).

Table 2

The Threshold Sensitivity (Trypanosome/ml Blood) of Blood Films for the Detection of T. congolense, T. vivax and T. brucei

(From Paris et al, 1982)

Method	T. congolense	T. vivax	T. brucei
Thin Film	2.5×10^4	5×10^4	2.5×10^4
Wet Film	1.2×10^4	8.3×10^3	8.3×10^3
Thick Film	8.3×10^3	6.25×10^3	5×10^3

(ii) Thin Stained Blood Films

A thin blood film is prepared by spreading a drop of blood on a clean grease free microscope slide to give a continuous single red blood cell film. After the film has air-dried, it is fixed with water-free methanol by either covering the film with/ or dipping the whole slide in methanol for 1 - 2 minutes. The film is then stained as for thick smears with Giemsa's stain.

Since the fixation preserves the morphological characteristics of the trypanosomes, species identification is possible. Overall length (size), shape of the posterior end and presence of free flagellum are commonly used in species identification. Size and position of kinetoplast and the distribution of cytoplasmic granules are less used, however they are valuable in confirmation of the identified trypanosome species.

Trypanosomes Concentration Techniques

Trypanosomes in a given volume of blood are concentrated so as to increase the probability of their detection, particularly in cases of low parasitaemia. The techniques usually involve some form of centrifugation and separation of the trypanosomes from the host cells.

(a) Silicone Centrifugation Technique

The technique which was developed by Ogbunude and Magaji (1982) is based on the differences between the host's red blood cells and the trypanosomes' densities. The erythrocytes being of greater density than the trypanosomes are pelleted by centrifugation through a layer of silicone fluid, whereas the

trypanosomes remain in the plasma supernatant.

In the technique, 25 µl of blood is dispensed into a 1.5 ml tapered microfuge tube containing 500 µl of silicone fluid (specific gravity 1.075) and centrifuged at 150 g for five minutes. The plasma supernatant fraction on the top of the silicone fluid is then examined as in unstained wet blood films.

Ogbunude and Magaji (1982) reported that the technique compared favourably with other microsensitive techniques such as the miniature anion exchange centrifugation (m-AECT) and microhaematocrit buffy-coat microscopy methods.

(b) Anion-Exchange Centrifugation Techniques - m-AECT

These techniques make use of the difference in charges between the host's erythrocytes and trypanosomes.

(i) Large volumes of about 5 - 10 ml of infected blood are passed through a 25 - 100 g column of diethylaminoethyl (DEAE) cellulose equilibrated to pH 8.0 with phosphate buffered saline. The more negatively charged erythrocytes are adsorbed by the positively charged cellulose particles, whereas the trypanosomes pass through the eluate. The eluate is examined directly as for unstained wet blood films.

(ii) Miniature anion/exchange centrifugation technique (mAECT) developed by Lumsden et al (1977) uses small volumes of blood (50 - 100 µl) which is passed through a column of DE52 in a disposable 2 ml plastic syringe. The eluate is collected in a centrifuge tube made from a drawn-out sealed pasteur pipette. It is then centrifuged at 525 g for five minutes, and microscopy examination for trypanosomes is made at the sealed end as for

Haematocrit Centrifugation technique (HCT).

Kimber (1984) reported on how to prepare buffer salts which can be stored at room temperature and reconstituted before using. He also showed that sterile prepared DEAE-Cellulose can be maintained at high temperatures for field use. The main set-backs of the method are the costs and the provisions for the availability of reagents for field use bearing in mind that different animal species require different buffer systems.

(c) Haematocrit Centrifugation Technique - HCT (Woo, 1969)

3 - 10 ml of blood is collected from the animal's jugular vein into a heparinised/EDTA vacutainer tube. After a gentle and thorough mixing, blood is drawn into a plain capillary tube (75 mm long, 1.2 - 1.5 mm internal diameter) up to three quarters of its length and its outside cleaned with a clean cloth or tissue paper. One end of the capillary tube is sealed by a very fine flame (without charring the blood column) or with 'cristaseal'. Following a 5 - 8 minutes centrifugation at 12,000 rpm in a microhaematocrit centrifuge, the capillary tube is placed on a groove of a specially prepared microscope slide (two rectangular microscope slide pieces of 25 x 10 x 1.2 mm thick fixed with DPX on a clean microscope slide 1.5 mm apart). A cover slip is then put on and the interphase flooded with water. The buffer coat-plasma junction is then examined using a Leitz SM distance objective, a Zernicke condenser and periplan 10 x eyepieces, or its equivalent.

(d) Capillary Concentration Technique - CCT (Walker, 1972)

The method is designed to create a large differential density between the erythrocytes and the trypanosomes to counter the tendency of T. congolense to be retained amongst the red blood cells.

Equal volumes of blood and a strong hypertonic non-toxic medium (9% glycerol, 9% magnesium sulphate, 0.1% TRIS buffer, pH 8.0 - 8.2, and phenol red 1 : 100,000) are mixed and allowed to stand for a minimum of 15 minutes. Plain capillary tubes are used to draw the blood mixture and treated as for HCT.

(e) Darkground/Phase Contrast Buffy Coat Technique - DG (Murray et al, 1977)

A buffy coat zone is prepared in a micro-haematocrit capillary tube as for HCT. With a diamond pencil, the tube is cut 1 mm below the buffy coat to incorporate the uppermost layer of red cells, and 1 cm above to include some plasma. With an aid of a micro-haematocrit capillary tube holder, the contents are expressed on to a clean slide, mixed and covered with a 22 x 22 mm coverslip. The preparation is examined using a phase contrast or darkground microscopy. Improved contrast can be obtained with a normal bright field microscope system with the condenser top out and/or the diaphragm closed to increase defraction.

Samples must be examined as soon as possible, at least within 4 - 6 hours after collection, otherwise the number of detectable trypanosomes falls off particularly with T. congolense.

The authors (Murray et al, 1977) showed this method to be consistently more reliable than other standard parasitological techniques for detecting trypanosomes in cattle blood. The advantage DG offers of sensitivity, speed of detection, species identification, quantification of parasitaemia and PCV estimation all from one sample makes it the best of all parasitological methods for the diagnosis of African animal trypanosomiasis (Murray et al, 1979; Paris et al, 1982; Kalu et al, 1986).

Table 3

The Threshold Sensitivity (Trypanosome/ml Blood) of Concentration Methods and Animal Inoculation for the Detection of T. congolense, T. vivax and T. brucei (From Paris et al, 1982)

Method	T. congolense	T. vivax	T. brucei
HCT	6.25×10^3	1.25×10^3	5×10^2
m-AECT	2.5×10^3	2.5×10^3	-
CCT	6×10^2	6×10^2	-
DG	2.5×10^2	5×10^2	5×10^2
Mouse Inoculation	-	-	1.25×10^2

Animal Inoculation

Laboratory rodents, particularly the readily available mice and rats have been used most frequently and they have been useful in revealing subpatent infection with Trypanozoon parasites in animals (Stephen, 1986).

The susceptibility of laboratory rodents is increased by immunosuppression with cyclophosphamide (300 mg/kg) or irradiation. Up to 1 ml or 5 ml of infected blood is injected intraperitoneally into individual mice or rats respectively. These animals are then checked for trypanosomes by using tail blood at 2 - 3 day intervals up to 30 days post inoculation before being declared uninfected (negative).

Paris et al (1982) observed that inoculation of suspected infected blood into mice proved to be the most sensitive technique for the detection of T. brucei. However, the results vary depending on the phase of parasitaemia and the ratio of infective to non-infective forms present in the donor's blood. The value of inoculation of suspected T. congolense or T. vivax infected blood/material is more limited as not all field isolates become established in rodents.

Stephen (1986) drew attention to scientists to further examine/study an unpublished technique employed by a technical assistant in a Nigerian laboratory. In the technique, 2 - 3 mls of heparinised blood is injected intraperitoneally into a laboratory-bred white rat. The hair coat is shaved off to allow the spleen to be easily seen. 10 - 15 minutes post inoculation, a splenic puncture is made through the thin body wall and the aspirate is examined as for wet or stained blood films. He observed that the originator of this technique often enough discovered trypanosomes when other rapid tests were negative.

Serological Tests

The preceding parasitological techniques rely on the demonstration of the trypanosomes in the host tissues particularly the blood, and this is possible during the acute stages of the disease when large numbers of the parasites are in circulation. However, during the early stages of infection or in chronic disease when the parasitaemias are low and sporadic, it is usually difficult to detect the trypanosomes in the blood. In such cases, diagnosis of infections with T. vivax and some isolates of T. congolense is further hampered by lack of infectivity for laboratory rodents. In such situations, serological tests can be very useful for diagnosis and particularly when conducting large scale trypanosomiasis surveys in which detailed microscopic examinations may not be possible.

Serological tests are based on the detection of antibodies against trypanosome antigens and/or trypanosome antigens in serum. Antibody detection tests have been used for the detection of trypanosome infected animals, and have been able to diagnose the disease as early as seven days post infection. However, various studies in laboratory animals and naturally or experimentally infected livestock showed that antibody levels decline slowly and remain higher than pre-infection levels several weeks after the parasites have been eliminated following trypanocidal treatment (Woo and Soltys, 1972; Staak and Lohding, 1979; Luckins, 1977; Luckins and Mehlitz, 1978; Luckins et al, 1978; 1979).

The persistence of antibodies against trypanosomes in serum complicates the evaluation of the infection status of individual animals as it is difficult to differentiate between a current and past infection. As it has been argued by Urquhart and Holmes (1987), antibody detection tests seem to be more valuable in assessing the distribution and incidence of infection in areas where the disease is uncommon or suspected, and in screening of potential experimental animals destined for trypanosomiasis research.

Tests which demonstrate circulating trypanosome specific antigens or products in the host enables active infections to be diagnosed and appear to offer the desired sensitivity and specificity; and they can also be useful in the evaluation of trypanocidal treatments (Rae and Luckins, 1984; Nantulya et al, 1987; Liu and Pearson, 1987; Liu et al, 1988; Nantulya and Lindquist, 1989).

In their experiments with rabbits and goats, Rae and Luckins (1984) demonstrated that, circulating antigens can be detectable in serum as early as 4 - 14 days post-infection and disappear within ten days after trypanocidal treatment. In cattle, Nantulya and Lindquist (1989) detected circulating trypanosomal antigens between 10 - 12 days post-infection with T. congolense and T. vivax; and 8 - 14 days with T. brucei infected animals. The antigens were cleared from the blood within two weeks following Berenil treatment in T. vivax and T. congolense infected cattle, but clearance was slower and more variable in T. brucei infections.

Nantulya et al (1987) cautioned that, at certain stages of infection, an antigen-antibody equilibrium in tissue fluids may shift to the extent that, free antigens may be undetectable. Therefore, assays for both circulating antigens and antibodies should be developed.

Immunodiagnostic tests fall mainly in two categories, that is, in vitro and in vivo tests.

In Vitro Tests

These assess the outcome of antigen-antibody interaction directly. The tests which have been used in the diagnosis of African animal trypanosomiasis include; enzyme immunoassays, fluorescent antibody tests, precipitation reactions, haemagglutination reactions, complement fixation test and radio-immunoassay.

Enzyme Immunoassays (EIA)

Enzyme immunoassays are qualitative and quantitative immunological procedures in which the extent of antibody-antigen reaction is evaluated by enzyme measurements (Avrameas, 1981). They take advantage of and make use of the biological properties of antibodies and enzymes (Kurstak, 1986).

The general principle employed in EIA is that an appropriate enzyme is conjugated to one of the immunoreactants, that is, antigen (Ag) or antibody (Ab). Following an immunological reaction (Ag-Ab interaction), a substrate is added and the substrate-product enzyme conversion is determined. The product concentration indicates how much enzyme-labelled immunoreactant

has been incorporated in the immunological reaction complex. Therefore, EIA can be employed to detect and quantify antigens and antibodies in body fluids (Avrameas, 1981; Kurstak, 1986).

The positive advantages EIA have over other serological tests include the easiness of performing the tests and reading of results; adaptability to simple tests and to automation (detection systems range from visual to photometric with coloured, fluorescent or luminescent substrates); use of cheap and stable reagents with a long shelf life, and have minimal risk of contamination thus free from limiting legislation. EIA are suitable under field conditions and particularly for sero-prevalence surveys of large populations (Anon, 1976; Voller et al, 1979; Avrameas, 1981; Kurstak, 1986).

There are basically two types of EIA, homogenous and heterogenous, and in each type the tests can either be competitive or non-competitive.

(a) Homogenous EIA

These assays are performed in solution and are most often used for the detection of small compounds (haptens). They are based on the principle that the enzyme activity is altered during the immunological reaction of the labelled reagent (Vollier et al , 1976; Kurstak, 1986).

(b) Heterogenous EIA

These assays are particularly used in the detection of large molecules such as infections agents. They are based on the principle that one immunoreactant (antigen or antibody) can be immobilised by being passively adsorbed on to a solid phase

without losing its reactivity, and the complementary immunoreactant can be conjugated to an enzyme in such a way that it still retains both its immunoreactivity and enzymatic reactivity. It is essential in these assays that non-specific binding is avoided and free immunoreactants are separated from the bound (Voller et al, 1979; Kurstak, 1986).

Heterogenous EIA, especially Enzyme Linked Immunosorbent Assay (ELISA) has attracted most attention and has been employed in the diagnosis of trypanosomiasis (Voller et al, 1979; Luckins, 1977; Sillayo et al, 1980; Townsend et al, 1982; Duffus, 1982; Rae and Luckins, 1984; Liu and Pearson, 1987; Nantulya et al, 1987; Liu et al, 1988; Nantulya and Lindquist, 1989).

Fluorescent Antibody Tests (FAT)

Unlike in EIA, these tests employ antibodies labelled with a fluorochrome. The commonly used fluorochromes are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). After an immunological reaction, the antigen-antibody (Ag-Ab) complex is observed under ultra-violet stimulus using a fluorescence microscope. Two types of FA staining are distinguished, that is, a direct technique and an indirect technique.

Direct FAT

In this technique, the fluorochrome is linked to an antigen-specific primary antibody.

Trypanosomes are made to adhere to a glass slide and then coated with a specific fluorochrome labelled primary antibody. If the antibody combines with the trypanosome (antigen), it

remains attached even after washing of the preparation and the trypanosomes will be seen to fluoresce under a UV-microscope (Stephen, 1986). These methods are suitable in identifying unknown antigens using antigen specific antisera.

Indirect FAT (IFAT)

For this technique, the antigen adhered to a glass slide is first located with an unlabelled primary antibody in a test sample (serum) and a labelled second antibody (anti-species IgG) is used to detect the Ag-Ab complex.

IFAT also referred to as "sandwich methods" are more sensitive than the direct method (Stephen, 1986). The antigen used in IFAT is simpler to prepare than in ELISA; usually consisting of smears of blood stream trypanosome forms from a suitable animal either unfixed or commonly fixed in acetone, and they can be stored desiccated at -20^{o}C. The test sera can be applied as a spot dilution or as a series of dilutions and the end-point is judged by the intensity of the fluorescence. The judgement of the end-point needs considerable experience and may vary from one investigator to another, hence making the interpretation of the results subjective (Anon, 1976).

IFAT has been used in the diagnosis of trypanosomiasis, but it has little value for individual animals and finds its greatest usefulness when employed on a herd basis (Stephen, 1986). IFAT has been found to be a highly sensitive method in detection of trypanosomal antibodies and compares favourably with ELISA. The ability of IFAT to distinguish between infections caused by the subgenera of salivarian trypanosomes, though controversial, it is

generally difficult (Wilson, 1969; Zwart et al, 1973; Luckins, 1977; Luckins and Mehlitz, 1978; Wells et al, 1982; Stephen, 1986). Since antibody levels in trypanosomiasis do not reach detectable levels for a few days after infection, early infections can be missed; while the persistent high antibody levels following trypanocidal treatment also poses a problem in assessment of infections. False positives due to T. theileri antibodies may be encountered, however they can be eliminated by suitable dilution (1 : 160) of the test sera (Zwart et al, 1973).

It is virtually impossible to mechanise IFAT, thus fewer samples can be screened per day. Also, the use of expensive, electric current dependent UV-microscope makes the relative cost per test to be very high and renders it unsuitable for use under field conditions (Anon, 1976).

Complement Fixation Test (CFT)

This is essentially a two phase test based on the principle that when an antigen reacts with a specific antibody in presence of complement (usually in added guinea pig serum), the complement is fixed to the immunoreactants and loses its ability to act further. A second system is an indicator system usually consisting of sheep red blood cells and anti-sheep red blood cell serum. Following addition of the indicator, haemolysis of the sheep red cells will indicate the presence of free complement, that is, the antigen and antibody did not combine and therefore the test is negative. The test is positive when there is no haemolysis (Stephen, 1986).

There have been claims that CFT compares in sensitivity and specificity with IFAT in the diagnosis of experimental T. congolense infections in cattle and it was easier to read than IFAT. Staak and Lohding's (1979) experiment in cattle showed that the specificity of the result after the first infection was low in T. congolense, fair in T. brucei and high in T. vivax infected cattle. Reinfection with the same trypanosome species resulted in high specificity of CF results in all cases. Following trypanocidal treatment, the fastest drop in CF antibody titres was observed in T. congolense cases followed by T. brucei cases, while persistence over prolonged periods (more than 56 days) was observed in T. vivax infected animals.

Due to the cumbersome nature of the test, it has not gained any routine use in the diagnosis of African animal trypanosomiasis.

Radio-immunoassay (RIA)

Solid-phase RIA can be carried out in a similar way as ELISA using intact parasites centrifuged then fixed on microtitre plates. However, RIA has not been employed as a diagnostic test in trypanosomiasis due to the complicated nature of using radioisotopes in labelling of the immunoreagents which does not only present a health hazard but the labelled reagents have also a short shelf life. Also, the expensive, electrical dependent equipment needed makes the test unsuitable and not feasible under field conditions.

Haemagglutination Reactions

These rely on the observation that various inert particles with soluble antigenic material adsorbed to their surface agglutinates in the presence of an appropriate antibody. Therefore, red blood cells which are treated, for example, with tannic acid, then coated with trypanosome antigens will agglutinate in an observable manner in the presence of a homologous antibody. Haemagglutination tests are sensitive, however, the haemagglutination inhibition reaction is even more sensitive (Stephen, 1986). Woo and Soltys (1972) found in experimentally T. rhodesiense infected rabbits and T. brucei infected sheep that indirect haemagglutination test may be a simple and a reliable serodiagnostic test.

For the diagnosis of trypanosomiasis in man and animals, various tests have been tried, such as a red cell adhesion, trypanolytic reactions, charcoal agglutination, latex agglutination, capillary tube agglutination, immuno-conglutinin level etc., however they all detect antibody and in most cases they lack the necessary specificity required, particularly where multiple infections occur. Therefore, they have largely remained of value, in research/experimental procedures (Stephen, 1986).

In Vivo Tests

The result of antigen-antibody interaction in these tests are assessed by inoculation of the reactants into laboratory rodents. One of such tests is "Neutralisation of Infectivity" described by Lumsden et al (1963), however it has never gained any practical routine use as a diagnostic test in African animal

trypanosomiasis.

CONTROL OF AFRICAN ANIMAL TRYPANOSOMIASIS

Several control measures have been developed and used over the past years and others are still under experimentation. All in all, the control methods can be grouped into two main categories; control of trypanosomiasis in the vertebrate host, and the control of the vectors, tsetse flies.

Control of Trypanosomiasis in the Vertebrate Host

Chemotherapy and Chemoprophylaxis

This is discussed extensively in the next section.

Use of Trypanotolerant Animals

Trypanotolerance or reduced susceptibility to African trypanosomiasis has been observed in some breeds of cattle, sheep and goats in Africa. It has been suggested that it results from a complex mixture of innate non-specific and acquired specific immune mechanisms mediated by physiological, biochemical and immunological factors (Vickerman and Barry, 1982).

The resistance to trypanosomiasis has been concluded to be genetic in nature as it has apparently been produced by natural selection, and therefore there is a possibility of breeding for tolerance (Finelle, 1980; Murray et al, 1981; Murray and Trail, 1984).

Reports by ILCA (1979) and Murray et al (1981) show that, despite the relative small size of the trypanotolerant breeds, they can survive and be productive in trypanosomiasis endemic

areas where Zebu perish. Those living under low or zero tsetse challenge are at least as productive as the other less tolerant breeds. The exploitation of trypanotolerant breeds offers therefore, one of the most important strategies to the control of African animal trypanosomiasis. However such breeds are largely restricted to West Africa.

Vaccination

It is not yet feasible to vaccinate animals against trypanosomiasis and no encouraging results from attempts to induce immunological protection under field conditions have been reported. Researchers have been compiling a "wardrobe" of all possible variations of glycoproteins. However, even though such a collection called a serodeme could be identified, synthesised and injected into an animal, it is unlikely to be effective since the animal's immune system would not respond adequately to so many different antigens.

Vaccination may be possible only after knowing how the process of antigenic variation takes place and how to disrupt it. Extension of studies such as those of Tetley et al (1981) and Jenni and Brun (1981), may be helpful.

Vector Control

Vector control is one of the important measures used to eliminate trypanosomiasis from an area. Different methods have been applied, however total elimination of tsetse has always been difficult to achieve due to the re-invasion of the treated zones by tsetse from adjacent infestations or by recrudescence of small

foci that have been difficult to eliminate. This has also been aggravated by the absence of well defined area development projects (Finelle, 1980).

The methods employed for controlling the vectors can be grouped into two main categories, viz. biological and chemical control.

Biological Control

This involves methods such as the use of predators, parasites, pathogens, release of sterile males, cytogenetical manipulations (genetical control) and destruction of tsetse habitat (ecological control).

Bush clearing to deprive the vectors of shade, moisture and breeding sites, which are essential for its survival, replaced after the second world war, the ill-conceived, if well meaning massacre of wildlife (Allsopp et al, 1985). Bush clearing has been extensively used and may still be useful, however the labour costs involved and the risk of soil erosion precludes its application on a large scale.

Promising results have been obtained with applied research on the release of sterile males (Sterile Insect Techniques - SIT) in Tanzania, Nigeria and Zimbabwe, however its effectiveness and economic implications have not yet been fully determined (Finelle, 1980; House, 1982; Takken et al 1986; Brandl, 1988).

The use of predators, parasites and pathogens of the vector's puparial and adult stages are possibilities which have been attempted but are yet to be put into practice (Nantulya,

1986).

Chemical Control

The use of insecticides still remains as the most reliable and practical alternative for large scale tsetse control (Finelle, 1980; Allsopp, 1984). Insecticides have been in use since the 1940s following the successful early trials with DDT and other chlorinated hydrocarbons around Lake Victoria in Tanzania, and later on in Kenya, Uganda, Nigeria and Zimbabwe by ground spraying using pressurised knapsack sprayers (Allsopp et al, 1985).

The low dosage aerosal technique which proved highly successful and environmentally acceptable was later adopted. Sequential aerial spraying became widely established as an effective technique for rapid and extensive control of tsetse (Allsopp et al, 1985); and it is considered to be more economical than the trapping of tsetse and SIT in long term, for programmes covering large areas (Brandl, 1988). While the application of conventional ground spraying is hampered by high rainfall, aerial spraying has its own logistic problems. The spraying at night to overcome meteorological conditions (convection and high wind) results in the inability to deposit the insecticide effectively on the ground in hilly areas and on tall forest trees. Aerosol spraying also demands efficient ground support and expensive, sophisticated equipment (Allsopp et al, 1985).

Simple, cheaper and environmentally more acceptable methods came into use following the appearance of the "Challier-Laveissiere biconical trap" in 1973. Traps baited with acetone

or carbon dioxide and 1-octen-3-ol, and impregnated with a pyrethroid insecticide, detamethrin, have been in use with very promising successes in Zimbabwe, Malawi, Zambia, Cote d'ivoire and Nigeria (Allsopp et al, 1985; Brandl, 1988). Takken et al (1986) found that insecticide impregnated targets adequately controlled tsetse population in marginal habitats in central Nigeria, and the same targets were also efficient barriers in preventing reinvasion of the treated area.

As it has been argued by Allsopp (1984), an integrated insecticidal approach combining the benefits of various chemical, and possibly non-chemical control methods, could provide a solution to the persistent tsetse and trypanosomiasis problems in Africa.

CHEMOTHERAPY AND CHEMOPROPHYLAXIS OF AFRICAN ANIMAL TRYPANOSOMIASIS

Chemotherapy and chemoprophylaxis are of particular importance as practicable means of combating trypanosome infections in animals. Trypanocides are widely used and it is estimated that, over 25 million treatments are carried out annually in Africa, the majority being for the treatment and control of bovine trypanosomiasis (Tacher, 1982). The trypanocides that have been developed and used for curative and prophylactic purposes for the control of animal trypanosomiasis are categorised in the following text.

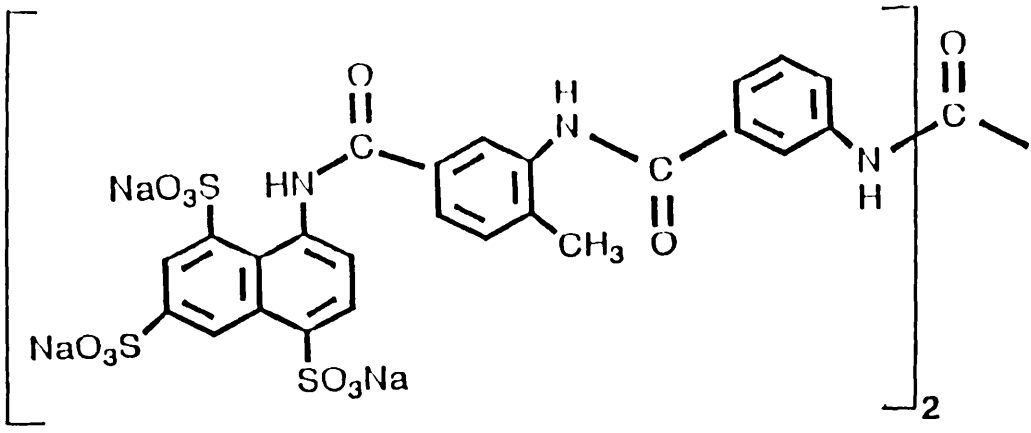
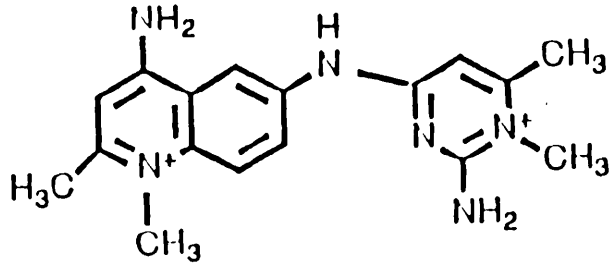


Figure 1. Suramin Sodium



$(CH_3SO_4)_2$

Figure 2. Quinapyramine Dimethosulphate

Arsenic Compounds

These were in use as early as the middle of the 19th Century to combat trypanosomiasis in Africa, however they are no longer of significance in animal trypanosomiasis (Ruchel, 1975).

Antimony Compounds

Potassium-antimony tartate (tartar emetic) or its sodium analogue was first shown by Plimmer and Thomson in 1908 to be able to eliminate infections with T. brucei and T. evansi in laboratory rodents. Due to its severe tissue reaction, it was administered intravenously in cattle at a dosage of 1.0 - 1.5 g in a 5% aqueous solution and sometimes repeated at daily or weekly intervals. However, it was later proved to be effective only against T. vivax and T. congolense but not T. brucei infections in cattle. Though the drug caused about 6% mortality due to its toxicity in treated cattle, it was used on a large scale in Africa, and it continued to be in use in some areas until the early fifties (Leach and Roberts, 1981).

Pentasodium antimonybiscatechol-3,5 disulphate (Antimosan^{R}, Bayer Company) was used in 6.3% tissue-isotonic, almost neutral solution to treat T. congolense and T. vivax infections in cattle (Ruchel, 1975).

Sulphonated Naphthylamine Compounds

Suramin (Bayer 205^{R}, Antrypol^{R}, Moranyl^{R}) was developed in Germany between 1914-1918 and became available in 1920. Its development began with the synthetic cotton dye, Congo Red, from which the first metal-free trypanocides, trypan red and trypan

blue emerged.

More active and colourless compounds were produced by substituting urea for benzidine as the central link for the two halves of the symmetrical polycyclic dye molecule.

Suramin (Figure 1) was shown to be effective against experimental infections with T. equiperdum and naturally occurring T. evansi infections in camels. To date, suramin has remained as the drug of choice in the treatment of Trypanozoon infections in equines and T. evansi infections in camels as it is less toxic in these species than quinapyramine. It is given intravenously as it can cause severe local reactions when administered by other routes (Leach and Roberts, 1981).

Quinoline and Phenanthridine Compounds

The development of the drugs contained in this group followed the findings of the trypanocidal activity in acridine dye, notably acriflavine. Dissection of the linear 3-ring molecular structure gave rise to compounds consisting of the 2-ring portion containing a nitrogen atom (i.e. quinoline), or of the isometric non-linear 3-ring phenanthridine derivatives which all proved to be trypanocidal.

4-aminoquinoline derivative, surfen-C, showed marked activity against T. congolense. Nevertheless, the field trials of the 1930s in Africa led to its unacceptability due to its toxic reactions when administered either intravenously, intramuscularly or subcutaneously. Further developments of this drug subsequently led to the synthesis in the early fifties of quinapyramines (Antrycide^{R}).

Quinapyramine dimethosulphate (Figure 2) is a yellow powder with up to 33% solubility in water. It is active against T. congolense, T. vivax and T. brucei, and is used for curative purposes in all domestic animals at a recommended dose of 4.4 mg/kg bodyweight (BW) subcutaneously. Sometimes, it may provoke toxic reactions particularly in equines, nonetheless this may be avoided by dividing the dose into two halves and giving them at 5 - 6 hours interval (Ruchel, 1975; Leach and Roberts, 1981).

Quinapyramine chloride is sparingly soluble in water, and subcutaneous administration was shown to result in a formation of a depot at the injection site with a slow release of the drug. Thus, though the trypanocidal levels are reached, they are too low for curative purposes. A mixture containing three parts by weight of the quinapyramine dimethosulphate and four parts by weight of quinapyramine chloride was made and marketed as Antrycide Prosalt^{R}, with a recommended dosage of 11 mg/kg giving a 5 mg/kg quinapyramine dimethosulphate. Subsequently a revised formulation (RF) in which the quinapyramine chloride is halved and the recommended dosage reduced to 7.4 mg/kg BW was introduced and proved to be equally effective and less costly. In this mixture the therapeutic and prophylactic actions and effects of the two basic preparations are combined, providing a 2 - 4 month protection (Ruchel, 1975; Leach and Roberts, 1981; Finelle, 1983b).

Phenidium chloride was the first in the aminophenanthridine series to be used in the treatment of animal trypanosomiasis in

1931, and its effectiveness against T. congolense and T. vivax infections in cattle was reported by Browning et al (1938). However, due to its low solubility and a narrow therapeutic index, attention was directed to other phenanthridines with an eventual development of dimidium bromide.

Dimidium bromide proved to be effective against T. congolense and able to eliminate the majority of infections at a dosage of 1 mg/kg BW subcutaneously. The drug was used for mass treatment campaigns in East and Central Africa, however by 1952 drug resistance became widespread and the use of higher doses resulted in severe toxicity exhibited by local reactions and photosensitisation after treatments in cattle at 1 - 2 mg/kg BW subcutaneously (Evans, 1948).

Homidium bromide (Ethidium^{R}, CAMCO) was developed by substituting an ethyl for a methyl group on the quaternary heteroatom of dimidium (Watkins and Wolfe, 1952), and the drug proved to be as equally effective as dimidium against T. vivax and T. congolense infections at 1 mg/kg BW intramuscularly, and less toxic. Ethidium is an almost odourless, dark purple, crystalline or amorphous powder with a little solubility in cold water (1 : 29) increasing to 1 : 10 in hot water. The preparation has mainly a curative effect, and overdosages result in damage to the liver, oedemata or even death (Ruchel, 1975; Leach and Roberts, 1981).

Homidium chloride (Novidium^{R}, RMB Animal Health Ltd.) is equally active therapeutically at a dose of 1 mg/kg BW intramuscularly and is soluble in cold water. Photosensitisation

of the skin is possible with light-coloured animals or local swellings may occur (Ruchel, 1975). Homidium compounds (Figure 3) have been shown to have a selective effect on T. vivax infections in cattle and have also been used with success in treating T. vivax infections in the horse. The trypanocidal action of homidium compounds is related to the ability of the drug to bind with trypanosome's kinetoplast DNA (Wagner, 1971; Henderson et al, 1977).

Pyrithidium bromide (Prothidium^{R}, May and Baker Ltd.) was produced by the substitution of the pyrimidyl moiety of quinapyramine for a hydrogen of the 7-amino group of 2,7-diamino-9-p-aminophenyl-10-methyl phenanthridium chloride (Watkins and Woolfe, 1956). The drug (Figure 4) is an odourless, red or reddish purple powder. Solutions of 2% are made by dissolving the drug in boiling water, and when used, have been shown to eliminate T. congolense infections in the field when given at 0.2 - 0.4 mg/kg BW intramuscularly or subcutaneously (Whiteside, 1960). Intramuscular injections of 2.0 mg/kg BW have been shown to give protection in cattle against T. vivax and T. congolense for 2 - 4 months (Finelle, 1983b).

Wragg et al (1958), developed metamidium by following the same principles used in the synthesis of pyrithidium. They combined the diazotized p-aminobenzamide moiety of the diminazine molecule with homidium chloride in the presence of sodium acetate giving rise to two isomers both of which have trypanocidal action. It was later revealed that the more active of the two products, that is, metamidium, was itself a mixture of

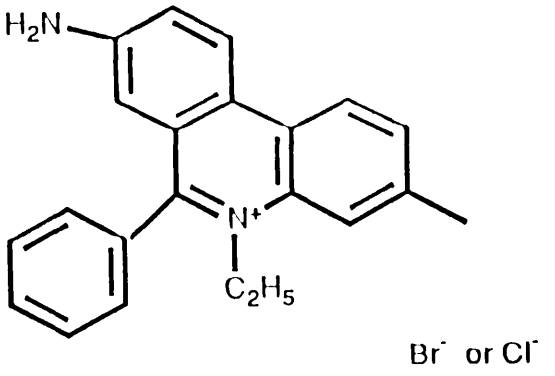


Figure 3. Homidium Bromide or Chloride

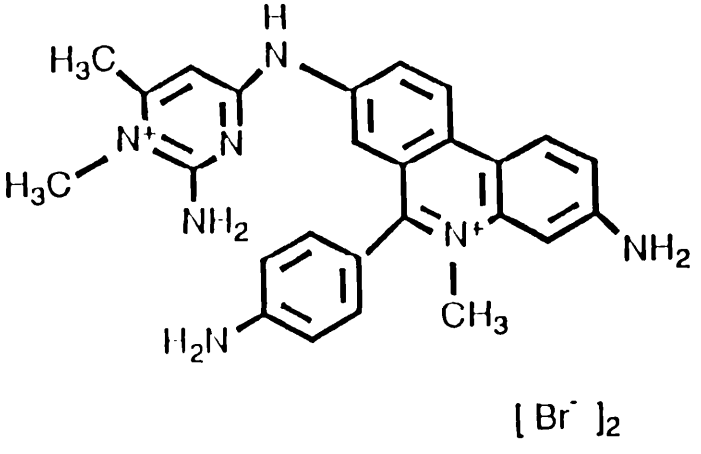


Figure 4. Pyrithidium Bromide

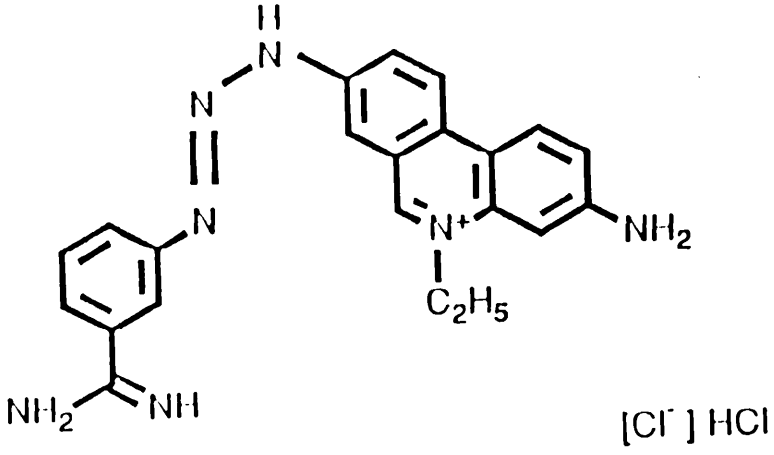


Figure 5. Isometamidium Chloride

two isomers. Berg (1960; 1963) clarified the structure of the soluble, highly active red isomer and was able to isolate it in sufficient quantities for field trials; it was named isometamidium (Samorin^{R}, RMB Animal Health Ltd.).

Isometamidium (Figure 5) was first marketed in 1961 and it was recommended to be administered intramuscularly at a dose of 0.5 mg/kg BW for curative purposes of sensitive strains, 1 - 2 mg/kg BW for drug resistant strains and 2 mg/kg BW for prophylaxis.

Intramuscular injection of isometamidium can cause severe local reactions and extensive fibrosis (Boyt, 1971; Finelle, 1983a). Kinabo and Bogan (1988d) showed that the necrotic tissue at the injection site to be well defined, ovoid in shape measuring about 8 cm in length, with a cross section diameter of 2.5 cm at the middle and surrounded by a markedly oedematous muscular tissue. At six weeks following drug administration, the necrotic tissue was surrounded by a dense large mass of fibrous tissue, and despite the lesion appearing to contract grossly, microscopically the lesion was similar to that observed at three weeks.

Finelle (1983a) advised dividing the dose so that the injection volume does not exceed 15 ml per one injection site. Toure (1973a) showed that intravenous doses of 0.5 mg/kg BW can be well tolerated by goats and cattle. Intravenous injection can be an alternative method of isometamidium administration provided that the injection is made slowly, leakage to subcutaneous tissue and solutions of high strength (more than 2% w/v aqueous)

avoided, and the correct dosage made (Toure, 1973b; Kalu, 1983; Kalu et al, 1983; Ali and Hassan, 1984; Dowler et al, 1989).

Isometamidium, like homidium, displays antitumour effects, apparently related to the inhibition of nucleic acid synthesis due to the drugs binding to DNA (Wagner, 1971; Henderson et al, 1977). Kinabo and Bogan (1987) reported that isometamidium has a higher affinity for DNA as compared to homidium. The interaction of isometamidium with anionic lipids may also account for some of its actions as lipid requirements of trypanosomes are considered to be largely exogenous and incorporated into phospholipids upon uptake. It is probable that isometamidium interferes with the uptake mechanism or the intermediary metabolic steps.

Aromatic Diamidines

Diminazine aceturate (Berenil^{R}, Farbwerke Hoechst A.G.) was developed from surfen C as detailed by Jensch (1958). The drug is an odourless, yellow powder, with a solubility of 1:14 in cold water (20^{o}C) and only slightly soluble in alcohol, ether and chloroform. Solution can be used for up to 10 - 15 days when stored at room temperature.

Berenil is administered intramuscularly at a recommended dose of 3.5 mg/kg BW. This has been shown to eliminate infections with T. vivax and T. congolense, but a dose of 5 mg/kg BW is required to eliminate infections with T. brucei. The drug is also effective against Babesia spp (Leach and Roberts, 1981). Berenil is well tolerated by most domestic animals, for example in cattle, Fairclough (1963) showed that

doses of up to 21 mg/kg BW could safely be given.

Almost all of a parenterally administered dose of Berenil is rapidly excreted through the kidneys and cleared from blood within 24 hours after injection, this makes the drug unsuitable for prophylactic use. The characteristic of quick clearance has been thought to reduce the risk of development of resistance. However, resistance to Berenil has been observed since 1967 in T. vivax and T. congolense infections, notably in the Central African Republic, Chad, Nigeria, Kenya and Uganda (Finelle, 1983a) and recently reported in Tanzania (Mbwambo et al, 1988). Finelle (1983a) advised the use of another trypanocide such as isometamidium when resistance occurs rather than increasing the dosage of Berenil.

A summary of the aforementioned trypanocides currently employed for the treatment of animal trypanosomiasis is given in Table 4, whereas Table 5 shows the drugs that are used for prophylactic purposes.

Isometamidium chloride is currently the only available drug with both curative and prophylactic activity against salivarian trypanosomes in domestic animals (Kinabo, 1988a). Strict adherence to the treatment regimens so as to avoid the development of drug resistance is essential. This has not been helped by fluctuations in availability of the drugs in Africa. Moreover, chemoprophylaxis has been limited under conditions of traditional animal production. The problems associated with chemotherapy and chemoprophylaxis of African animal trypanosomiasis, namely, pharmacological (range of drugs

Table 4

Curative Trypanocides in Animal Trypanosomiasis

Drug	Trade Name	Condition of Use		Activity on Trypanosomes		Toxicity			Treatment of Relapses
		Solution	Dose[a]	Very Active	Less Active	Well Tolerated	Possible Local Reactions	Possible General Reactions	
Homidium bromide	Ethidium[b]	2% hot water	1 i.m.	T. vivax		Bovines			Diminazene
Homidium chloride	Novidium[c]	2% cold water		T. congolense		Ovines Caprines	Equines		Diminazene
Diminazene aceturate	Berenil[d]	7% cold water	3.5 s.c. or i.m.	T. congolense T. vivax	T. brucei T. evansi	Bovines Ovines Caprines	Equines	Equines Camels Dogs	Isometamidium
Quinapyramine sulfate	Trypacide Sulphate[e]	10% cold water	5 s.c.	T. congolense T. vivax T. brucei T. evansi		Bovines Ovines Caprines Camels	Equines	Dog	Isometamidium
Isometamidium chloride	Samorin[c] Trypamidium[f]	1 or 2% cold water	0.25 to 1 deep i.m.	T. vivax T. congolense	T. brucei	Bovines Ovines Caprines Equines Dogs	Bovines		Diminazene
Suramin		10% cold water	10 i.m.	T. evansi T. brucei		Camels Equines			Quinapyramine

a The dose is stated in mg per kg of body weight; i.m. = intramuscular, s.c. = subcutaneous
b CAMCO, UK.
c RMB Animal Health Ltd.
d Farbwerke Hoechst A.G.
e RMB Animal Health
f Specia

Toure (1973) and Dowler et al (1989) showed that isometamidium can also be administered intravenously

Table 5

Trypanocides Used for Chemoprophylaxis

Drug	Trade Name	Conditions of Use: Treatment Solution	Conditions of Use: Dose[a]	Activity: Trypanosomes	Activity: Protection (Months)	Toxicity: Well Tolerated	Toxicity: Possible Local Reactions	Treatment of Relapses
Isometamidium chloride	Samorin[b] Trypamidium[c]	1 to 2% cold water	0.5 to 1 deep i.m.	T. vivax T. congolense T. brucei	2-4	Bovines Ovines Caprines Equines Dogs	Bovines	Diminazene Diminazene
Pyrithidium bromide	Prothidium[d]	2% boiling water	2 deep i.m.	T. vivax T. congolense	2-4	Bovines Ovines Caprines Dogs	Bovines Equines	Diminazene Isometamidium
Quinapyramine chloride+ sulfate	Trypacide Pro-salt	3.5 g for 15 ml cold water	7.4 s.c.	T. brucei T. evansi	2	Equines Camels Bovines	Equines	Suramin
Suramin-quinapyramine complex		5% cold water	40 (quinpyramine) s.c.	T. simiae T. evansi	Young pigs 3 Adults 6	Pigs Equines Camels		Isometamidium 12.5 to 35 mg/kg Isometamidium Diminazene

a The dose is stated in mg per kg of body weight; i.m. = intramuscular, s.c. = subcutaneous
b RMB Animal Health Ltd.
c Specia
d CAMCO, U.K.
e RMB Animal Health

available, drug resistance, toxicity, duration of prophylaxis) and logistical and financial problems (communications and facilities, personnel, education, finance, political structure) have been well discussed by Holmes and Scott (1982).

In tsetse infested areas, chemoprophylaxis using isometamidium chloride has been shown to be economical and effective method of control of animal trypanosomiasis (Ford and Blaser, 1971; Wilson et al, 1975; 1976; Logan et al, 1979; Owero Wafula and Mayende, 1979; Morrison et al, 1981; Holmes and Scott, 1982; Trail et al, 1985; Dowler et al, 1989).

The precise duration of prophylaxis with isometamidium chloride is still debatable and varies widely. The factors which may influence the duration of prophylaxis have been thought to include drug dosage, stress and plane of nutrition, concurrent diseases, breed susceptibility and/or tolerance, level of parasite challenge, trypanosome species and the host immune system (Toure, 1973b; Njogu et al, 1985; Whitelaw et al, 1986; Peregrine et al, 1988; Jibbo et al, 1988; Ogunyemi and Ilemobade, 1989). However, most of these factors have not been thoroughly investigated.

It can be drawn from Table 6 that higher drug dosages give a longer period of prophylaxis than lower dosages. This is supported by the work of Peregrine et al (1988) using T. congolense metacyclics, who demonstrated a direct relationship between drug dosage and the duration of chemoprophylaxis. It has been suggested by Whitelaw et al (1986), drug residues at the level of the skin may play an important role in the mode of

Table 6

Effect of Drug Dosage on the Duration of Isometamidium Chloride Prophylaxis

(From Ogunyemi and Ilemobade, 1989)

Drug Dosage (mg/kg)	Experimental Condition	Minimum Duration of Protection	References
1.0	Field. Trypanosomiasis risk unstated	3 months (95 days)	Kirkby, 1961 (Nigeria)
2.0	Field. Trypanosomiasis risk unstated	4 months (116 days)	Kirkby, 1961 (Nigeria)
2.0	Field. Medium risk	3 months (93 days)	Robson, 1962 (Tanzania)
4.0	Field. Medium risk	10 months (304 days)	Robson, 1962 (Tanzania)
0.5	Medium to high risk	3.5 months	Fairclough, 1963 (Kenya)
1.0	Low risk	3 months	Wiesenhutter et al, 1968 (Tanzania)
1.0	Laboratory condition	5 months	Whitelaw et al, 1986 (Kenya)
0.5	Laboratory condition	3 months	Ogunyemi, 1986 (Kenya) Peregrine et al, 1988 (Kenya)
1.0	Laboratory condition	4 months	Ogunyemi, 1986 (Kenya) Peregrine et al, 1988 (Kenya)

action of isometamidium chloride. It is possible that the higher the dosage, the higher is the amount of drug in depot tissues; nonetheless, other factors such as the injection site and/or the mode of administration, the degree of tissue reaction at the injection site and physical exercise of the animal may also be important. Since dosages higher than 1.0 mg/kg BW are normally not recommended, formulations which have shown to provide longer duration of prophylaxis without adverse effects to the animal, such as isometamidium-dextran sulphate complex (Aliu and Sannusi, 1979) or liposomal formulation of isometamidium chloride (Fluck and Hopkins, 1987) may be useful in the future.

In contrast to laboratory or well controlled experimental conditions, animals in the field are subjected to various stresses and periods of poor nutrition. The relationships between such stresses or varying levels of nutrition and the duration of prophylaxis have not been fully investigated.

In their experiment (Whitelaw *et al*, 1986), no anti-trypanosome antibodies were detected in the animals which successfully resisted challenge and they concluded that antigenic priming of the immune system does not occur during chemoprophylaxis. Peregrine *et al* (1988) also found that antibodies to metacyclics did not appear in any of the cattle as long as chemoprophylaxis was effective, and in a group (challenged with 5×10^5 *in vitro* derived metacyclic trypanosomes) where low antibody titres were found, they proved to be non-protective. They concluded that antibody is unlikely to play any significant protective role in animals maintained by

a chemoprophylactic regime with isometamidium.

Comparative studies pertaining to the relative duration of prophylaxis by isometamidium against T. vivax and T. congolense in cattle under controlled conditions are lacking. The results obtained by various studies under laboratory conditions (Table 7) indicate that the duration of prophylaxis may be shorter in T. vivax than in T. congolense infections at a dose of 0.5 mg/kg BW; however, at 1.0 mg/kg BW the duration is similar.

Earlier studies by Whiteside (1962), Trail et al (1985) and Jibbo et al (1988) appear to indicate that the duration of chemoprophylaxis with isometamidium was reduced in areas of high tsetse challenge. However, they lack the evidence of an existing relationship between high tsetse density and high trypanosomiasis risk or level of challenge. It was found out by Peregrine et al (1988) that the duration of prophylaxis afforded by isometamidium in the two T. congolense serodemes examined were the same, irrespective of the method or weight of challenge. In the same experiment, they showed that when isometamidium is used to treat an existing infection in cattle, it offers the same degree of chemoprophylactic activity against an unrelated serodeme, thus a concurrent infection does not appear to affect the duration of prophylaxis.

In principle, it would be advisable to make preliminary trials in order to determine the treatment rate per year in a given locality, and Berenil should be used as a "sanitive" if relapses occur before a second prophylactic dose of isometamidium is due (Finelle, 1983b).

Table 7

The Duration of Isometamidium Prophylaxis Against Different Trypanosome Species in Cattle under Laboratory Conditions

Trypanosome	Drug Dosage (mg/kg BW)	Duration (Months)	Reference
T. vivax	0.5	2	Aliu and Sannusi (1979)
T. vivax (IL2968)	0.5	2	ILRAD (1987)
T. vivax (IL2969)	0.5	1	ILRAD (1987)
T. congolense	0.5	3	Peregrine et al (1988)
T. vivax	1.0	4	Toro et al (1983)
T. congolense	1.0	4	Peregrine et al (1988)
T. congolense	1.0	5	Whitelaw et al (1986)

All in all, as advised by Lee and Maurice (1983), the treatment with prophylactic drugs should be carried out; firstly, when animals are to be exposed to tsetse challenge for a specific and limited period of time, which is shorter than the protective period of the drug; and secondly, when the animals are stationary (i.e. not nomadic) and are available throughout the year for regular inspection and treatment.

Drug Resistance in Chemotherapy and Chemoprophylaxis

The extensive use of trypanocidal drugs for the treatment and control of African animal trypanosomiasis has resulted in the appearance of drug-resistant trypanosomes in many parts of Africa (Evans, 1948; Whiteside, 1961; Jones-Davies, 1967; Anon, 1979; Gitatha, 1979; Morrison et al, 1981; Finelle, 1983a; Lee and Maurice, 1983; Mbwambo et al, 1988). The problem of drug resistance became so prevalent in some areas, for example, Nigeria (Na'Isa, 1967) so as to warrant the withdrawal of homidium chloride (Novidium) from general use. Drug resistance may have also been advocated by having a limited number of effective trypanocides considering that no new drugs have been brought to market for more than 30 years.

Under field conditions, drug resistance refers usually to the failure to cure an infection with a normal curative dose of the drug concerned. This is of practical importance as the curative dose is near the maximum tolerated dose with many trypanocidal drugs (Leach and Roberts, 1981). The resistance can either be natural or acquired/developed.

Natural resistance of a trypanosome strain or species to a drug is that variation in drug sensitivity shown by trypanosomes that is not dependent on previous exposure to the particular drug. For example, homidium is more effective against T. vivax than against T. congolense; whereas West African strains of T. vivax have a higher level of natural resistance to diminazine than T. congolense (Leach and Roberts, 1981).

Development of drug resistance in the field is thought to occur as a result of underdosing, whereby the concentration of the trypanocide in the animal's body is below the effective level to ensure destruction of the trypanosomes. This can result from frank underdosing (incorrect dosage) particularly in mass treatments, irregular prophylactic regimes or stopping the use of a trypanocide while the animals are still at risk especially in areas of high trypanosome challenge and the occasional use of prophylactic drugs in curative treatments (Whiteside, 1961; Finelle, 1983b).

Cross-resistance in trypanosomes to many of the trypanocides used has also been reported (Whiteside, 1961; Na'Isa, 1967 Williamson, 1982; Williamson et al, 1982; Finelle, 1983a; Lee and Maurice, 1983) arising from the close relationships of the drugs' chemical composition. Table 8, illustrates the cross-resistance existing between trypanocides (Whiteside, 1961). The analysis of cross-resistance led to the proposition of the term "sanitive", that is, for a drug which cures infections resisting treatment with other drugs. For example, isometamidium is effective

Table 8

Drug Cross-Resistance Analysis (From Whiteside, 1961)

Trypanosomes Resistant to	Response of Trypanosomes to: Quinapyramine	Homidium	Prothidium	Metamidium	Diminazene
Quinapyramine	R	++	+	+	++
Homidium	+	R	+	+	O
Prothidium	+	++	R	O	?
Metamidium	+	++	+	R	O
Diminazene	O	O	O	O	R

R	Direct Resistance
+	Cross-Resistance to Curative Dose
++	Cross-Resistance to Higher Dose
O	No Cross-Resistance

against trypanosomes resistant to quinapyramine, homidium, diminazine and pyrithidium. "Sanitive pairs" are thus pairs of potent curative drugs which do not provoke resistance the one to the other, such as; homidium and diminazine or metamidium and diminazine, or isometamidium and diminazine (Whiteside, 1961; Toure, 1973b; Anon, 1979; Finelle, 1983a).

The alternate use of two drugs that do not cause mutual cross-resistance may therefore furnish a reasonable prospect of controlling African animal trypanosomiasis provided the standards of veterinary supervision and animal husbandry are high.

CHAPTER TWO

THE EXTENT OF ISOMETAMIDIUM BINDING TO SERUM PROTEINS IN TREATED ANIMALS

INTRODUCTION

Following an intramuscular injection, isometamidium chloride primarily accumulates at the injection site followed by the liver, kidney and spleen from where it is slowly released into the circulation. However, when the drug is injected intravenously, large amounts tend to accumulate in the parenchymatous organs, particularly liver and kidneys (Philips et al, 1967; Braide and Eghianruwa, 1980; Kinabo and Bogan, 1988a).

Philips et al (1967) reported that isometamidium is bound by serum albumin, deoxy- and ribonucleic acids, heparin and hyaluronic acid. The binding to DNA is further supported in the studies by Wagner (1971) and Kinabo and Bogan (1987) who advocated an existence of two binding sites with different affinities for isometamidium. A strong binding seems to be due to intercalation, and a weaker binding involves electrostatic interaction between the drug and phosphate groups of DNA. Insoluble complexes are also formed with nucleic acids and mucopolysaccharides when there is neutralisation of the cationic drug and the polyanionic molecules. However, further studies pertaining to the acclaimed extensive binding of the drug to plasma proteins have not been reported.

It has been shown following treatment that isometamidium is cleared rapidly from serum to concentrations of 0.7 and 1.7 μg/ml plasma 24 hours after an intravenous administration of 0.5 and 1.0 mg/kg BW respectively in camels (Ali and Hassan, 1984); 2.17 μg/ml serum 24 hours after an intramuscular administration

of 0.5 mg/kg BW in goats (Braide and Eghianruwa, 1980); and below 10 ng/ml serum within 24-48 hours after either an intravenous or intramuscular administration of 0.5 mg/kg BW in cattle (Kinabo and Bogan, 1988b). The kinetic analysis of the serum concentration versus time data by Kinabo and Bogan (1988b) in cattle, revealed a terminal phase elimination half-life of 1.3 hours and a volume of distribution of 1.2 l/kg.

The major constraint in studying chemoprophylaxis of African animal trypanosomiasis, and consequently the possible significance of drug resistance has been the lack of suitable sensitive tests which are able to detect very low levels of isometamidium chloride in serum and tissues. The main problems associated with the assay of isometamidium chloride in biological matrices are the low drug concentrations in plasma of treated animals, its apparent extensive binding to plasma and tissue macromolecules, and its instability in extreme pH conditions and high temperatures (Kinabo and Bogan, 1988a).

The existing assays for isometamidium chloride in serum and tissues include, spectrophotometric assays (Philips et al, 1967; Braide and Eghianruwa, 1980), HPLC assay of Perschke and Vollner (1985), radioimmunoassay (Kinabo and Bogan, 1988c) and a solid phase extraction followed by ion-pair reversed-phase HPLC described by Kinabo and Bogan (1988b).

The spectrophotometric assay is not specific and sufficiently sensitive to determine isometamidium chloride concentrations lower than 1 μg/ml in plasma or 2 μg per gram of tissue. The HPLC assay (Perschke and Vollner, 1985) has a better

sensitivity with a detection limit of 100 ng/ml serum. However, it uses large amounts of sample (about 1 ml of serum) and is indirect in that isometamidium is first converted to homidium before separation and detection. The radioimmunoassay for isometamidium using anti-isometamidium antibodies raised in sheep and ^{14}C-labelled isometamidium as a radioligand has a limit of detection of 29 ng/ml serum without extraction although it probably has potential for improvement. The limits of detection of the ion-pair HPLC method with fluorimetric detection (Kinabo and Bogan, 1988b) are 10 ng/ml of serum and about 400 ng/g of wet tissue.

Recently, an ultrasensitive ELISA capable of detecting isometamidium as low as 5 - 10 pg/ml in sera of treated cattle has been developed by Whitelaw et al (in press). The assay utilises anti-isometamidium antibody raised in sheep and functions on the basis of competition. Isometamidium conjugated to egg albumin is adsorbed on to the solid phase, and this competes with isometamidium in the test sample for binding sites on the anti-isometamidium antibody. At high sample drug concentration, the anti-isometamidium antibody complexes predominantly with the drug in the liquid phase and to a much lesser extent to that on the solid phase, hence low optic density (OD) results on the development of the assay with a chromophore. Conversely, a high OD is obtained at low sample concentration of isometamidium.

The study described in this chapter was aimed at determining the extent of binding of isometamidium chloride to serum proteins

by employing TCA precipitation and ultra-filtration methods for the separation of free and bound drug. The levels of the isometamidium were measured by the recently developed ultra-sensitive ELISA.

MATERIALS AND METHODS

Production and Purification of Sheep Anti-Isometamidium Antibody (IgG)

Materials

Isometamidium antiserum produced in sheep was kindly donated by Elizabeth Gault (Joint ODA Project, Departments of Veterinary Physiology and Parasitology, Glasgow Veterinary School). The immunogen used for challenge was isometamidium conjugated to porcine thyroglobulin (PTG).

An LKB fraction collector (LKB Instruments Ltd. 6522 A) was used to collect the separated fractions of the purified antibody and a protein estimation kit was obtained from the Sigma Chemical Company.

The chemicals and reagents used were of analytical grade purchased from British Drug House.

Methods

A saturated ammonium sulphate solution was prepared by heating at 50°C and stirring a mixture of 1000 g $(NH_4)SO_4$ in 1000 ml distilled water until most of the salt was dissolved. This was left to stand overnight at room temperature before the pH of the solution was adjusted to 7.4 by addition of sulphuric

acid.

A multi-step method described by Catty and Raykundalia (1988) to precipitate the IgG fraction of the immunoglobulin was followed albeit, with modifications, as described below.

Saturated ammonium sulphate solution at $0^{o}C$ was added drop wise to 45 ml of whole antiserum on ice with constant stirring until a 33% saturated (v/v) final concentration was reached. The mixture was then left to stand for one hour on ice with mixing (without frothing) before being centrifuged at 3,000 rpm for 15 minutes. The precipitate was washed in ice-cold 33% (v/v in distilled water) ammonium sulphate, by centrifugation and resuspension.

The final precipitate was dissolved without frothing in 0.01M phosphate buffer, pH 7.2 to give about 15 ml of an euglobin solution which was then dialysed against the same buffer overnight.

A DEAE column was prepared and equilibrated with the same phosphate buffer allowing 15 - 20 ml as settled volume per 5 ml euglobin solution. The column was well washed and when the packing was completed, excess buffer was removed from the top and the euglobin layered-on. When the euglobin entered the matrix, the top-end of column was attached to a gradient buffer reservoir (0.01M - 0.71M phosphate buffer pH 7.2). Using a LKB fraction collector, the column was run at about 20 ml/hour and 5 ml samples collected.

Three peaks which were collected were then concentrated using polyethylene glycol 20,000.

Sephadex G200 was equilibrated overnight using 0.1M TRIS-HCl buffer pH 7.2. It was then washed using the same buffer, degassed and transferred to a column. The same buffer was passed for one hour to make the column settle before the concentrated fractions I, II and III were separately run through using a homogeneous TRIS-HCl buffer (0.1M pH 7.2).

The protein contents of the eluted sub-fractions were determined by using the reagents of and the method given by Sigma Chemical Company (Procedure No. P5656 of 1987) based on Peterson's modification of the micro-Lowry method.

The purity of the subfractions was determined by immunoelectrophoresis, using 5 µl of the eluate per well and a donkey anti-sheep IgG antiserum.

Trichloracetic Acid (TCA) Precipitation and Ultrafiltration of Serum Samples from Isometamidium-Treated Cattle

Materials

Centriflo^{R} membrane cones type CF25 with polyethylene support cones (CS1A) and polycarbonate collecting tubes (CT1) were purchased from Amicon Ltd., Uppermill, Stonehouse, Great Britain. The membrane cones are inert, non-cellulosic polymer laminated on tough inert substrate and have more than 95% retention of molecules with molecular weight above 25 kD.

Protein estimation kit reagents (TCA, sodium deoxycholate and Lowry's reagent) obtained from Sigma Chemical Company were used for TCA precipitation of proteins in the samples.

The samples included a normal bovine serum (NBS) pooled from

calves (347, 41, 42, 43. 44, 45) at day 0; sera of the same calves from day 30 after being treated with isometamidium (Samorin^{R}) at 0.5 mg/kg BW. and bovine serum albumin (30 mg/ml).

Methods

To 1 ml of serum from each calf treated with isometamidium (CSS), normal bovine serum (NBS) and bovine serum albumin (BSA), 0.1 ml of sodium deoxycholate was added, mixed and left to stand at room temperature for ten minutes. 0.1 ml of TCA was then added and immediately thoroughly mixed by agitation and left to stand for 30 minutes at room temperature.

The tubes were then centrifuged for 15 minutes at 3,000 rpm before the supernatants were separated from their respective precipitates. The precipitates were then redissolved using Lowry's reagent. The supernatants and the redissolved precipitates were separately dialysed overnight in 0.1M phosphate buffer pH 7.4. All the samples were done in duplicates. The concentration of isometamidium chloride in the dialysed samples was determined by ELISA.

Centriflo membrane cones were soaked in distilled water for about one hour before use. The membrane cones were placed into CS1A supports by rotating the support counter clockwise, pushing the cone down firmly until its tip protruded through the bottom and the cone was fully seated in the support, locking it in place. The supports were pushed into the CT1 tubes, seating the flanges on the tubes, and excess water was removed from the membrane cones by centrifuging the sets for a few minutes prior to insertion of the samples.

For ultrafiltration, duplicates of 2 ml each of serum from the calves treated with isometamidium (347, 41, 42, 43, 44, 45), NBS, and BSA were separately put in the membrane cone sets and centrifuged at 1000 g for two hours to achieve complete ultrafiltration. Isometamidium levels were determined in the ultra filtrates by ELISA.

Determination of Isometamidium Levels in Samples by Enzyme Linked Immunsorbent Assay (ELISA)

Materials

Isometamidium chloride (Samorin^{R}) was obtained from RMB Animal Health Limited. The buffer salts were of analytical reagent grade from British Drug House. Microtitre plates obtained from Alpha Laboratories were used for the ELISA and absorbance was measured on a Multiskan MCC at 405 nm. Samples were normal bovine serum (NBS) pooled from calves (347, 41, 42, 43, 44, 45) at day 0; sera of the same calves from day 30 after being treated with isometamidium at 0.5 mg/kg body weight, bovine serum albumin and their respective ultrafiltrates and dialysed TCA redissolved precipitates and supernatants. Other control samples included, 1 ml distilled water (H_2O); 1 ml H_2O and 0.1 ml sodium deoxycholate (DOC); 1 ml H_2O, 0.1 ml DOC and 0.1 ml TCA; 1 ml H_2O, 0.1 ml DOC, 0.1 ml TCA and 1 ml Lowry's reagent. The DOC, TCA and Lowry's reagent were purchased from Sigma Chemical Company contained in a protein estimation kit. Other reagents, unless specified, were purchased from Sigma Chemical Company.

Methods

The microtitre wells were coated by passive adsorption with egg albumin-isometamidium conjugate (0.0125 ratio) diluted 1 : 1000 with 100 mM carbonate/bicarbonate buffer pH 9.0. The plates were washed with distilled water following an overnight incubation at 4°C, dried at 37°C then stored desiccated at 4°C until they were used. Prior to assay the plates were washed four times with (PBST) phosphate buffered saline-Tween (1000 ml 0.15M phosphate buffered saline pH 7.2, 500 ul Tween-20).

Two sets of isometamidium standards ranging from 500 ng/ml to 50 fg/ml were prepared by diluting isometamidium in 0.1M phosphate buffer pH 7.4 and normal bovine serum.

5 µl of the isometamidium standards or test samples were added to the wells followed immediately by 100 µl of the purified sheep anti-isometamidium antibody diluted 1 : 5000 with PBST. The assay was performed in triplicate for the standards and duplicate for the test samples.

The plate was incubated overnight at 4°C, the wells emptied and washed three times with PBST. 100 µl of alkaline phosphatase labelled donkey anti-sheep IgG diluted at 1 : 1000 in PBST was then added to the microtitre wells, and the plates incubated for one hour at 4°C.

After washing the plate three times with PBST, 100 ul of substrate, that is, 2 mg/ml p-nitrophenyl phosphate disodium in substrate buffer (97 ml diethanolamine, 101 mg $MgCl_2$, 800 ml distilled water) was added to each well.

The plate was read in a Multiskan MCC at 405 nm after 15 - 30 minutes incubation at room temperature. Standard curves using the standard isometamidium dilutions were drawn and used for determining the isometamidium concentrations of the samples.

RESULTS AND DISCUSSION

The euglobin obtained by ammonium sulphate precipitation yielded three peaks, namely MGI, II and III (Figure 6) following a molecular weight gradient separation with 0.01M - 0.71M phosphate buffer pH 7.2 through a DEAE column. The three peaks were concentrated to 12 ml for MGI and 5 ml for MGII and MGIII. When these three peaks were further purified by passing through a Sephadex G200 column each yielded two subfractions, that is, 19.5 ml and 18.0 ml for MGI fraction I and II respectively; 9.0 ml and 10.5 ml for MGII fraction I and II respectively; and 4.5 and 13.0 ml for MGIII fraction I and II respectively, as shown in Figures 7 - 9.

By employing immunoelectrophoresis, the purity of MGII fraction II proved to be the best (Figure 10) and it was the one that was used for various analyses of isometamidium chloride.

It will be noted from the isometamidium standard curves drawn (Figure 11) that the optic densities of the standards in normal bovine serum were lower than those in phosphate buffer at the same concentrations. This can be due to the effect of the serum proteins in the assay, probably brought about by the binding of the drug to the serum proteins. However, in both curves, the assay shows to be most sensitive between 500 fg and 500 pg. The two standard curves were important when determining

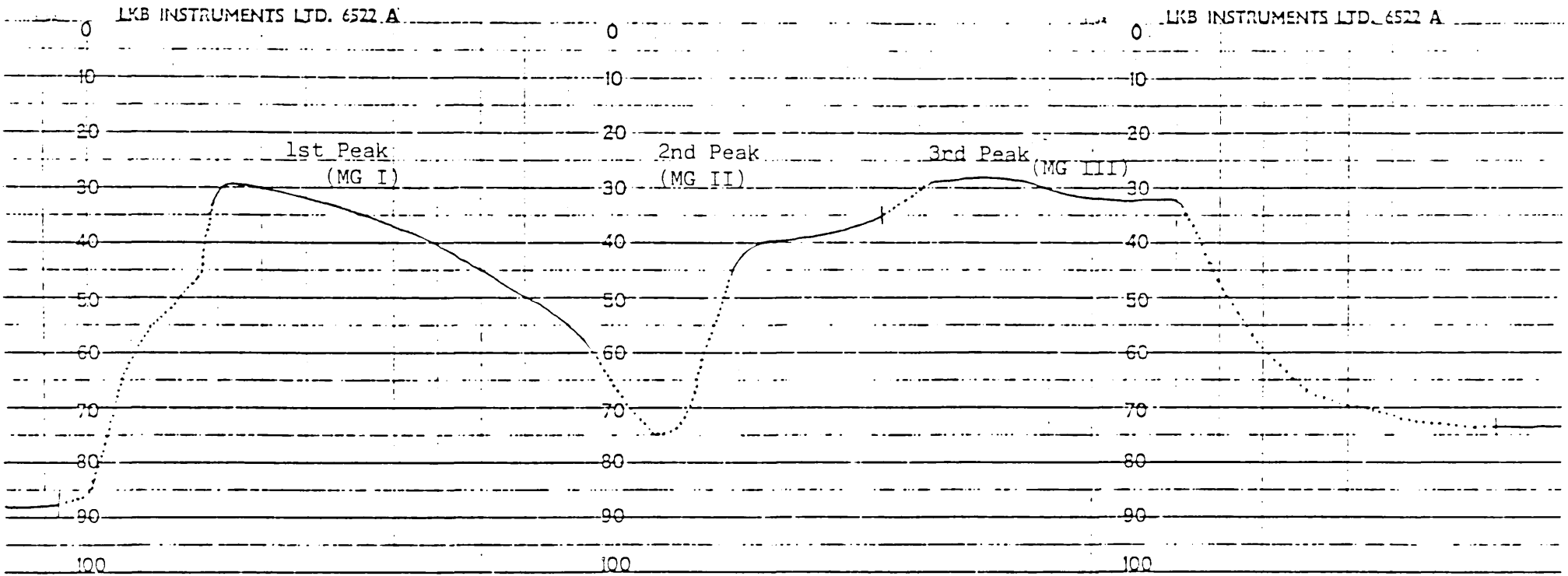


Figure 6

Sheep Anti-Isometamidium Molecular Gradient Euglobin Fractions through DEAE.
(DEAE Column 63 x 3 cm; PBS Buffer 0.01 - 0.7M, pH 7.2)

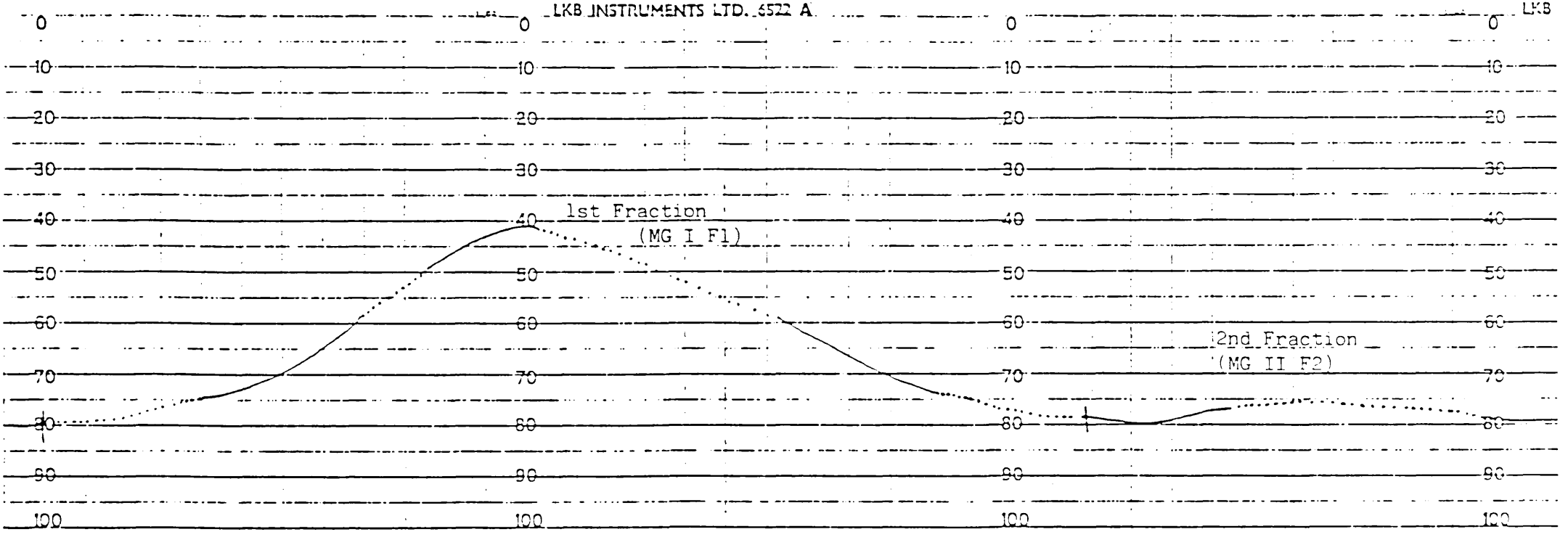


Figure 7

Purified Anti-Isometamidium IgG Sub-Fractions of MG I Through Sephadex G200.
(DEAE Column 90 x 3 cm; TRIS-HCl Buffer 0.1M, pH 7.2)

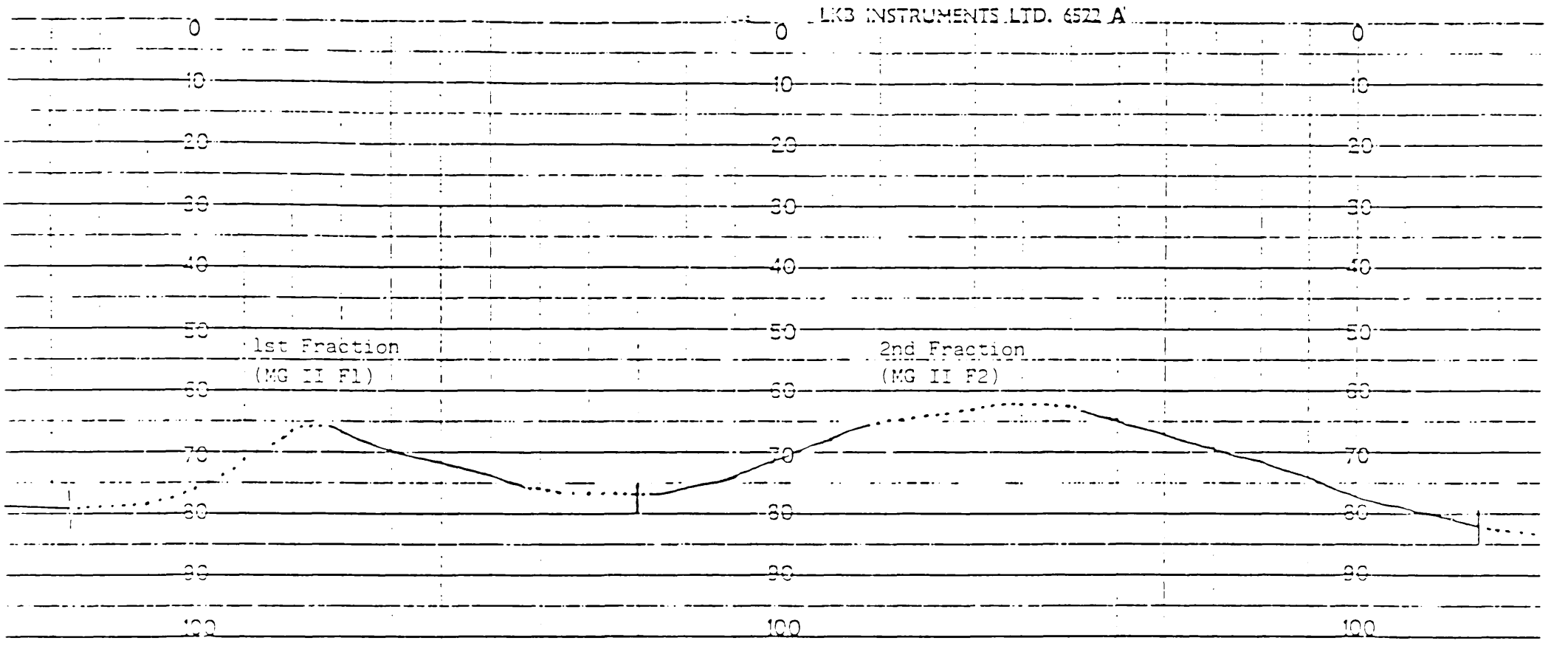


Figure 8

Purified Anti-Isometamidium IgG Sub-Fractions of MG II Through Sephadex G200
(DEAE Column 90 x 3 cm; TRIS-HCl Buffer 0.1M, pH 7.2)

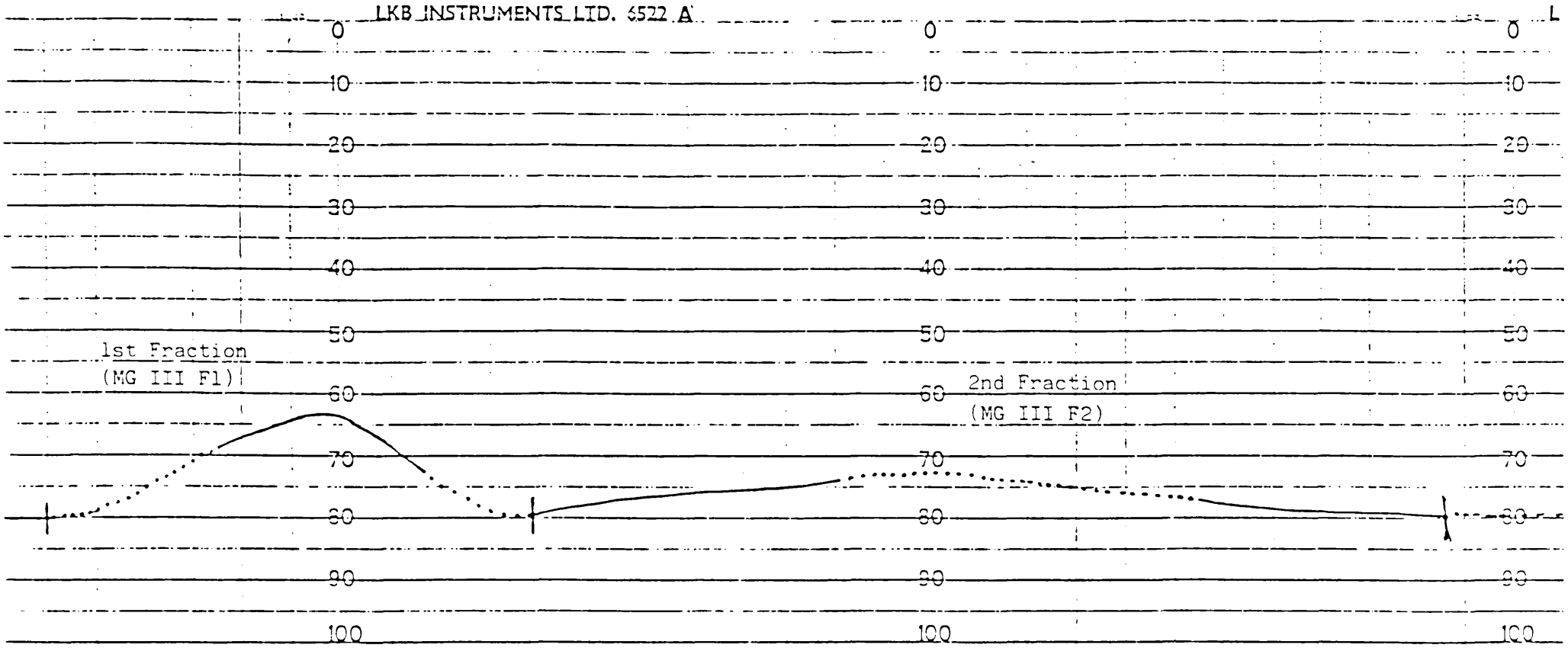


Figure 9

Purified Anti-Isometamidium IgG Sub-Fractions of MG III Through Sephadex G200
(DEAE Column 90 x 3 cm; TRIS-HCl Buffer 0.1M, pH 7.2)

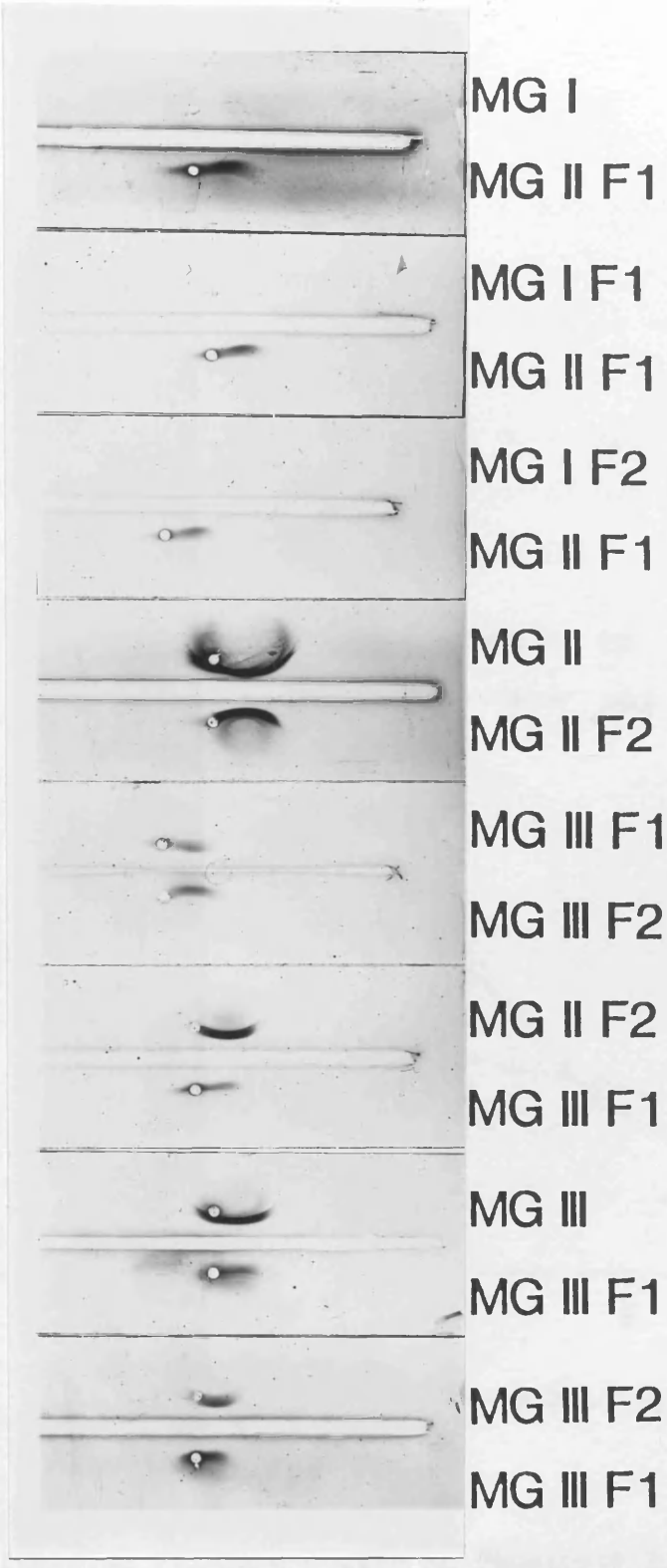


Figure 10

Immunoelectrophoretic Analysis of Anti-Isometamidium Fractions and Sub-Fractions Using Donkey Anti-Sheep IgG

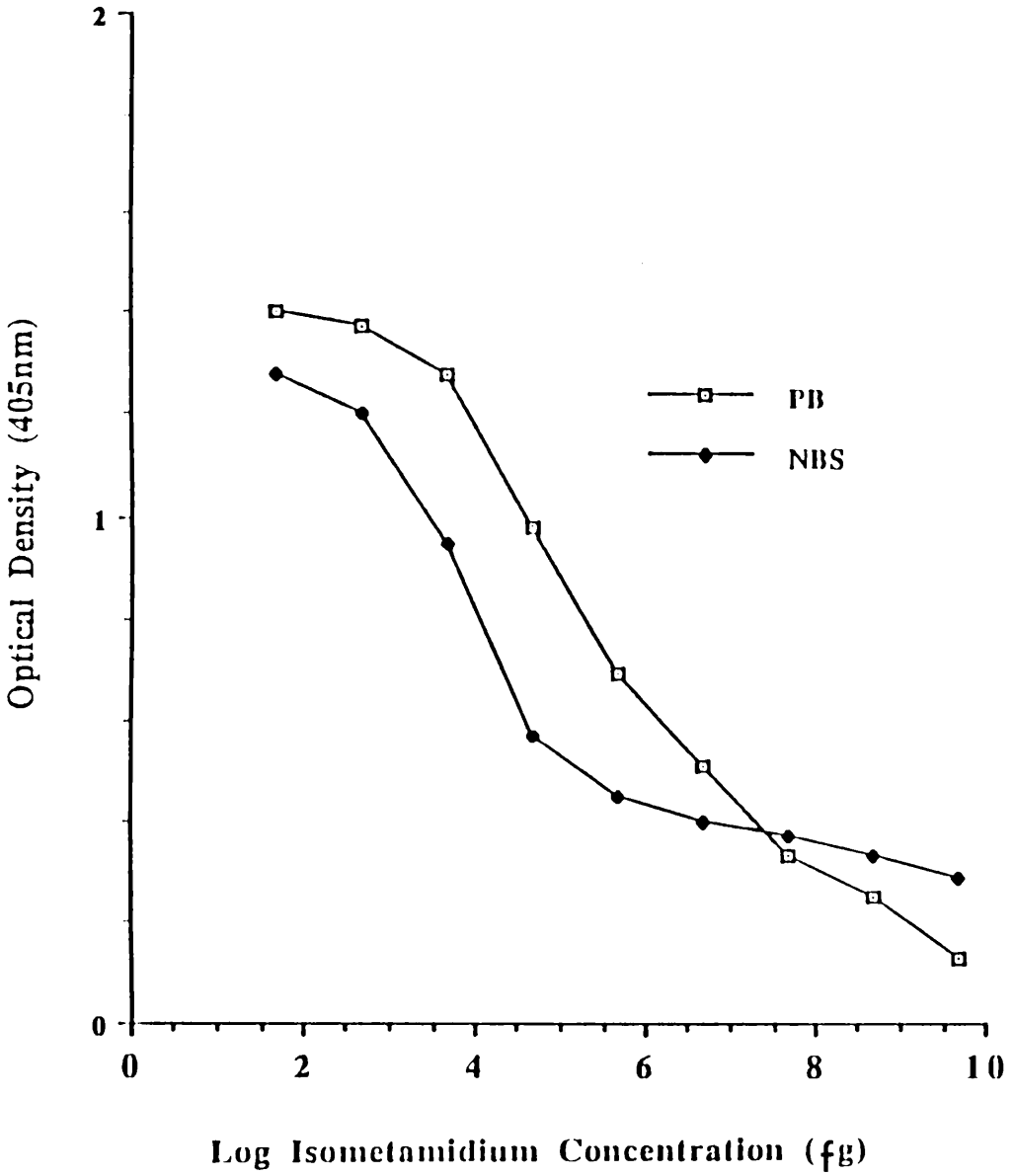


Figure 11

Isometamidium Standard Curves in Phosphate Buffer (PB) and Normal Bovine Serum (NBS).

the sample isometamidium concentration so as to take into effect the medium in which isometamidium was contained.

Table 9 shows the isometamidium concentrations in the samples analysed. It seems that TCA affects the assay of isometamidium by lowering the optical density, hence giving false high isometamidium results as depicted by comparing the results obtained with the redissolved TCA precipitates of CSS, NBS, BSA and the control samples containing TCA. Lowry's reagent also seems to have a similar effect as TCA. To this effect, the results obtained by TCA precipitation were disregarded and not considered in making any conclusions.

Analysis of the ultrafiltrates from the (CSS) isometamidium containing sera (347, 41, 42, 43, 44, 45) by ELISA showed that about 99% of the isometamidium remained in the protein matrix. However, the precentage recovery of isometamidium in the ultrafiltrates of the control isometamidium solutions in distilled water were 95% and 98%, indicating that some of the drug may have been retained or bound to the ultrafilter membrane. Thus, from the results it can be concluded that 95-99% of isometamidium chloride was bound to serum proteins in isometamidium treated animals.

The studies on the binding of various drugs to bovine serum albumin (Birkett and Wanwimolruk, 1985), human α_1 — acid glycoprotein (Muller et al, 1985), lipoproteins (Urien, 1985), gammaglobulins (Albengres, 1985), leucocytes and platelets (Lemaire, 1985) and erythrocytes (Ehrnebo, 1985) have indicated that serum proteins have the priority and higher capacity to bind

Table 9

Sample Concentrations of Isometamidium Chloride as Determined by ELISA

Description of Contents	Mean O.D.	Samorin Concentration per ml	% Recovery or Unbound Isometamidium
Serum with Isometamidium CSS*1	0.97	5 pg	-
TCA Supernate of CSS*2	1.352	1.7 pg	34.0
Redissolved TCA precipitate of CSS*3	1.329	2.75 pg	-
Normal bovine serum (NBS)	1.379	< 50 fg	-
TCA supernate of NBS	1.434	< 50 fg	-
Redissolved TCA precipitate of NBS	1.297	4 pg	-
Bovine serum albumin (BSA)	1.391	< 50 fg	-
TCA supernate of BSA	1.393	< 50 fg	-
Redissolved TCA precipitate of BSA	1.326	2.75 pg	-
Control samples:			
(a) 1.0 ml H_2O + 0.1 ml sodium deoxycholate	1.408	< 50 fg	-
(b) As (a) + 0.1 ml TCA	1.340	2 pg	-
(c) As (b) + 1.0 ml Lowry's reagent	1.289	4.25 pg	-
(d) Distilled water	1.497	< 50 fg	-
Ultrafiltrate of CSS	1.369	< 50 fg	0.01
Ultrafiltrate of NBS	1.408	< 50 fg	-
Ultrafiltrate of 500 pg isometamidium in water	0.745	475 pg	95.0
Ultrafiltrate of BSA	1.427	< 50 fg	-
Ultrafiltrate of CSS	1.370	< 50 fg	0.01
Ultrafiltrate of 2.5 ug/ml isometamidium solution in water	0.198	2.45 µg	98.0

*Mean values for calves 347,41,42,43,44,and45 with a range of 4.9-5.15pg (*1); 1.6-1.9pg (*2); and 2.5-3.0pg (*3).

drugs than the cells found in blood. Thus, evaluation of the binding of isometamidium to serum proteins is of importance when considering its pharmacokinetics in treated animals. It is probable that this extensive binding of isometamidium chloride to serum proteins has contributed to the problems faced in the development of analytical methods for its detection in sera of treated animals.

The extensive binding of isometamidium to serum proteins has important implications in the mechanism of uptake of the drug by trypanosomes *in vivo* in treated animals, which is currently unknown. The trypanosome cell coat is composed of a single glycoprotein molecule of about 65 kDa (Vickerman and Barry, 1982; Pays, 1986; Barbet *et al*, 1989) and it is possible that this surface coat covering the trypanosome plasma membrane is capable of binding the drug.

Reports of studies of different drugs binding to albumin (Birkett and Wanwimolruk, 1985) and human α_1-acid glycoprotein (Muller *et al*, 1985) indicates that drugs are bound to hydrophobic areas on these molecules. Isometamidium chloride exhibits some hydrophobic properties due to the phenyl moieties present in the molecule (Kursch *et al*, 1983; Kinabo and Bogan, 1988a). It is possible that the trypanosome surface coat has hydrophobic areas which are capable of binding the drug and eventual absorption into the cell which may be rapid as evidenced by its rapid clearance from serum and accumulation into parenchymatous organs in treated animals.

It is interesting to note that the prophylactic effect of isometamidium is due to the characteristic retention of the drug at the injection site and the accumulation in parenchymatous organs to be released slowly into the circulation. It is apparent that the amount of the drug released into the circulation is very small and for this to have an effect on the trypanosome, it is suggested that the trypanosome surface coat must have sites with higher affinity to bind isometamidium than the plasma proteins.

Comparative studies of isometamidium binding to the trypanosome surface coat in isometamidium-sensitive and resistant strains might also be of great value. It is possible that there is mutation or genetic change which could result in the reduction of hydrophobic binding sites or the affinity of the trypanosome surface coat to isometamidium in drug resistant trypanosomes.

CHAPTER THREE

THE DEVELOPMENT OF A CHEMILUMINESCENT WESTERN-BLOT TECHNIQUE FOR THE DETECTION OF PROTEINS BINDING ISOMETAMIDIUM IN THE SERUM OF TREATED ANIMALS

INTRODUCTION

The phenomenon of chemiluminescence and bioluminescence has been known for a long time, however it is only in the 1980s that its applicability has been introduced into clinical laboratories.

Chemiluminescence is the phenomenon observed when the vibronically excited product of an exoergic chemical reaction reverts to its ground state with the emission of photons (Shaw, 1985). The photophysical process involved is illustrated by the Jablonski diagram (Figure 12) which also serves to differentiate chemiluminescence from fluorescence and phosphorescence.

The types of chemical reaction involved in chemiluminescence are usually oxidation reactions and one of the best known examples is the reaction of luminol (5-amino-2, 3-dihydrophtalazine-1, 4-dione) with peroxide or perborate (Kricka and Thorpe, 1984; Shaw, 1986). In this reaction (Figure 13) the product, aminophthalate is produced in an excited state and it decays to the ground state with the emission of light (λ max 425 nm).

In chemiluminescent reactions mediated by luminol, only 1% of the reacting molecules produce light emission, as such they are less efficient as compared to natural bioluminescent reactions (Kricka and Thorpe, 1984). Bioluminescent reactions are also oxidation reactions involving catalytic enzymes (luciferases) and their substrates (luciferins) which are responsible for the light emitted by the firefly and marine bacteria such as Vibrio fischerii.

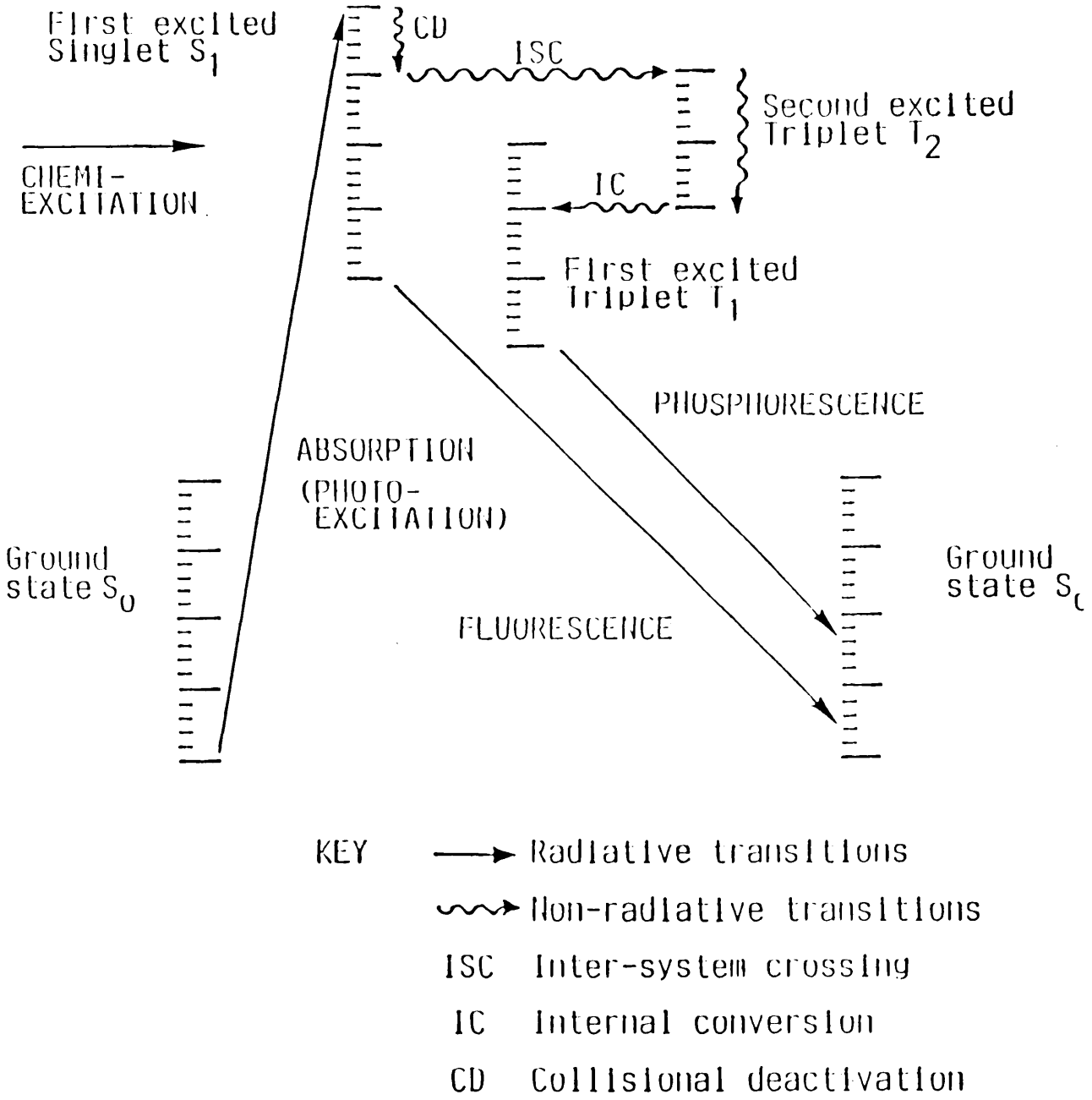


Figure 12

Jablonski Diagram. It illustrates that fluorescence and phosphoresence processes require excited state population by irradiation of ground state molecules; whereas, chemiluminescence involves the formation of the products of a chemical reaction in excited state. Light emission occurs with formation of ground state molecules.

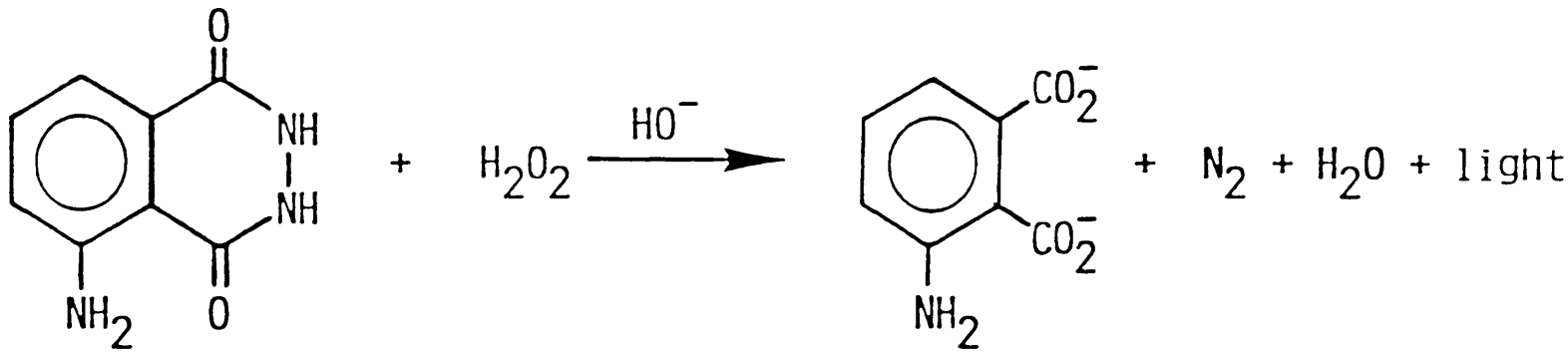


Figure 13

Chemiluminescent Oxidation of Luminol

Acridinium esters reaction also proceeds in the presence of dilute alkaline hydrogen peroxide, however, unlike luminol it does not require a catalyst. Thus, levels of background chemiluminescence and interference which are characteristically high in complex oxidation systems are very low (Shaw, 1985; 1986).

In analytical luminescence, a sample and a luminescent reagent are mixed together and placed in front of a light detector, either a silicon photodiode or a photomultiplier tube. The total amount of light or the peak light intensity is related to the concentration of the analyte in the sample (Kricka and Thorpe, 1984).

Luminescence analyses have been employed in the measurement of cellular ATP using firefly luciferase-luciferin reaction in a pre-transfusion viability of red blood cells and platelets, ATP levels in bacteriuria, and red cell ATP levels in studies of various disease states such as diabetes, uraemia and hexokinase deficiency. In immunology, chemiluminescence has been used to study the phagocyte function in conditions such as chronic glanulomateous disease, burns, renal disease and myeloperoxidase deficiency. Luminescent reaction has also been used to serve as an indicator reaction for peroxidase, ATP or NADH produced in an initial reaction in the assays for glucose, creatine kinase and alcohol (Kricka and Thorpe, 1984). Chemiluminescent immunoassay has also shown to be a logical and viable alternative to systems using radioisotopes. It is a "non-radiometric", sensitive and

rapid immunoassay for a wide range of analytes including alpha-fetoprotin, ferritin, choriogonadotropin, prolactin, thyrotrophin and thyroxine (Shaw, 1985).

Scheneppenheim and Rautenberg (1988) adapted a luminescence assay for the detection of peroxidase-labelled secondary antibodies which react specifically with antibodies to human immunodeficiency virus (HIV) on Western-blots. They demonstrated that the method was one hundred times more sensitive than either commercial ELISA or the chromogenic peroxidase assay on the blots, and detects seroconversion earlier than any other technique.

Western-blotting, that is, the transfer and immobilisation of proteins fractionated on the basis of molecular weight in sodium dodecyl sulphate polyacrylanide gel electrophoresis (SDS-PAGE) on to membranes has proved to be a useful technique for the detection and characterisation of a wide range of proteins (Burnette, 1981; La Rochelle and Froehner, 1986; Andrews, 1987; Catty and Raykundalia, 1988). Polyacrylamide gels are formed by co-polymerisation of acrylamide and bis-acrylamide (N, N'-methylene-bis-acrylamide). The reaction is a vinyl addition polymerisation initiated by a free radical generating system of the incorporated ammonium persulphate and TEMED (tetramethyl-ethylenediamine). The resulting polymer chains are randomly cross-linked by bis creating closed loops and a complex "web" polymer with a characteristic porosity which depends on the polymerisation conditions and monomer concentrations (Hames, 1985).

SDS-PAGE is able to separate the individual components of a sample not only on the basis of molecular size but also molecular shape. The technique therefore, permits determination of the molecular weights of proteins having different molecular shapes by splitting the proteins' s-s covalent bonds into their individual polypeptide chains by denaturation with SDS and a thiol. These chains are negatively charged in proportion to their length such that their electrophoretic mobility (Rf) depends on their molecular weight alone. With SDS-PAGE therefore, the molecular weight of a sample protein(s) can be determined by comparing the electrophoretic mobilities with those of known protein markers (Laemli, 1970; Sigma, 1982; Hames, 1985; Andrews, 1987).

Several different types of immobilising matrix are employed in Western-blotting, however, nitrocellulose (NC) is widely used. The mechanism of protein binding to NC is complex and it is still not fully understood; but hydrophobic interactions are probably important since the addition of non-ionic detergents such as Triton X-100 aids subsequent elution of protein from the matrix (Andrews, 1987). The incorporation of methanol tends to stabilise the geometry of the gel during transfer and also increases the binding capacity of transfer membranes for protein, but reduces the efficiency of protein elution from the gel particularly SDS-PAGE gels. Thus, long electroelution (more than 12 hours) times may be required for efficient transfer of large proteins. For Western blotting, Immobilon-P (teflon) transfer membrane, a strong hydrophobic polyvinyl difluoride (PVDF) has

been shown by the manufacturer (Millipore, 1987) to be superior to nitrocellulose and nylon.

About 5 ng of transferred and immobilised proteins in blots can be detected with biotin-avidin technology, the sensitivity and efficiency being comparable to that of silverstaining in polyacrylamide gels (La Rochelle and Froehner, 1986; Pierce, 1989). The biotin molecule can easily be activated and coupled to either antigens or antibodies, usually with a complete retention of activity. N-hydroxysuccimide (NHS) esters react with primary amines to form amide bonds. The reaction occurs via nucleophilic attack by an unprotonated amine (generally lysine epsilon groups) towards NHS ester, resulting in a stable amide bond formation and release of N-hydroxysuccimide (Figure 14). The reaction is favoured in alkaline pH which keeps primary amines in an unprotonated state; also the hydrolysis of the NHS ester is favoured in dilute protein solutions.

Streptavidin (MW 60,000) is more commonly used than avidin. Unlike its glycoprotein counterpart, streptavidin does not contain carbohydrate moieties thus, it has a lower isoelectric point (pI = 5) resulting in reduced non-specific binding (Hoffman et al, 1980; Haeptle et al, 1983). Streptavidin is isolated from Streptomyces avidini and purified over an affinity column to assure the integrity of the four biotin-binding sites (Pierce, 1989). An enzyme-streptavidin conjugate is able to bind any immobilised biotinylated antibody or antigen in an assay, and the resulting colour, fluorescence or luminiscence after the addition of an appropriate substrate can

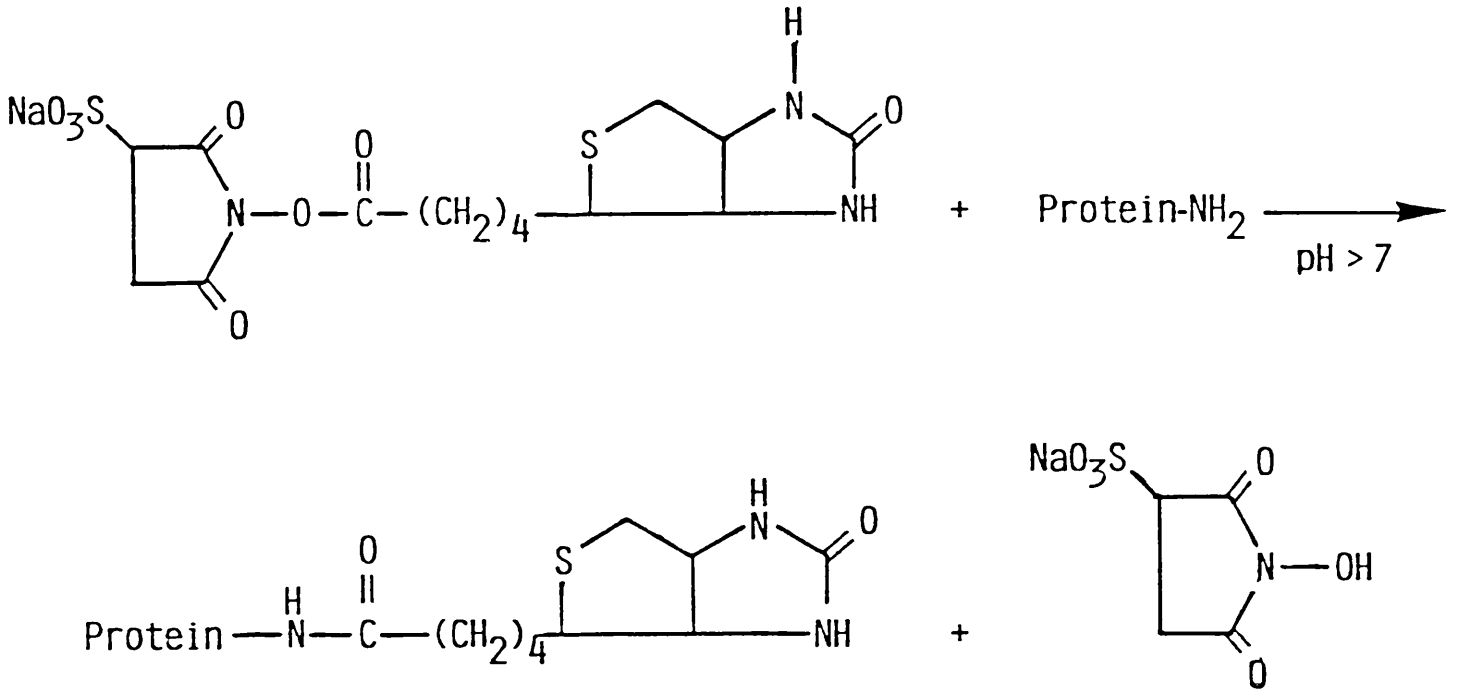


Figure 14

The Reaction of NHS-Biotin or Sulfo-NHS-Biotin with Protein Amines

be correlated to the concentration of the antibody or antigen present.

The work detailed in this chapter was aimed at identifying serum proteins to which isometamidium chloride binds and characterise the molecular weight of these proteins in the sera of isometamidium-treated animals. To achieve these objectives, the principles of SDS-PAGE, Western-blotting, avidin-biotin system and chemiluminescence were employed with modifications where necessary. For example, X-ray plates were used to detect light emitted during the chemiluminescent reaction and therefore determine the molecular weight of the isometamiium binding proteins and their distribution in the SDS-PAGE gels.

MATERIALS AND METHODS

Production and Purification of Sheep Anti-Isometamidium Antibody (IgG)

The sheep anti-isometamidium antibody was raised and purified as described in Chapter Two.

Biotinylation of the Purified Sheep Anti-Isometamidium (IgG)

Chemicals and Reagents

N-hydroxysuccinimide ester (NHS-biotin) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company; the other chemicals and reagents were of analytical grade obtained from British Drug House.

Methods

The purified anti-isometamidium antibody was dialysed

against 0.1M $NaHCO_3$ pH 8.2 - 8.6 for 16 hours and the protein content was adjusted to 1.0 mg/ml using distilled water.

N-hydroxysuccinimide ester (NHS-biotin) was dissolved in dimethyl sulphoxide to give a concentration of 1 mg/ml. It was immediately added to the antibody at a rate of 120 µl per 1 ml of the protein, mixed and left to stand for four hours at room temperature.

This mixture was dialysed against phosphate buffered saline with azide, pH 7.0 - 7.4 for 16 hours, after which the buffer was changed and dialysed further with stirring for 15 minutes at room temperature.

SDS-PAGE and Western-Blotting of Protein Samples

Materials

A Bio-Rad power pack and accessories were used. Immobilin-P (PVDF teflon transfer membrane) was purchased from Millipore; molecular weight protein standards and a silver staining kit for proteins from Sigma Chemical Company.

All other chemicals and reagents were of analytical quality from British Drug House.

Samples included:-

(a) Sera from animals treated with isometamidium at 0.5 mg/kg BW in:-

(i) Cattle Nos. 42, 43, 44, 347, 348 : 2, 16 and 30 days post treatment;

(ii) Goats Nos. 123, 129, 145, 149 : 2, 16 and 30 days

post treatment;

(iii) Pooled sera from eight mice, four days post treatment.

(b) Sera from the same animals prior to isometamidium treatment;

(c) PTG (porcine thyroglobulin) and EA (egg albumin) solutions, 4 mg/ml;

(d) PTG-isometamidium and EA-isometamidium conjugates of 4 mg/ml protein and protein-isometamidium ratio of 0.005 and 0.0125 respectively;

(e) Isometamidium spiked normal bovine serum (500 µg/ml).

Methods

The samples were diluted using a loading buffer (5 g SDS, 5 ml 1M TRIS pH 7.5, 2 ml 100 mM EDTA, 10 ml glycerol, 2 ml 0.2% bromophenol blue all made to a final volume of 95 ml with distilled water pH 6.8) to give dilutions of 1 : 4 for cattle, goats and mouse samples; 1 : 80 for EA and 1 : 160 for PTG samples. They were then boiled in a water bath for five minutes to denature the proteins.

Ten percent homogenous polyacrylamide slab gels were prepared by mixing 1.25 ml of solution 'A' (3M TRIS-HCl, 0.8% SDS, 0.46% TEMED), 2.5 ml of solution 'N' (40% acrylamide, 0.54% bis-acrylamide), 6.2 ml of distilled water; the mixture was then degassed before 700 µl of 10% ammonium persulphate was added, and the mixture poured on to vertical gel casters immediately. Propan-1-ol was poured on the setting gel to avoid drying and following gel polymerisation (5 - 10 minutes), was removed and

the gel top rinsed with distilled water.

A 5% stacking gel prepared by mixing 1.25 ml of solution 'N', 1.25 ml of solution 'S' (1M TRIS-HCl, 0.8% SDS, 0.4% TEMED) and 7.5 ml distilled water, degassed and 100 µl of 10% ammonium persulphate added, was poured on the main gel and gel combs inserted to form the wells. The gel was left for 5 - 10 minutes to set. The function of the stacking gel is to separate the samples and concentrate them on to the running gel.

Cast gels were placed in an electrophoresis tank containing PAGE running buffer (15 g TRIS base, 72 g glycine, 2.5 g SDS in 1 litre distilled water). Ten microlitre samples were applied on the formed wells, and electrophoresis carried out at 200V for about one hour and terminated when the bromophenol blue dye front neared the end of the gel.

A set of protein standards were run alongside the samples routinely to calibrate the gels and hence determine the molecular weights of the unknown sample proteins. Gels were silver stained as per instruction kit (Sigma Tech. Bulletin No. P3040[2-87]. A standard curve of $\log_{10}$ (known molecular weights) against relative mobilities (Rf) was plotted and used to determine the molecular weights of the unknown sample proteins.

After running the samples in SDS-PAGE, the gels were removed from the cassettes (casters) by careful detachment from the glass plates, and equilibrated in a transfer buffer (9.1 g TRIS, 43.2 g glycine in 2.4 litres distilled water, and 600 ml methanol) for about 15 minutes to remove electrophoresis buffer salts and detergents, and to allow the gel to shrink to its final size.

Immobilon-P (teflon membrane) was cut at a slightly larger size than the gel, soaked in methanol for 2 - 3 minutes, then transferred to the transfer buffer for about 15 minutes. The gel was overlaid on to the teflon membrane then both were sandwiched between buffer soaked double thickness Whatmann No. 1 filter papers and fibre pads assembled in a gel holder transfer cassette (Figure 15). Care was taken to remove all air bubbles during the assembly. The cassette holder was placed in the blotting tank with the teflon paper on the side of the positive electrode. The tank was filled with transfer buffer and a transfer effected by running for 16 hours at 30V and 0.1A.

After the transfer, the teflon membrane was removed and placed in a small plastic tray containing "wash buffer" (5.35 g Na_2HPO_4, 1.95 g $NaH_2PO_4.2H_2O$, 42.5 g Nacl, 2.5 ml Tween-20 all in five litres of distilled water) to remove any adherent gel which may still be attached to the teflon membrane by continuous shaking for 15 minutes. The blotted membrane was then stored in wash buffer containing 5% marvel at 4^oC for up to 16 hours; or chemiluminescent assay carried out forthwith. Marvel is a protein carrier acting as a blocker to prevent non-specific binding during the chemiluminescent assay.

Chemiluminescent Assay for Isometamidium on the Blotted Membrane

Materials

Biotinylated sheep anti-isometamidium IgG (as described previously). X-ray plates were obtained from Du Pont de Nemours (Deutschland) GMBH. Streptavidin peroxidase, luminol (5-amino-2,

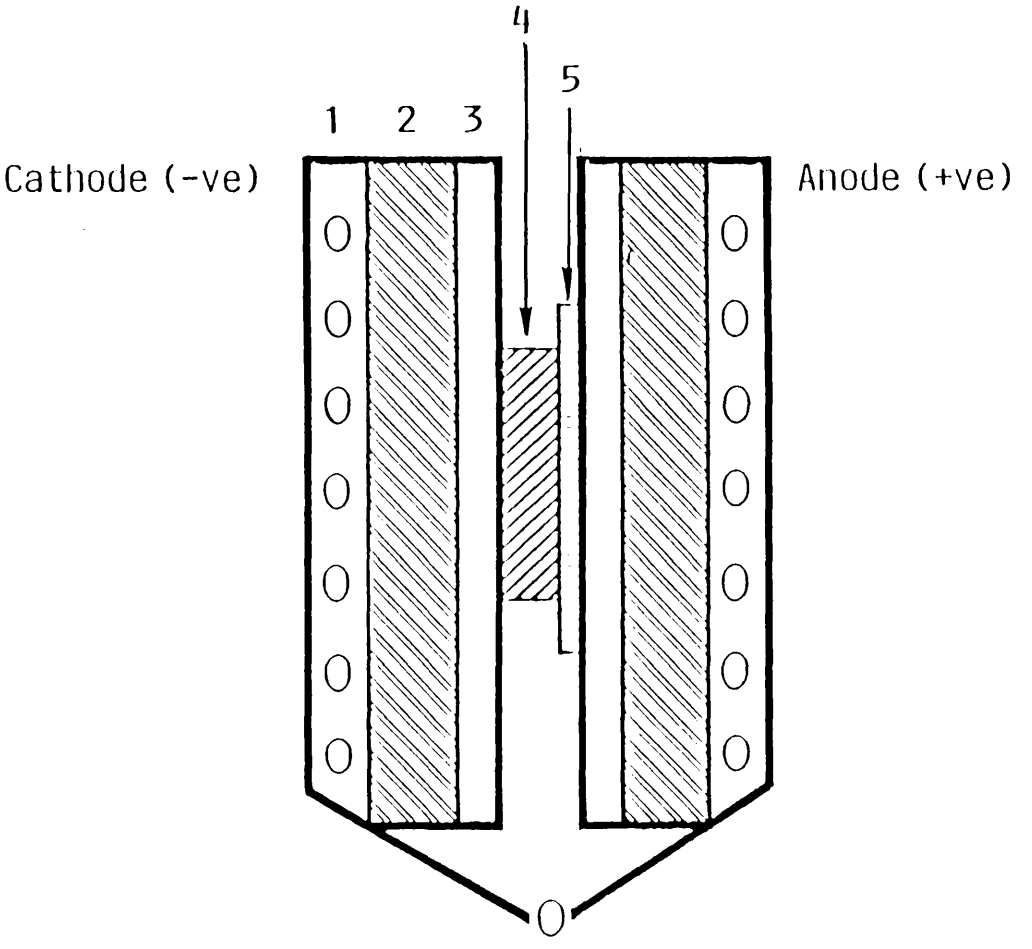


Figure 15

Diagrammatic Presentation of an Assembly of a Gel Holder Cassette During an Electrophoretic Transfer. Porous Cassette Holder (1), Fibre Pads (2), Filter Papers (3), Gel (4) and PVDF Teflon Membrane (5)

3,-dihydro-1, 4-phythalazinedione) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Company, and 4-iodophonenol from Aldrich Chemical Company. All other chemicals were of analytical grade from British Drug House.

Methods

The blotted membrane was further washed with continuous agitation in a diluting buffer (5.35 g Na_2HPO_4, 1.95 g $NaH_2PO_4.2H_2O$, 42.5 g Nacl, 25.0 g Marvel, 2.5 ml Tween-20 all in five litres of distilled water) for 45 minutes to block non-specific binding.

Biotinylated sheep anti-isometamidium IgG was diluted at a ratio of 1 : 30 in diluting buffer and the blotted membrane incubated in this solution with shaking for one hour followed by three washes each for three minutes with wash buffer (5.35 g Na_2HPO_4, 1.95 g $NaH_2PO_4.2H_2O$, 42.5 g Nacl, 2.5 ml Tween-20 all in five litres of distilled water).

The blotted membrane was then incubated for 45 minutes in streptavidin peroxidase (1 : 400 in diluting buffer) and followed by 3 x 3 washes in wash buffer.

A chemiluminescent substrate (iodophenol-luminol solution) was prepared by dissolving 40 mg luminol in 5 ml of 1M TRIS base then mixed with 2.5 ml 1M TRIS HCl to obtain a solution with a pH of 6.5, and made up to 100 ml using 150 mM NaCl solution. Ten milligrams of 4-iodophenol was dissolved in 1 ml DMSO, added to the luminol solution and thoroughly mixed. Immediately before use, 32 μl of 30% H_2O_2 solution was added to the iodophenol-

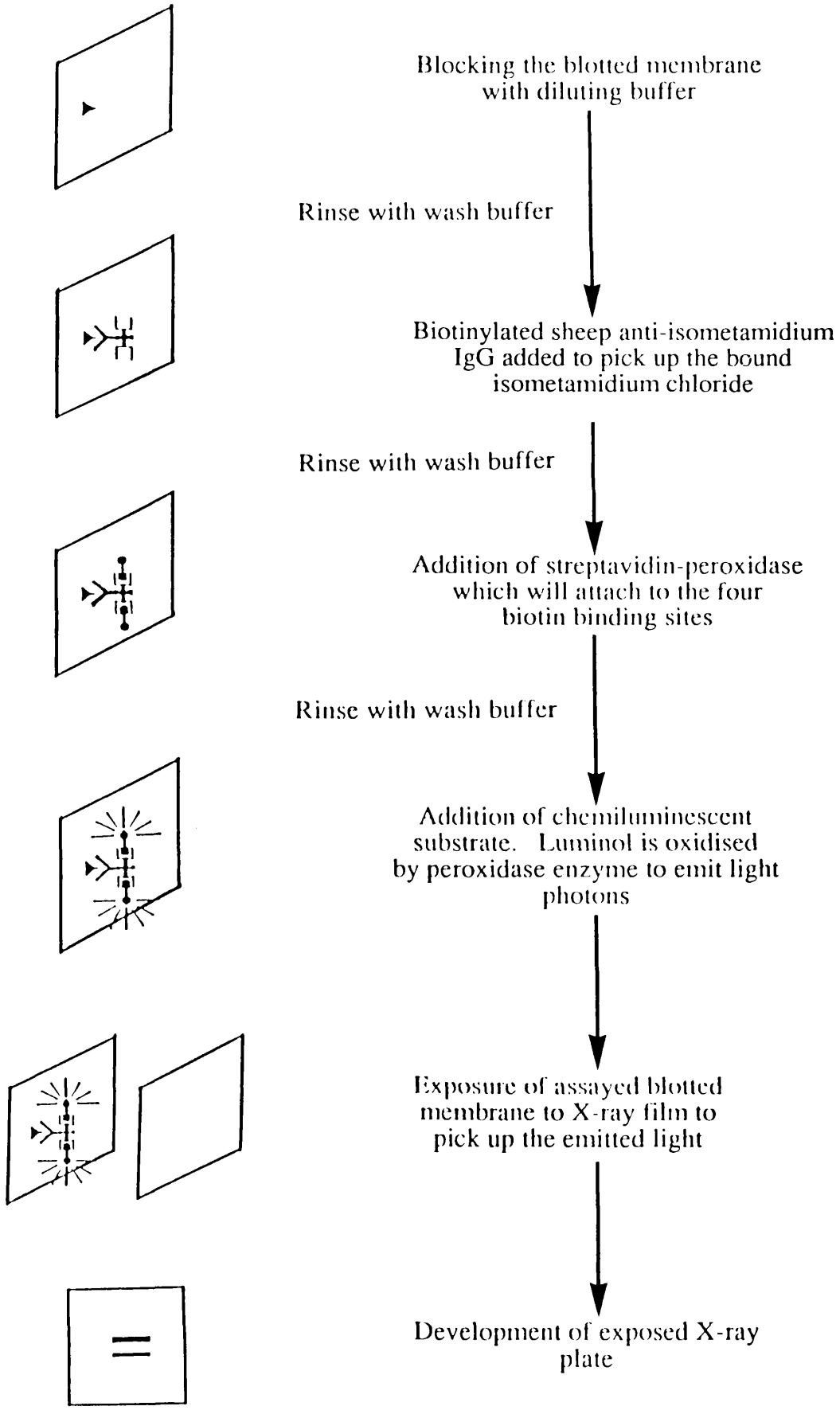


Figure 16

The General Steps Followed During a Chemiluminscent Assay

luminol solution.

The blotted membrane was immersed in the fresh substrate and removed after one minute, excess solution being shaken off, and placed in a polythene snap-bag taking care to exclude all air bubbles.

The snap-bag (containing the blotted membrane) was exposed to X-ray plates in a dark room for periods of 10 - 30 seconds, and the plates developed.

The relative mobility (Rf) of each band on the X-ray plate was calculated by dividing the distance that the band had migrated from the point of application by the distance the dye-front had migrated from the point of application.

RESULTS AND DISCUSSION

The relative mobilities (Rf) of the molecular weight protein standards (Figure 17) were used to draw the standard curve (Figure 18).

Isometamidium was detected in isometamidium conjugated porcine thyroglobulin (PTG) and egg albumin (EA) by chemiluminescence as evidenced by the bands observed in the developed X-ray plates (Figure 19). No bands were observed in the control runs of neat PTG and EA. The PTG-isometamidium conjugate remained mainly at the point of application and this is due to the high molecular weight of PTG (600 kD). The big smear observed may either be diffusion of the protein from the application point, breakdown complexes of the PTG-isometamidium during its denaturing with SDS, excess application or diffusion of photons. This may also be the case for the diffuse band

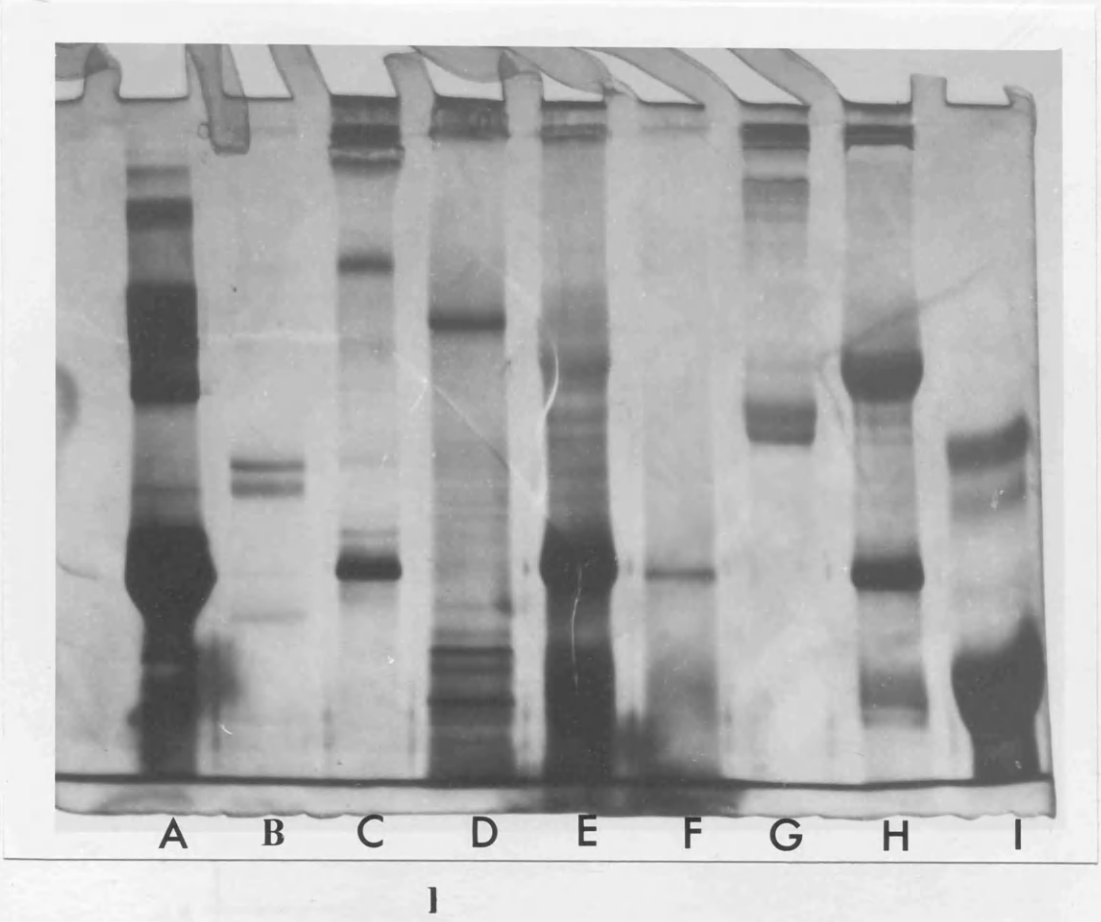


Figure 17

Silver stained protein standards in a 10% homogeneous SDS-PAGE gel. Bovine serum albumin (A), Plasminogen (B), Fibrinogen (C), Myosin (D), Mixed standards (E), Haemoglobin (F), Phosphorylase (G), β-galactosidase (H) and Egg albumin (I).

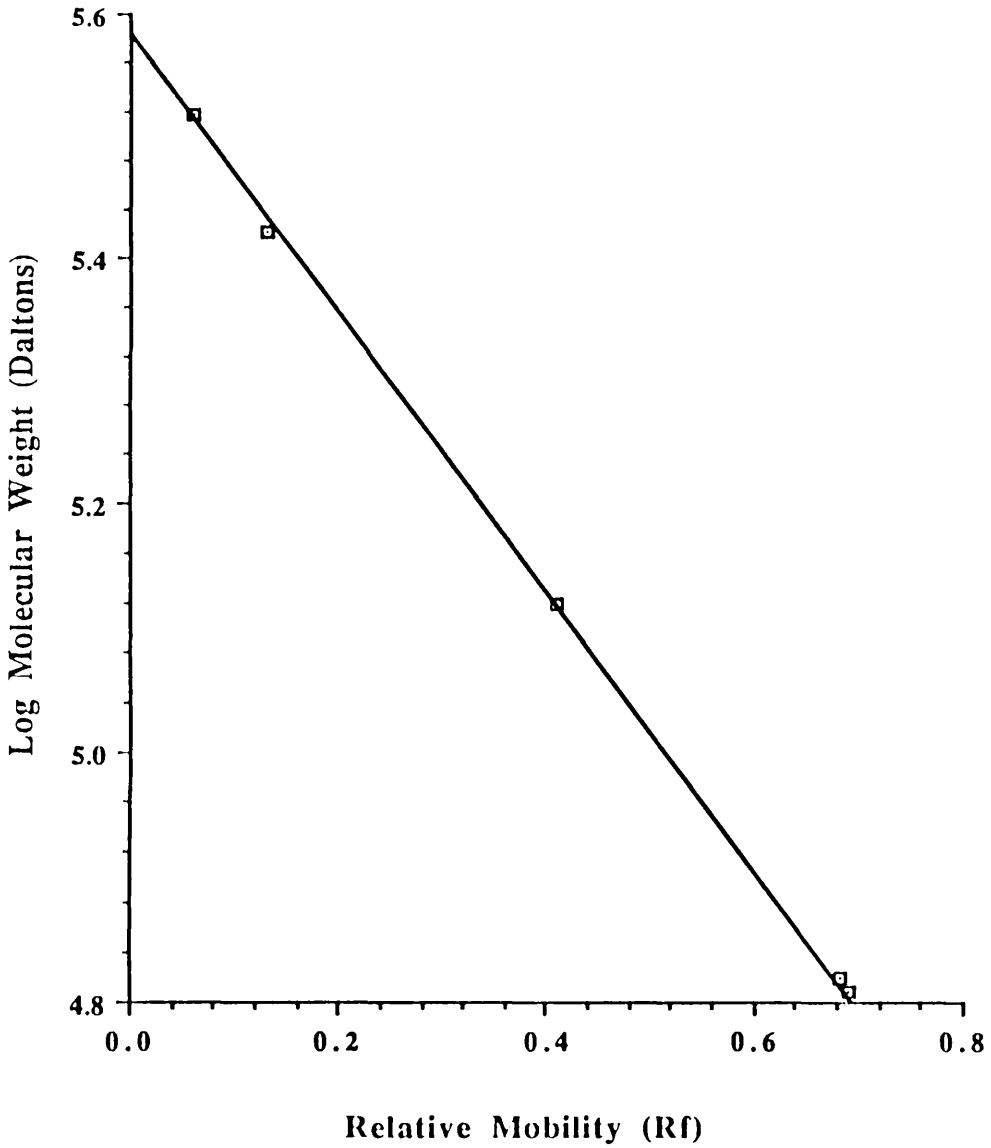


Figure 18

A Standard Curve using Molecular Weight Protein Standards in a 10% Homogeneous SDS-PAGE gel.

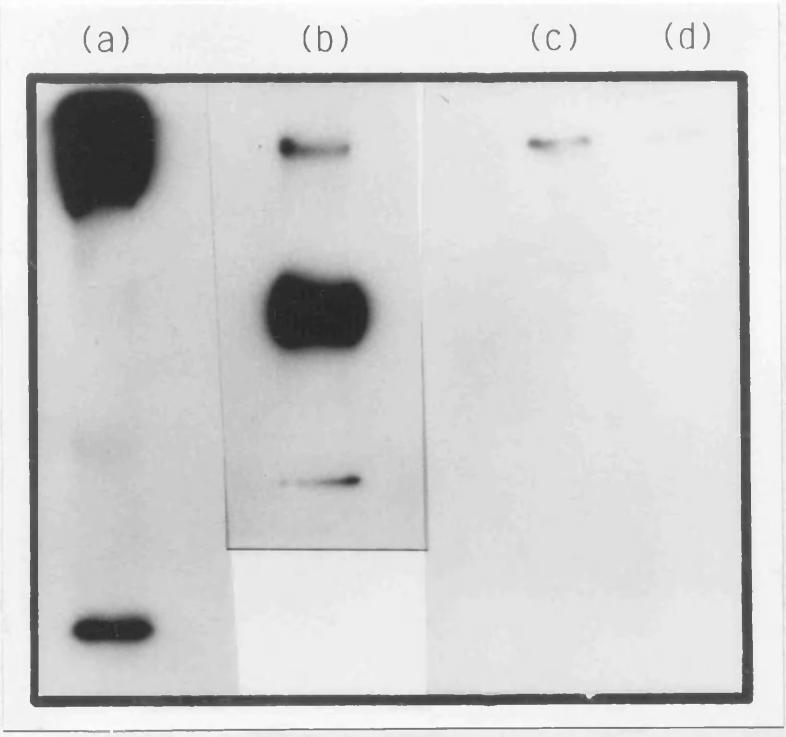


Figure 19

Chemiluminescent Western-blot for Isometamidium, Isometamidium-conjugated porcine thyroglobulin (a), Isometamidium conjugated Egg albumin (b), Porcine thyroglobulin (c), Egg albumin (d).

observed with EA-isometamidium.

Likewise, the drug was also detected by chemiluminescence in sera of isometamidium treated cattle, goats and mice, whereas no bands were observed in the control sera of the same species (Figure 20).

In sera of isometamidium treated cattle, one strong band with two weaker bands were observed (Figures 20 and 21) in the blots of denatured non-reduced PAGE gels. Even at times when all the bands were weak, at least one seemed to be stronger (Figure 21). Since the strength of the bands is determined by the amount of photons emitted by oxidised luminol, which is in turn influenced by the levels of streptavidin peroxidase-antibody (anti-isometamidium IgG) and antibody-isometamidium interactions; then sera with low amounts of the drug will show weaker bands. The stronger band in cattle sera correlate to the band obtained with isometamidium spiked albumin and isometamidium spiked normal bovine serum (Figure 22) with a relative mobility (Rf) of 0.41 which corresponds to molecular weights of about 140 kD. The weaker bands have Rfs of 0.36 and 0.46 which correspond to about 155 and 122 kD respectively.

In sera of isometamidium treated goats and mice, only one band was observed with a relative mobility (Rf) of 0.09 which corresponds to a molecular weight of about 300 kD.

Philips et al (1967) reported the binding of isometamidium to albumin, DNA, RNA, heparin and hyaluronic acid and further presumed albumin to be the major binding component in rat serum. The chemiluminescent results in this study are suggestive that

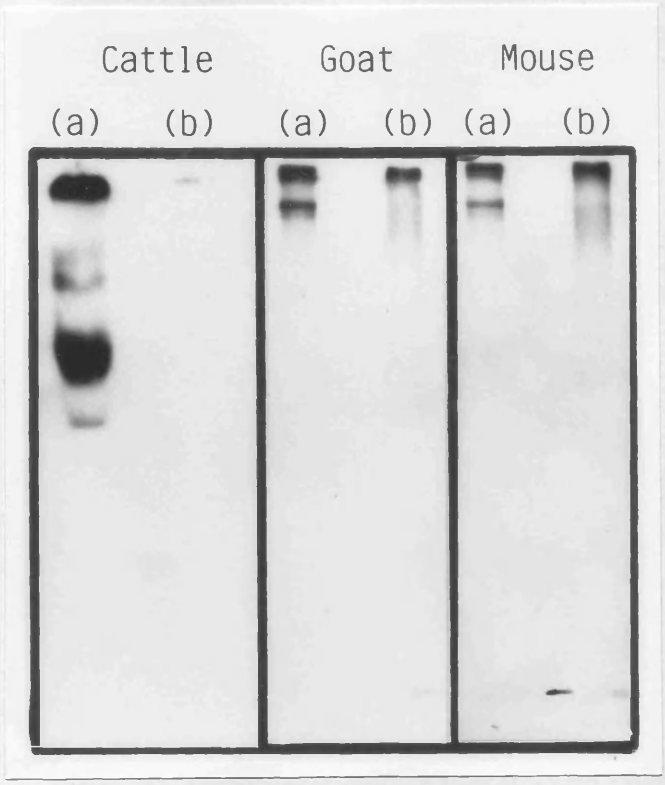


Figure 20

Chemiluminescent Western-blot for Isometamidium in serum from treated animals (a) and untreated animals (b) Serum of calf No.347, 16 days post treatment is shown for cattle.

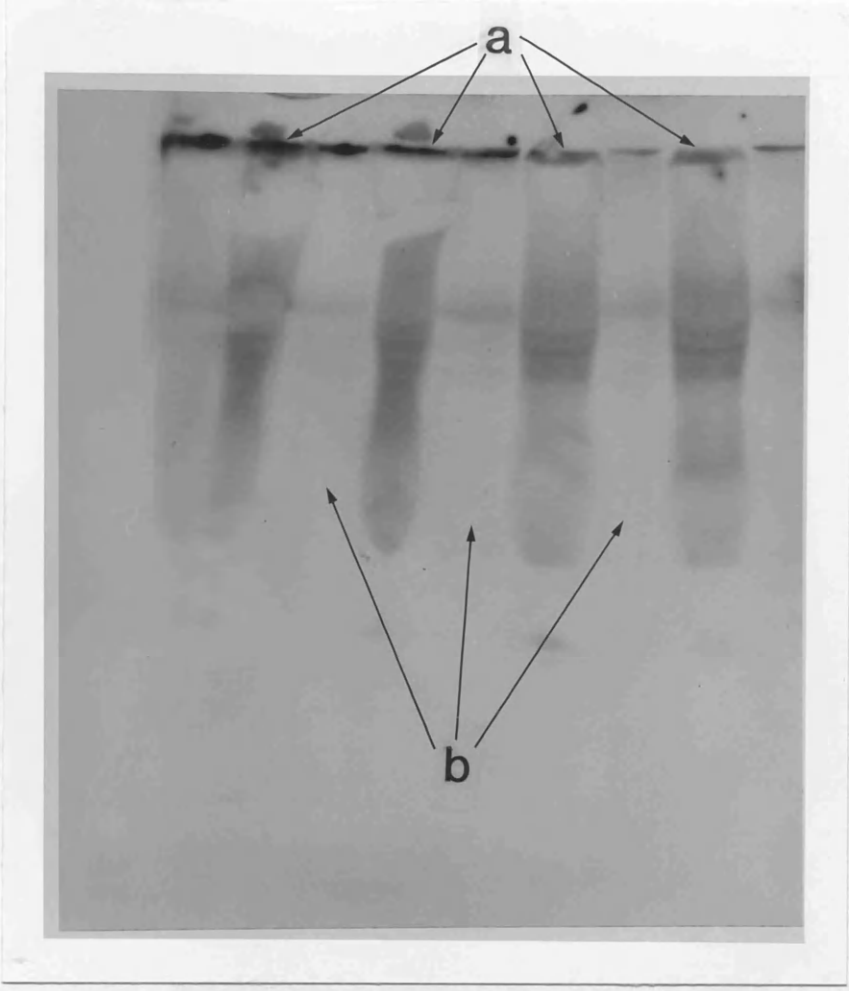


Figure 21

Chemiluminescent Western-blot for Isometamidium in bovine from treated animals (a) and untreated animals (b)

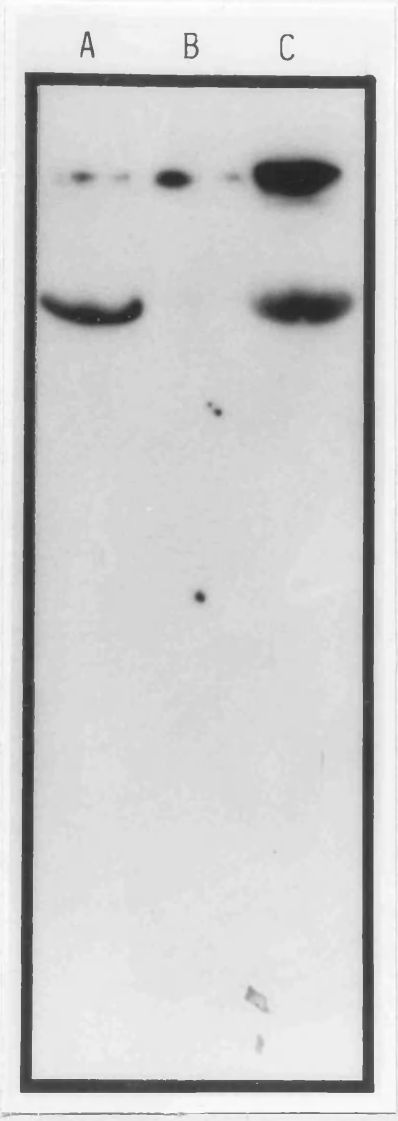


Figure 22

Chemiluminescent Western-blot for Isometamidium in isometamidium-spiked normal bovine serum (A), normal bovine serum (B), isometamidium spiked bovine serum albumin (C).

the main protein that does bind the drug in sera of treated cattle has a molecular weight of about 140 kD and it is probably a dimer of bovine serum albumin. In mouse and goats, the protein with about 300 kD which binds isometamidium cannot be identified with certainty as an albumin quadruplet or a pentamer. Preabsorption of isometamidium treated sera with monoclonal antibodies against albumin will be an aid in ascertaining if albumin is the major protein that binds isometamidium chloride.

Other workers have identified six binding sites for various drugs on the albumin molecule. However Birket and Wanwimolruk (1985) have suggested there may be additional binding sites for drugs and endogenous compounds. The albumin binding sites are hydrophobic areas with a positive charge located at the entrance to the binding site. The hydrophobic properties of isometamidium chloride (Kinabo and Bogan, 1988a) may explain the binding of this drug to albumin. A feature of all albumins is the presence of adjacent cysteine-cysteine residues which are the basis of a repeating loop structure formed by disulphide bridges. These loops stabilised by disulphide bonds make this molecule stable in acids, bases and denaturing solutions. Proteolytic fragments of bovine serum albumin also retain helical structure, some binding sites and antigenic sites (Birket and Wanwimolruk, 1985).

Studies employing equilibrium dialysis and circular dichroism in other drugs by Muller et al (1985) confirmed that human α_1-acid glycoprotein (α_1-AGP) has only one common binding site for most drugs. The site is a remote high affinity hydrophobic area within the protein component of the glycoprotein

molecule. Also, the basic character of the drugs does not appear to be obligatory for high-affinity binding to the site, and that hydrophobic interactions are more important than electrostatic interactions. In this study, no bands were observed by chemiluminescence in the sera of isometamidium treated animals corresponding to α_1-AGP (molecular weight 41.1 kD), thus either very little or no isometamidium is bound to this protein. It is also important to note from the studies by Birket and Wanwimoluruk (1985) and Muller et al (1985) that albumin has a higher capacity to bind drugs than α_1-AGP. It is possible that the other isometamidium binding proteins of about 122 and 155 kD could be glycoproteins.

Other blood components that bind drugs include erythrocytes (Ehrnebo, 1985), leucocytes and platelets (Lemaire, 1985), plasma lipoproteins (Urien, 1985) and immunoglobulins (Albengres, 1985). The major binding components in erythrocytes are haemoglobin, carbonic anhydrase and the cell membrane. In circulating blood, there is a competition between plasma proteins and blood cells for the free drug available. High plasma binding may result in low distribution to erythrocytes in spite of high affinity of the drug to red blood cells. Generally, the binding of a drug to erythrocytes is only important for the total body distribution if the binding is extremely strong or if the drug has a low distribution volume (Ehrnebo, 1985).

Ehrnebo (1985) suggested that erythrocytes would exhibit the lowest priority pharmacokinetically to bind drugs compared to albumin and α_1-AGP. There is no evidence of isometamidium

erythrocytes
binding to Λ, but an investigation of isometamidium-erythrocyte interaction is worthwhile when studying its complete pharmacokinetic analysis bearing in mind that erythrocytes form their own compartment in the circulating blood. Also, the major protein of the erythrocytes, haemoglobin, amounts to 7 - 8 times as large as the amount of albumin in the whole blood.

Drug-lipoprotein complexes may function as vehicles for drug absorption or for drug delivery to specific tissues particularly parenchymal cells via the lipoprotein receptor-mediated pathway and play a role in degradation of drugs in plasma. Nearly all types of drugs are capable of binding to isolated lipoproteins provided they exhibit a certain degree of hydrophobicity (Urien, 1985). It is possible that isometamidium is bound to lipoproteins; if so, it may form a basis in explaining its rapid clearance from serum following parenteral administration and its accumulation in specific tissues especially the parenchymatous organs. Another mechanism, receptor independent is responsible for removal of lipoproteins from plasma by the scavenger cells of the reticulo-endothelial system (Urien, 1985). The removal of the drug from plasma and its subsequent accumulation in the liver, kidneys and spleen is also suggestive of isometamidium binding to lipoproteins.

In humans, the high density lipoproteins (HDL), α_1-lipoproteins have molecular weights ranging from 148 - 154 kD for VHDL; 166 - 175 kD for HDL; 195 kD for HDL_3 and 435 kD for HDL_2; whereas low density lipoproteins (VLDL and LDL), the α_2-lipoproteins have higher molecular weights ranging from

3.2×10^3 - 20×10^3 kD. It is possible that what is retained at the point of application as seen on the developed X-ray plates of sera of isometamidium treated animals, could be lipoproteins of higher molecular weights. Also, it is possible for the characterised isometamidium binding proteins of about 122 and 155 kD to be lipoproteins (HDL). The existence of an endogenous carrier-mediated mechanism or an active transport system for isometamidium chloride in vivo has been hypothesised by Kinabo (1988a). It is possible that lipoproteins play the role of endogenous carriers and if it is so, they may also aid in the uptake of the drug by the trypanosomes. It is therefore important in future work to ascertain whether isometamidium binds to serum lipoproteins.

Leucocytes and platelets also have a high affinity but low capacity to bind drugs. The high affinity is due to an interaction with pharmacological factors located on the cell membranes. These cells are capable of accumulating drugs by specific transport or binding systems, and have necessary enzymes for metabolising drugs (Lemaire, 1985). Some degree of binding of isometamidium chloride to leucocytes has been observed (I. Sutherland, personal communication) thus, further studies in this area may help to explain in part the metabolism of the drug in vivo.

Gammaglobulins (γ-G) are large polypeptide molecules which bind to drugs much less than albumin. Drugs bind to the ends of the two arms of γ-G, the site located at a crevice between V_L (variable region of light chain) and V_H (variable region of heavy

chain) fragments, the shape and size of the binding site varying mainly due to the exchange, deletion or addition of amino acids in the hypervariable regions. The drug binding functions of immunoglobulins are drug recognition of foreign matter and drug binding to cell receptors. Generally, the amount of the drug which binds to γ-G is less than 1% of the total blood binding when related to physiological concentrations of blood proteins, thus, immunoglobulins play no relevant role in drug transport or in the drug interaction and have no effective function in carrying therapeutic amounts of drug towards a specific target (Albengres, 1985). However, considering that hypergammaglobulinaemia is a common feature in trypanosome infections, gammaglobulins may also play a role in binding of isometamidium in treated cases. It has been shown in this study that one of the drug binding proteins has a molecular weight of about 155 kD, which is within the range of gammaglobulins (150 - 160 kD), thus, gammaglobulins should be considered among the possible isometamidium binding proteins.

For the chemiluminescent assay, teflon membrane strips instead of gel-sized membranes can be used so as to minimise wastage of reagents. The use of biotinylated purified sheep anti-isometamidium IgG eliminates the use of a second antibody, for example, HRPO-conjugated donkey anti-sheep IgG. This increases the sensitivity of the assay due to the four binding sites available on the biotin molecule for the HRPO conjugated streptavidin. It also reduces the probability of species-cross reactions and non-specific binding, thus increasing the

specificity of the assay.

The ability of the purified anti-isometamidium antibody to bind to isometamidium in sera of treated animals and isometamidium-protein conjugates or isometamidium-spiked proteins in the chemiluminescent assay indicates that the antibody is highly specific for isometamidium. Thus, chemiluminescent assays using this antibody could be performed in studies of the uptake of isometamidium chloride by trypanosomes *in vitro* and *in vivo*; and the antibody can also be used in assays for the quantification of isometamidium in body fluids.

CHAPTER FOUR

THE DEVELOPMENT OF A "CHEMILUMINESCENT DOT-BLOT TECHNIQUE" FOR THE QUANTIFICATION OF ISOMETAMIDIUM IN THE SERUM OF TREATED ANIMALS

INTRODUCTION

The work explained in this chapter was developed from the findings, reported in the preceding chapters, that about 99% of isometamidium is bound to serum proteins in treated animals. It has also been shown that a purified anti-isometamidium antibody is highly specific for the drug and it can be employed in a chemiluminescent assay to detect isometamidium bound to serum proteins.

In chemiluminescent assays, the product of luminol oxidation, aminophthalate in an excited state decays to a ground state with the emission of light, λ max 425 nm (Kricka and Thorpe, 1984). In samples containing higher amounts of the drug, the levels of interactions between anti-isometamidium antibody to isometamidium, and antibody-streptavidin peroxidase will increase, conversely more luminol will be oxidised resulting in emission of more light photons. The antithesis is also true.

It has been shown that photographic detection of chemiluminescent and bioluminescent reactions is less expensive and simpler than measuring the light emission by a photomultiplier tube or a silicon photodiode (Kricka and Thorpe, 1986). Also the use of Polaroid Type 612 instant film in chemiluminescent and bioluminescent photographic assays, where luminol is the analyte, utilising horseradish peroxidase-peroxide system offers a limit of detection of 500 fmol.

This study attempted to take advantage of the ability of immobilon-P (a PVDF teflon membrane) to bind to proteins by applying the serum samples directly on the teflon membrane, and

carrying out the chemiluminescent assay thereafter. It was the objective of this study to examine future possibilities of utilising the chemiluminescent assay for rapid quantification of isometamidium chloride in sera of treated animals.

MATERIALS AND METHODS

Materials

Samples included sera from cattle (Nos. 41, 42, 43 and 45) on days 2, 16 and 30 after treatment with isometamidium at 0.5 mg/kg BW and their respective controls (Day 0).

Biotinylated sheep anti-isometamidium IgG (described in Chapter Three). X-ray plates were obtained from Du Pont de Nemours (Deutschland) GMBH, Immobilin-P from Millipore, Streptavidin peroxidase, luminol and dimethyl sulfoxide from Sigma Chemical Company, and 4-iodophenol from Aldrich Chemical Company. Other chemicals and reagents were of analytical grade from British Drug House.

Methods

Serum samples were diluted serially using loading buffer (5 g SDS, 5 ml 1M TRIS pH 7.5, 2 ml 100 mM EDTA, 10 ml glycerol, 2 ml 0.2% bromophenol blue all made to a final volume of 95 ml with distilled water, pH 6.8) and boiled for five minutes in a water bath.

A teflon membrane was soaked in methanol for three minutes then transferred to a transfer buffer (9.1 g TRIS, 43.2 g glycine, 600 ml methanol made up to three litres with distilled water) and left to soak for another 5 - 10 minutes. Excess fluid

was shaken off the teflon membrane, and the moist membrane laid on a 96 well micro-titre plate. The samples were applied as 5 µl dots on the teflon membrane arranged in an increasing dilution series. The membrane was left at room temperature for 30 minutes to enable the proteins to bind and the spotted samples to dry.

A chemiluminescent assay for isometamidium chloride on the teflon membrane was performed as detailed in Chapter Three.

RESULTS AND DISCUSSION

The density observed on the developed X-ray plate (Figure 23) decreases with increasing dilution of the serum samples, that is, the less isometamidium present in a sample results in a decrease of luminol oxidised and hence light photons emitted. The end-points of the different samples differ indicating that the sample which initially had a lower isometamidium concentration reached end-point detection by the polyclonal antibody first across the X-ray plate. Thus, visually a judgement can be made on which sample or which animal has a low level of isometamidium chloride and the vice-versa.

It is possible therefore, by utilising an end-point dilution of a standard isometamidium sample to estimate the amount of the drug contained in test samples run alongside, since the degree of exposure in a chemiluminescent assay is proportional to the intensity of light emission. The estimations of isometamidium in test samples are illustrated in Figure 24.

Scheneppenheim and Rautenberg (1988) demonstrated in their study of human immunodeficiency virus (HIV) that,

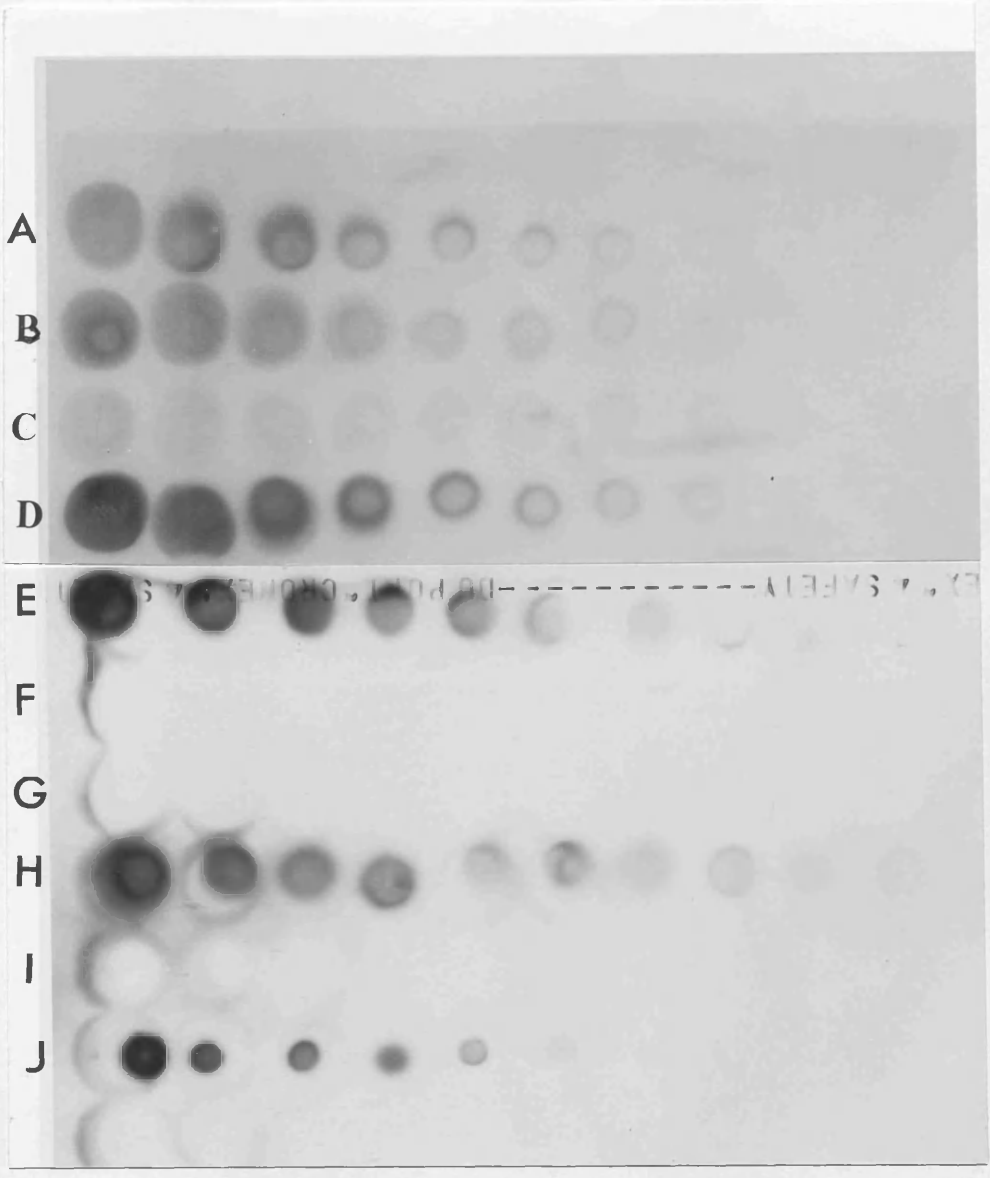


Figure 23

Chemiluminescent Dot-blot Technique. Serially diluted serum samples after an exposure to x-ray film showing those without isometamidium chloride (F, G, I, K, L), with very low amount (C) and with fairly high amounts (A, B, D, E, H, J).

Figure 24

Hypothetical Estimation of Isometamidium in Serum of Treated Animals (based on results obtained in Figure 23)

Assume (J) to be the standard sample:

The end-point of detection of isometamidium in (J) is 1/16 which corresponds to 50 ng/ml isometamidium.

The original concentration of the sample is therefore 16 x 50 = 800 ng/ml.

Likewise, the concentration in the unknown samples applied can be calculated, that is:-

Samples (D) and (H) contain: 128 x 50 ng/ml = 6.4 μg/ml.

Samples (A), (B) and (E) contain: 64 x 50 ng/ml = 3.2 μg/ml.

chemiluminescent assays on blots are one hundred times more sensitive than either commercial ELISA or chromogenic peroxidase assay and detects seroconversion earlier than any other technique. For quantification of isometamidium, the sensitivity of the assay can be improved by the use of Polaroid Type 612 (ASA 20,000) instant, black and white film. As regards the kinetics of light emission in this reaction, that is, a prolonged emission instead of a flash, then the use of Polaroid films will not pose any problems.

The improvement of chemiluminescent assay in the determination of isometamidium in blood of treated animals may offer advantages over other assays which utilise plasma or serum only. Since there are possibilities of isometamidium being bound to leucocytes and erythrocytes, then haemolysed blood samples can be used instead of plasma or serum in the chemiluminescent dot-blot, which may have otherwise interfered in the other assays for isometamidium chloride (spectrophotometric assay, HPLC, radioimmunoassay, ion-pair HPLC and ELISA). Likewise, comparative studies on levels of the drug bound to cellular components and serum proteins can be undertaken.

The use of a Polaroid camera for this assay will offer the advantage of performing multiple assays simultaneously using a low cost portable instrument requiring no power source, and a permanent visual record can be obtained and stored. Thus, under field conditions, the photographic detection of the chemiluminescent dot-blots could provide rapid information regarding drug levels in blood of isometamidium treated animals

and therefore the presence of drug-resistant trypanosomes indirectly. The drawbacks of the photographic detection are that the film is less sensitive than a photomultiplier tube and the results are semiquantitative rather than quantitative unless neutral density filters or a densitometer is used (Kricka and Thorpe, 1986).

A chemiluminescent dot-blot technique could also be employed in serodiagnosis of African animal trypanosomiasis by using biotinylated monoclonal antibodies for the detection of trypanosomal antigens in the blood. Thus, from a single blood sample, disease diagnosis and determination of isometamidium levels could be carried out at minimal time in the field.

CHAPTER FIVE

GENERAL CONCLUSIONS

The foregoing studies have shown that in cattle treated by intramuscular injection of isometamidium chloride, the drug which is released into the circulation is bound to serum proteins (95 - 99%) and very little, if any of it, is free and unbound.

In the studies to determine the extent of isometamidium binding to serum proteins, the separation of free and protein bound isometamidium by ultrafiltration was shown to be a superior method to TCA precipitation when their products (ultrafiltrates, redissolved TCA precipitates and supernatants) were analysed by ELISA since TCA was shown to affect the optical densities of the analytes in ELISA.

The extensive binding of isometamidium chloride to serum proteins has important implications for future studies on the uptake of the drug by trypanosomes. The possibility exists that the trypanosome surface coat glycoprotein has sites with higher affinity to isometamidium than the plasma proteins. Comparative studies of isometamidium binding to sensitive and resistant trypanosome surface coats should also be conducted.

Chemiluminescent studies showed that the serum proteins which bind isometamidium have molecular weights of about 122, 140 and 155 kD in cattle and about 300 kD in goat and mice. In cattle, the protein of about 140 kD was suggested to be a dimer of bovine serum albumin. This molecule seems to be the main isometamidium binding protein compared to the other two which have not been fully characterised. Studies on binding of isometamidium to other serum proteins, particularly glycoproteins, lipoproteins and gammaglobulins, erythrocytes,

leucocytes and platelets may also show other binding sites of importance.

Chemiluminescent studies have also revealed that a purified anti-isometamidium antibody produced in sheep using an isometamidium conjugated porcine thyroglobulin, is highly specific for isometamidium chloride and can be used in immunological assays for the detection and quantification of the drug in sera of treated animals.

"Chemiluminescent Western-blot technique" is a sensitive and accurate method in characterisation of isometamidium binding proteins in serum, and may also be a useful technique in studying protein binding of other trypanocidal drugs, and possibly the uptake of these in vitro and in vivo.

A novel application of this technique "Chemiluminescent dot-blot" has shown a possibility of offering a simple, quick, less expensive and yet a sensitive method for the quantification of isometamidium in body fluids of treated animals under field conditions. The sensitivity and accuracy of the assay can be improved by using Polaroid Type 612 instant films in a polaroid camera incorporated with neutral density filters or using a densitometer. This technique also offers the possibilities of combining the determination of isometamidium levels with serodiagnosis of African animal trypanosomiasis from a single blood sample.

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