STUDIES OF CRUDE PROTEINS IN *LEISHMANIA DONOVANI* PARASITES THAT ARE RESISTANT TO SODIUM STIBOGLUCONATE (PENTOSTAM^{R})

By

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DECLARATION

I, Sammy M. Orinda hereby declare that this is my own work and it has not been presented to any other University.

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DEDICATION

To my mother and late father.

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ABBREVIATIONS

APS - Ammonium Persulphate

BSRC - Biomedical Sciences Research Centre

CPM - Count per minute

FBS - Foetal Bovine Serum

NLB - Nairobi Leishmania Bank

KEMRI - Kenya Medical Research Institute

Sb - Antimony

S.E.M - Standard error of mean

SD - Standard Deviation

SAMT - Semi Automated Microdilution Technique

SDS-PAGE - Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis

WHO - World Health Organisation.

UNIT ABBREVIATIONS

Ci - Curies

D - Dalton

KD - Kilodalton

Kg - Kilogram

ml - millilitre

mg - milligram

mM - millimoles

µg - microgram

µl - microlitre

v - Volume

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ABSTRACT

Leishmania donovani, the causative agent of visceral leishmaniasis (Kala-azar), has been known to develop resistance to sodium stibogluconate (Pentostam^R). The present study aimed at developing a Pentostam-resistant *L. donovani* promastigote strains *in vitro*. This was done by continually exposing the parent susceptible *L. donovani* promastigotes (NLB 065) to increasing concentrations of Pentostam^R for a period of 3 months until the promastigotes were multipying in 10 mg/ml of Pentostam^R. The differences in polypeptide composition of the crude proteins (antigens) of the parent susceptible *L. donovani* promastigotes and the *in vitro* induced Pentostam-resistant *L. donovani* promastigotes (NLB 065R) were detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The mean of inhibitory concentration of Pentostam^R which killed 50% (IC_{50}) of the parent susceptible *L. donovani* promastigotes was 0.084 mg/ml ± 0.01 (SD). The Pentostam-resistant *L. donovani* promastigotes had a mean of IC_{50} 2.90 mg/ml ± 0.83 (SD) and this was significantly different ($t_{0.05}$ 6=2.447, $P<0.01$) from the mean IC_{50} for Pentostam^R against the parent susceptible *L. donovani* promastigotes. The *in vitro* induced, Pentostam-resistant *L. donovani* promastigotes were 35 times more resistant to Pentostam killing than the parent susceptible *L. donovani* promastigotes and this resistance to Pentostam^R was maintained after 4 months of continuous culture in the absence of drug pressure. This indicated that the Pentostam^R resistance in NLB 065R was genetically stable. The Pentostam-resistant *L. donovani* promastigotes had in their polypeptide composition, a polypeptide band of molecular weight 85,000 ± 500 Daltons (D). This polypeptide band was observed in

both logarithmic and stationary phase cultures of NLB 065R promastigotes. These results suggest that the polypeptide of molecular weight 85,000 ± 500 D is unique only to the Pentostam-resistant *L. donovani* promastigotes.

CHAPTER 1

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW.

Leishmaniases is the name of a group of diseases caused by a variety of haemoflagellate protozoan organisms belonging to the genus *Leishmania* and the family trypanosomatidae. *Leishmania* parasites are transmitted by bites of infected female phlebotomine sandflies belonging to the genera *Phlebotomus, Lutzomyia* and *Psychodopygus* depending on geographical location (Minter, 1989). A large number of *Leishmania* species can infect a variety of vertebrates such as reptiles and mammals. The infections being particularly common in man (Jamnadas, 1983).

Human leishmaniasis is acquired during a sandfly bite. These parasites, occurring as highly motile flagellated promastigotes, are introduced into the human skin where they are engulfed by macrophages (Berman, 1988). Within the macrophages the parasites lose their flagella and assume a round oval amastigote forms and then multiples until the macrophage bursts open releasing the amastigotes which are again engulfed by other macrophages (Berman, 1988).

The *Leishmania* amastigotes within the macrophages are generally found in various parts of the body, thus presenting a wide spectrum of the disease. Some parasites are localized at the inoculation site, as in typical cutaneous leishmaniasis, while others metastasize from the inoculation sites into the lymph glands, nodules and other areas of the skin and mucocutaneous regions (nasal and buccal cavities), as in mucocutaneous leishmaniasis (WHO, 1990). Some parasitised cells and free parasites are taken up in the blood circulation and eventually get localized or trapped in the liver, spleen

and bone marrow, as in visceral leishmaniasis (WHO, 1990). Macrophages in these sites perpetuate the proliferation of parasites.

It has long been shown that there are a number of different species of *Leishmania* associated with different types of the disease in man. The different types of the disease range from mild self curing to severe fatal leishmaniasis (Jamnadas, 1983). *Leishmania* infection can lead to a variety of clinical conditions depending on the nature of the infecting species and the immune response developed by the host (Jamnadas, 1983). The infection caused by different infecting species, result in: Cutaneous, Mucocutaneous and Visceral leishmaniasis.

Cutaneous leishmaniasis, "Oriental sore" is a relatively innocuous, self curing localized lesion developing at an exposed site at the point where *Leishmania* parasites were introduced by the bite of the sandfly vector (Jamnadas, 1983). These lesions commonly occur on the face, arms and legs and appear 2 to 8 weeks following sandfly bites (Mahmoud and Warren, 1977). Lesions begin as small erythematous papules, which enlarge slowly and may attain a size of 1 to 2 centimetres (Mahmoud and Warren, 1977). Within a few months the nodules can develop into ulcers that remain as such for periods of up to two years, followed by slow healing and scar formation (Berman, 1988). Although the disease heals within two years, the lesions may be painful, become secondarily infected and cause facial deformity (Adams and Maegraith, 1964). Cutaneous leishmaniasis can be caused by *Leishmania aethiopica*, *Leishmania major* and *Leishmania tropica* in the old world (Minter, 1989). In the new world the parasites which cause cutaneous leishmaniasis include *Leishmania braziliensis panamensis*, *Leishmania mexicana mexicana*, *Leishmania* (*m*) *amazonensis*, *Leishmania* (*m*) *pifonoi* and *Leishmania braziliensis guyanensis* (Minter, 1989). Cutaneous leishmaniasis prevail in South

America, Middle East, Mediterranean countries and some parts of Africa (Manson-Bahr, 1964). For instance in 1969, an endemic focus of cutaneous leishmaniasis was found in the Eastern slopes of Mt Elgon in Bungoma District (Kungu *et al.*, 1972) and a sandfly *Phlebotomus longipalpis* was incriminated as the vector of this parasite (Mutinga and Ngoka, 1978).

Mucocutaneous leishmaniasis (espundia) is a condition initiated as in cutaneous leishmaniasis, but in some patients, the lesions may spread to the mucocutaneous borders of the nose and mouth and erode into the nasal septum, resulting in gross facial deformity (Mahmoud and Warren, 1977). This disease is caused by *Leishmania braziliensis braziliensis* and it is a disease mainly of the South America only (Jamnadas, 1983)

The third classical type of leishmaniasis, visceral leishmaniasis (kala-azar) is caused by *Leishmania donovani* and *Leishmania infantum* in the Old world and *Leishmania chagasi* in the New world Minter, (1989). Visceral leishmaniasis is widely prevalent in many parts of the tropical and sub-tropical world (Handman, 1986; Makherjee *et al.*, 1988). The clinical syndrome from infection with *L. donovani, L. infantum* and *L. chagasi* is due to the invasion of the reticuloendothelial cells in the spleen, liver, bone marrow, lymph nodes and skin (Mahmoud and Warren, 1977). The infection forms in Africa and Central Asia involve a cutaneous nodule which often develops at the site of the parasite inoculation within a few days and lasts for months (Mahmoud and Warren, 1977). After an incubation period of 2 to 6 months systemic manifestations usually develop insidiously. The earliest symptom is fever, which in more than 80% cases develops into a characteristic pattern with twice-daily elevations in temperature other less common symptoms are dizziness, weakness

and weight loss (Mahmoud and Warren, 1977). On physical examination the most characteristic sign of visceral leishmaniasis is enlargement of the spleen and liver which are firm and may reach to the iliac fossa (Mahmoud and Warren, 1977; Berman, 1988). Lymphadenopathy may be generalized and is commonly seen in patients from Africa, the Mediterranean region and China (Minter, 1989). Untreated infections become chronic and are complicated by anaemia, gastrointestinal bleeding tendencies, jaundice and hypoalbuminemia. The mortality rate in untreated infections is above 75% (Mahmoud and Warren, 1977; Chulay *et al.*, 1983) and is due to intercurrent infections such as pneumonia and pulmonary tuberculosis (Mahmoud and Warren, 1977).

The objectives of drug treatment are to cure the patient from intracellular parasitic infection, prevent relapse, avoid the development of unresponsiveness and to keep hospitalization and costs to a minimum (WHO, 1990). To achieve these objectives, appropriate drugs must be given at adequate doses and frequency for a suitable period of time. Grogl *et al.*, (1989) noted that the initial treatment of leishmaniasis should be adequate in terms of dose and frequency with the aims of eliminating the parasites and diminishing the possibility of antimony resistance developing in the field in response to minimal dose therapy.

The drug of choice for the chemotherapy of the three major forms of leishmaniasis viz cutaneous, mucocutaneous and visceral are the pentavalent antimonial compounds such as sodium stibogluconate (Pentostam^{R}) and Meglumine antimonate (Mahmoud and Warren, 1977; Steck, 1981; Chulay *et al.*, 1983; Berman *et al.*, 1987). The two pentavalent antimonials, (Sb) sodium stibogluconate and meglumine antimonates are chemically similar and their toxicity and efficacy in

visceral leishmaniasis are thought to be related to their content of pentavalent antimony (WHO, 1990); Meglumine antimonate solution contains about 8.5% of antimony (85mg/ml), where as sodium stibogluconate solution contains about 10% of antimony (100 mg/ml). In cases where Pentavalent antimonial compounds fail, other drugs such as pentamidine and amphotericin B are used (Sampaio *et al.*, 1971) though in some cases these drugs also fail to eradicate the parasite (Neva *et al.*, 1979). Peters *et al.* (1980) reported that relatively few drugs have been found to be effective against leishmaniasis in man and that none of these drugs used in the treatment of leishmaniasis is devoid of significant toxicity and all must be administered in large doses over long periods of time. This procedure is difficult and costly and may prove impractical in some situations (Berman and Wyler, 1980; Peters *et al.*, 1980). The availability, price, safety and efficacy of the drug coupled with the need for prolonged injection schedules of the pentavalent antimonials has been suggested by Grogl *et al.*, (1989) as some of the most important contributing factors to suboptimal treatment conditions in the developing countries.

The mode of action of pentavalent antimonials is unknown. Pentostam has been shown to interfere with nucleic acid and protein synthesis in *L. mexicana*, a process which may be attributed to decreased nucleoside triphosphate formation (Berman *et al.*, 1985). More recently, Berman *et al.* (1989) have shown that Pentostam specifically inhibits the glycolytic pathway and fatty acid beta-oxidation in *L. mexicana amazonensis*.

Leishmaniasis is characterized by a certain degree of resistance to antimonial treatment (Napier *et al.*, 1942; Marsden, 1949; Sen Gupta, 1953; Manson Bahr, 1959; Chulay *et al.*, 1983; Jha, 1983;

Bryceson *et al.*, 1985) and relapses are not exceptional (Chulay *et al.*, 1982; Wijers, 1971). There are increasing reports about either initial failures of patients to respond to antimony treatment or emergence of the disease after one or more apparently successful previous courses of treatment (Grogl *et al.*, 1989).

The development of drug-resistant promastigotes therefore provide a useful tool for the investigation of the mechanisms through which *Leishmania* develop resistance to Pentostam^R. The experimental use of drug resistant promastigotes of *L. donovani* may suggest chemotherapeutic strategies to overcome drug resistance and may make it possible to include Pentostam^R resistant strains in drug testing programs.

1.1 Chemotherapy of Leishmaniases

The initial treatment of a parasitologically proven case of visceral leishmaniasis has been based on a daily injection of 20 mg of antimony (Sb) per kg of body weight, to a maximum of 850 mg (WHO, 1990). This implies a maximum daily dose of 10 ml of meglumine antimonate or 8.5 ml of sodium stibogluconate. The injections are normally given for a period of 20 days. The duration of treatment varies from one endemic area to another, but treatment should be continued for 2 weeks after apparent parasitological cure. The exact length of treatment is individually determined for each country and for each patient. For example in India a 40 day regimen has been found to give best results, whereas in China, 6-day courses were reported to produce a cure rate of over 90% (WHO, 1990).

1.2 Drug resistance in Leishmaniasis

Parasitic protozoan infections have been shown to constitute a major problem to socioeconomic development in most of the developing countries (Grogl *et al.*, 1989). Resistance to drugs among the parasites and limited knowledge of the mechanisms by which the parasites acquire resistance account for an additional burden in the development of pragmatic public health policies for the control of these human parasitoses (Grogl *et al.*, 1989). Resistance to antiparasitic drugs has been reported for malaria (Moore and Lanier, 1961; Peters, 1970; Wyler, 1983; Peters, 1985; Geary *et al.*, 1986), trichomoniasis (Muller *et al.*, 1980) and trypanosomiasis (Dvorak and Howe, 1977; Leach and Roberts, 1981; Filardi and Brener, 1987). Resistance in *Leishmania* to antimonial chemotherapy has been recognised for decades (Napier *et al.*, 1942; Marsden, 1949; Sen Gupta, 1953; Wijers, 1971; Peters, 1974, 1976; Chulay *et al.*, 1983; Jha, 1983; Bryceson *et al.*, 1985).

Initial failures of *L. donovani* to respond to Pentostam^R or relapses after treatment is increasingly being reported (Grogl *et al.*, 1989; Ullman *et al.*,1989) and it is not known whether these cases of antimonial drug insusceptibility are due to the development of drug resistance in the parasite or due to a variety of host factors that might also contribute to drug unresponsiveness (Ullman *et al.*, 1989). Peters *et al.* (1980) reported that pentamidine isothionate is sometimes used successfully in the therapy of visceral leishmaniasis when antimonials fail, but this drug is of little value in East Africa. Wijers (1971) reported relapse rates of between 10% to 30% in a study done on the occurrences of relapses after Pentostam^R treatment of Kala-azar cases in Tharaka of Meru district. Manson-Bahr (1959) in his review of Kala-

azar in East Africa has reported a relapse rate of Pentostam^R (Sb) in 1 out of 21 cases (4.7.%); urea stibamine, 1 out of 19 cases (5.2%); Hydroxystilbamidine, 4 out of 20 cases (29)and for the combined course, 3 out of 19 cases (15.7%). Gachihi *et al.* (1987) in their study of 54 patients in Machakos Provincial Hospital reported a relapse rate of 9.7% in the group of patients treated with 20mg Sb/kg/day of Pentostam^R and 18.2% in the group treated with 10mg Sb/kg/day. Muigai *et al.* (1985) treated 50 patients with Pentostam^R at a dosage of 10 mg/kg/day of whom 25 responded well and had no relapse within a year of treatment; 18 responded but were not followed up; 2 did not respond to the treatment and 3 could not be evaluated. Chulay *et al.* (1982) reported that a 2 year old girl failed to respond to treatment of a dose of 10mg Sb/kg/ body weight of Pentostam^R every 12 hours for 15 days and when she was treated with high dose Pentostam^R 20mg Sb/kg/day for 90 days she relapsed 8 weeks after stopping treatment and she failed to respond to additional Pentostam^R (20mg Sb/kg twice daily). These patients who relapse often require prolonged and repeated treatment imposing a severe economic and logistic burden on health care facilities in areas where they occur (Gachihi *et al.*, 1987).

Mebrahtu *et al.* (1989) reported the first human visceral leishmaniasis caused by *L. tropica* in Africa. They characterized *L. tropica* isolates from two patients with visceral leishmaniasis who were unresponsive to treatment with sodium stibogluconate and further suggested the possible existence of genetic susceptibility or failure to respond to Sb.

Possible explanations for these clinical and parasitological unresponsiveness to organic pentavalent antimonials has been proposed by several authors. Berman (1988) suggested that the

reasons might include, parasite resistance or genetic, immunological or physiological defects in the host. Similarly Grogl *et al.*, (1989) has proposed that inherent resistance of the parasite to Sb could be immunologic, physiologic or pharmacokinetic defects in the host, a genetic susceptibility of failure of patient to respond to Sb or a particular combination of these factors could cause Sb unresponsiveness.

Zakrzewski (1973) has outlined three basic mechanisms that may be responsible for the development of drug resistance in any living organism as altered drug uptake, altered metabolism and inactivation of the drug by the parasite. Any one of these alterations could be the result of a variety of biological changes which had occurred during the development of resistance. Zakrzewski (1973) suggested that altered drug uptake may be the reflection of changes at the cell surface or the internal membranes or of enzymes responsible for the active transport of the drug into the cell or its efflux out of the cell. He further suggested that altered metabolism may be the result of changes in a single enzyme protein so that the interaction of the drug with the enzyme is decreased or it may result from the development of new enzymatic proteins that bypass the step sensitive to the drug. Finally he suggested that drug inactivity may be caused either by the development of enzymes capable of degrading the drug or by the formation of proteins with or without enzymatic activity, capable of binding the drug and thus removing it from the circulation.

One common mechanism by which *Leishmania* become resistant to drugs *in vitro* is by genetic lesions in their transport systems (Ullman *et al.*, 1989). Mutant *L. donovani* cell lines have been characterized with biochemically defined genetic lesions in various components of the purine salvage pathway (Iovannisci and Ullman,

1984) and Mutant *L. major* (Ellenberger and Beverly, 1987) and *L. donovani* (Kaur *et al.*, 1988) defective in folate/methotrexate transport have also been characterized.

Bryceson *et al.* (1985) in their results of the clinical and immunological observations in Kenyan kala-azar patients, suggested that *Leishmania* acquires resistance to pentavalent antimony as a result of drug pressure. This has been supported by Grogl *et al.* (1989) when they developed resistance to Pentostam^R in promastigotes of *Leishmania* species *in vitro* by exposure to gradually increasing concentrations of drug over several passages. Ullman *et al.* (1989) has also generated several *L. donovani* strains, resistant to Pentostam^R killing by exposing the strains to drug pressure and further concluded that Pentostam^R resistance in the mutant organisms was of a stable genetic trait.

The development of drug resistance *in vitro* could greatly facilitate studies into the molecular basis of drug resistance or drug insusceptibility (Ullman *et al.*, 1989). The advantages of using an *in vitro* system for the induction of drug resistance include the development of parasites with levels of drug resistance comparable to those found *in vivo* as well as the development of parasites with a higher level of drug resistance than can possibly be induced in animal models due to the toxicity of the drug to the host (Grogl *et al.*, 1989).

1.3 Differences between logarithmic phase and stationary phase of *Leishmania donovani* promastigotes *in vitro*.

When *Leishmania* species are grown *in vitro*, parasites from the stationary phase differs from those in logarithm phase growth in being

more infective and more resistant to complement and macrophage mediated killing (Grogl *et al.*, 1987). This enhanced infectivity of stationary phase, promastigotes may be the result of their relative ability to survive host defense mechanisms. For instance, *Leishmania braziliensis panamensis* develops during growth *in vitro* from complement susceptible logarithmic phase promastigotes to complement resistant stationary phase promastigotes (Franke *et al.*, 1985). Sacks *et al.*, (1985) have found differences in the ability of stationary phase promastigotes to survive the leishmanicidal mechanisms of normal resident macrophages. Biochemical changes have been reported by Grogl *et al.* (1987) to occur in *Leishmania braziliensis panamensis* during the switch from logarithmic phase promastigotes to stationary phase promastigotes. Some of these changes have been detected by electrophoretic separation of whole cell extracts of *L. braziliensis panamensis* using SDS-PAGE and silver staining. When comparing the polypeptide profiles of logarithmic and stationary phase promastigotes of 2 strains of *L. braziliensis panamensis*, Grogl *et al.* (1987) found that polypeptides unique to logarithmic phase promastigotes had molecular weights of 31, 66 and 102 KD, while a polypeptide of 79KD was detected only on stationary phase promastigotes.

1.4 *Leishmania* proteins.

Lepay *et al.*, (1983) reported three major proteins of molecular weights 65,000, 25,000 and 23,000 daltons on the surface profile of two isolates of *L. donovani* promastigotes from Brazil and Sudan. They further suggested that proteins of molecular weight 65,000 was a

glycoprotein and may be a common *leishmania* antigen shared by *L. donovani, L. braziliensis, L. mexicana* and *L. tropica.* This suggestion has been supported by several other authors. It was shown, for example that after radio-ioination of the proteins exposed at the surface of *Leishmania* promastigotes, a glycoprotein of molecular weight ranging from 62,000 to 65,000 was predominantly labeled in different isolates of *leishmania* species infective to humans (Fong and Chang, 1982; Gardiner *et al.*, 1983; Etges *et al.*, 1985; Colomer-Gould *et al.*, 1985; and Bouvier *et al.*, 1985). This protein provisionally termed p63 is a major amphilic constituent of the membrane and represent approximately 1% of the total cell protein in *L. major* (Etges *et al.*, 1985). The polypeptide maps of p63 of *L. major, L. donovani, L. tropica* (Etges *et al.*, 1985) and *L. major, L. donovani, L. braziliensis* (Colomer-Gould *et al.*, 1985) are practically identical indicating that the primary structure of these proteins has been substantially conserved among the widely separated *Leishmania* species (Etges *et al.*, 1986).

Purkis and Coombs (1986) cultured amastigotes and promastigotes of *L. mexicana mexicana, L. amazonensis, L. major* and *L. donovani* and analysed them by SDS-polyacrylamide gel electrophoresis. Polypeptide banding patterns of the promastigotes of the four species were quite similar, but distinct differences were detected between those of amastigotes. The results suggested that the various species of *Leishmania* are adapted differently for survival and growth in the mammalian host.

Approximately 20 externally displayed membrane protein ranging in molecular weights between 10,000 to 200,000 daltons have been identified in *L. tropica* promastigotes (Handman, 1986). Of these, 12 were identified as mannose containing glycoproteins and

seven as major components (Handman, 1986). Handman *et al.*, (1981) by analysis of the radio iodinate promastigotes membrane of *L. tropica* revealed 6 major and some minor acidic protein, for amastigotes, and 6 major acidic poorly resolved basic protein. Four of the major proteins appeared to be common, one specific for promastigotes (50,000 daltons) and two specific for amastigotes (94,000 and 43,000 daltons).

Dwyer (1980), worked on the cell surface pellicular membrane, which were isolated from promastigote forms of *L. donovani* by differential and discontinuous sucrose gradient centrifugation procedures. As determined by SDS-PAGE, isolate pellicular membranes contained approximately 40 peptide bands ranging in molecular weight 12,000 to 22,000 daltons, of these 19 were detected by periodic acid schiffs (PAS) reagent, suggesting that most of the pellicular membrane carbohydrate constituents were present as glycoproteins. Ramasamy *et al.*, (1981) while studying the proteins and surface antigens on a Kenyan strain of *L. donovani* reported five proteins of molecular weights ranging from 18,000 to 95,000 daltons. Of these, three major protein antigens of molecular weights 66,000, 53,000 and 43,000 were identified. Jamnadas (1983) reported that proteins in the whole lysate of *L. donovani* promastigotes, after SDS-PAGE analysis revealed a complex pattern of several polypeptide chains. Up to 32 polypeptides could be clearly distinguished after Coomassie blue staining and the chains that were identified had molecular weights ranging from 15,000 to 200,000 daltons.

1.5 Justification of the study.

Drug resistance in the treatment of leishmaniasis is causing problems with the control of the disease. The mechanisms through which *Leishmania* parasites develop resistance to anti-leishmania drugs is not known. So the development of the Pentostam-resistant *L. donovani* promastigotes *in vitro*, will greatly facilitate studies into the molecular basis of drug resistance. These Pentostam-resistant *L. donovani* promastigotes can be used to investigate the mechanisms through which *Leishmania* parasites develop resistance to Pentostam. The experimental use of Pentostam-resistant *L. donovani* promastigotes may suggest chemotherapeutic strategies to overcome drug resistance and makes it possible to include Pentostam-resistant strain in drug testing programs.

The analysis of crude protein of *L. donovani* promastigotes that are resistant to Pentostam^R may enable the identification of resistant cases before treatment.

1.6. Specific objectives:

i. To generate a resistant strain of *L. donovani* promastigotes to Pentostam^R from a parent susceptible strain of *L. donovani* ((NLB 065) *in vitro* by continuous drug pressure.

ii. To compare polypeptide profiles of logarithmic and stationary phase culture promastigotes of the parent susceptible *L. donovani* promastigotes and the Pentostam-

resistant *L. donovani* promastigotes thus by SDS-PAGE purification.

CHAPTER 2

2.0 CULTURE AND MAINTENANCE OF *L. DONOVANI* PROMASTIGOTES

2.1 INTRODUCTION:

Many different media exist which are suitable for the cultivation of *Leishmania* promastigotes *in vitro*. There are media which are appropriate for the isolation of *Leishmania* stock and some media suited to the bulk growth of *Leishmania* promastigotes. Liquid media are suited to large scale production of *Leishmania* promastigotes (Miles, 1982). Liquid media are clear and can be examined with an inverted phase microscope without opening the flask, thus minimising possibilities of contamination.

2.2 MATERIALS AND METHODS.

2.2.1 Origin of Parasites

The parent susceptible *L. donovani*, Nairobi Leishmania Bank 065 (NLB 065) was obtained from the Nairobi Leishmania bank, Kenya Medical Research Institute (KEMRI). Strains of NLB 065R and NLB 065R$_I$ were developed in the laboratories. The NLB 065R strain was the Pentostam-resistant *L. donovani* promastigotes developed from the parent susceptible *L. donovani* promastigotes. The NLB 065R$_I$ was the

resistant strain derived from the NLB 065R and was cultured in the absence of drug pressure for 4 months.

2.2.2 Preparation of Media.

A sachet of RPMI 1640, with L-Glutamine and 20 millimoles (mM) of Hepes buffer (Flow Laboratories Scotland, U.K) was dissolved in 800ml of distilled water and 20.2mM of sodium bicarbonate (May and Baker, England) was added. The pH was adjusted to 7.2 using one mole hydrochloric acid (HCl) (May and Baker, England) and IM sodium hydroxide (E.T. Monk, Kenya). The volume was made up to 1000ml by adding distilled water. The medium was sterilized using 0.2*u*m millipore filters (Nalgene filterware, Nalge Company, USA) in 100ml aliquots into previously autoclaved glass bottles. The bottles were screwed tightly, labeled and stored at 4°C. To make the complete medium for growing the parasites, the medium above was supplemented with 20% of sterile foetal bovine serum (FBS) (Flow Laboratories, Scotland, U.K). To guard against bacterial contamination, 100µg/ml streptomycin and 100 units/ml G-penicillin (Flow Laboratories) was added.

2.2.3 Maintenance of Promastigotes.

Cultures of NLB 065 and NLB 065R promastigotes were maintained in 25 cm^2 plastic tissue culture flasks (Flow Laboratories Scotland, U.K) using 5 mls of complete RPMI 1640 at 25°C. Under these conditions the parasites were found to grow well and after 72

hours their population were found to be above 70.0 x 10^6 parasites/ml. The cultures were maintained by sub-culturing after every 4 to 5 days of incubation by transfering 0.1 ml of the active culture into 5 ml of the new medium. This was done aseptically in a vertical-flow laminar hood to avoid contamination of the cultures. Strains NLB 065 and NLB 065R were maintained in 2 tissue culture flasks as a safety precaution in case some were lost through contamination.

2.2.4 Determining the growth rates of *L. donovani* strain NLB 065, NLB 065R and NLB 065R$_1$

The strain NLB 065 was the parent susceptible *L. donovani* from which NLB 065R was developed by continuous drug pressure. Strain NLB 065R$_1$ was derived from NLB 065R and was kept in complete medium RPMI 1640, supplemented with 20% FBS for a period of 4 months in the absence of drug pressure.

Three strains were cultured in 25 cm^2 sterile disposable flasks (Flow Laboratories Scotland, UK). For strains NLB 065 and NLB 065R$_1$, 4ml of complete RPMI 1640 supplemented with 20% FBS was added to the culture flask. One millilitre of 2.0 x 10^6 logarithmic phase promastigotes/ml of NLB 065 and NLB 065R$_1$ was added to the culture flask. For strain NLB 065R, 3.5 ml of complete medium was used, 0.5ml of Pentostam^R (100 mg Pentostam^R sb/ml, The Wellcome Foundation Ltd, London) was also added then mixed to give a concentration of 10.0mg/ml of antimony. One millilitre of 2.0 x 10^6 logarithmic phase promastigotes/ml of NLB 065R was added. The culture flasks were incubated at 25°C and haemocytometer counts were done after every 24 hours for 8 days.

2.3 RESULTS

The growth patterns of the *L. donovani* strains, NLB 065, NLB 065R and NLB $065R_1$ (Figure 1) illustrate that all the 3 strains had an exponential growth between day 1 and day 3. On day 3, the number of promastigotes per ml in all the 3 strains was above $7x10^7$. After day 3, the growth rate of all the 3 strains was lower, though on day 4, the number of promastigotes in all the 3 strains was higher than in day 3. The growth rate of strains NLB 065 and NLB $065R_1$ were drastically reducing from day 4 to day 8.

The stationary phase of strain NLB 065R was longer (3 days) as compared to the stationary phase of strains NLB 065 and NLB $065R_1$. The death phase of the strain NLB 065R started on day 6.

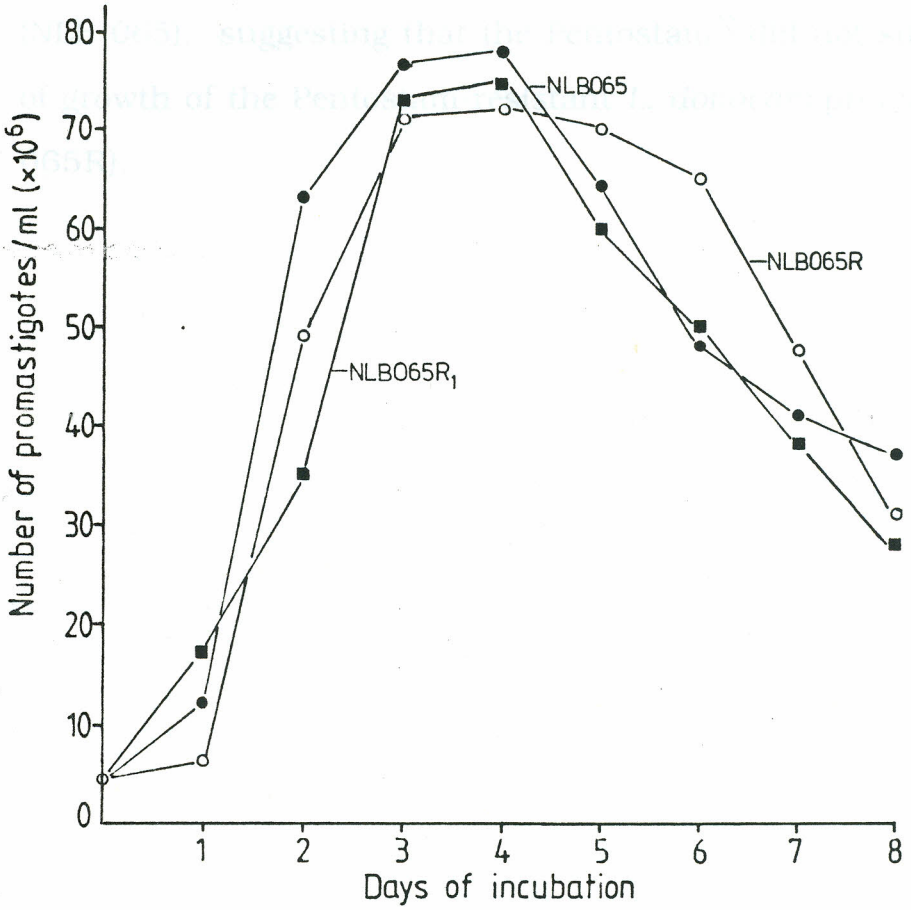

Figure 1. Growth patterns of *L. donovani* strains NLB 065, NLB 065R and NLB 065R$_I$. All the 3 strains were maintained at 25°C in RPMI 1640, supplemented with 20% heat-inactivated FBS. The growth media of NLB 065R was supplemented with 10.0 mg/ml of Pentostam^R.

2.4 DISCUSSION

The growth patterns of the Pentostam-resistant *L. donovani* promastigotes, cultures in the absence of drug pressure (NLB 065R$_I$) and those cultured under drug pressure (NLB 065R) had similar growth patterns to the parent susceptible *L. donovani* promastigotes (NLB 065), suggesting that the Pentostam^R did not suppress the rate of growth of the Pentostam-resistant *L. donovani* promastigotes (NLB 065R).

CHAPTER 3

3.0 DRUG SUSCEPTIBILITY TESTING.

3.1 INTRODUCTION

Susceptibility tests were performed *in vitro* using the methods of Grogl *et al.*, (1989) a modification of the semi-automated microdilution technique (SAMT) of Desjardins *et al.* (1979). Susceptibility tests to Pentostam^R were done on the following strains of *L. donovani*: NLB 065, NLB 065R, NLB 065R$_1$.

The assay for this test is based upon the incorporation of methyl [^{3}H] thymidine into DNA. The uptake of methyl [^{3}H] thymidine in DNA has widely been used as a measure of DNA synthesis and cell proliferation (Maurer, 1981). The suppression of the uptake of methyl [^{3}H] thymidine indicate response to Pentostam^R.

3.2 MATERIALS AND METHODS

3.2.1 PREPARATION OF *LEISHMANIA* PARASITES:

The *L. donovani* strains used in these experiments were the susceptible NLB 065, the Pentostam-resistant NLB 065R, which were maintained in RPMI 1640 supplemented with 20% FBS and containing 10mg/ml of Pentostam^R. The Pentostam^R resistant NLB 065R$_1$ promastigotes, were maintained in culture medium in the

absence of drug pressure. The NLB 065R promastigotes that were under drug pressure were washed 3 times with incomplete Schneider's medium, to remove any traces of Pentostam^{R}.

3.2.2. Preparation of Microtitration Plates.

The microtitre plate, (Costar^{R}) used consisted of 96 flat-bottomed wells arranged in a matrix of eight rows (A through H) and 12 columns (1 through 12) (Fig. 2)

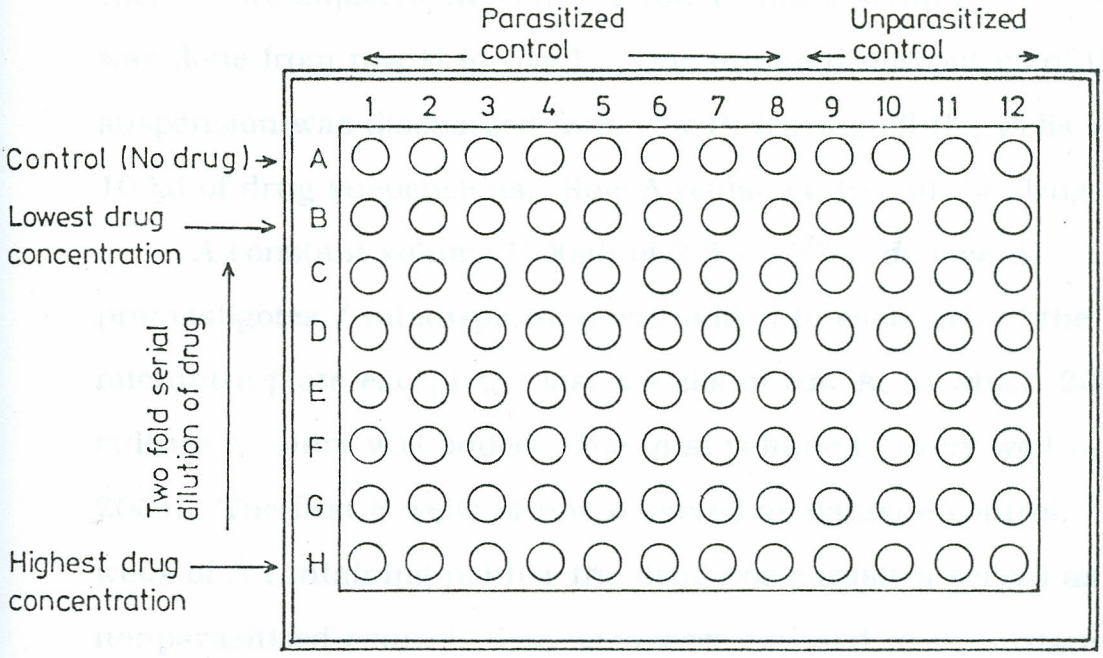

Figure 2. Representation of a microtiter plate with 96 wells arranged in 8 rows (A-H) and 12 columns (1-12). When prepared as described, wells 1 through 8 of row A served as a parasite control (no drug present) and wells 9 through 2 of row A serve as unparasitized control (no drug and no parasites). Each strain was present in duplicate columns. The highest concentrations of drug in row H, and in two fold dilution to the lowest concentration in row B.

The plates were prepared using aseptic techniques inside a laminar columns flow hood as described below. A 100µl Hamilton (Hamilton, Nevada) repeating dispenser was used to discharge 100µl of Schneider's medium supplemented with 10% FBS in wells from G through A. Two hundred microlitres of the drug solution was added to each of two adjacent wells in the row H and a serial two fold dilution was done from row H to row B. One hundred microlitres of the drug suspension was discharged from row B, leaving all the wells with 100µl of drug suspensions. Row A remained free of the drug.

A constant volume (100µl) of 2.5 x 10^6 *L. donovani* promastigotes / ml suspension was added to each well of the microtitre plate except the last 4 wells of row A, to which 200µl of the culture medium was added. The total volume in every well was then 200µl. The first 8 wells of row A served as parasite control. The last wells of A containing neither the drug nor parasites served as nonparasitized control. Parasites were exposed to Pentostam^R, with the highest concentration in row H and the lowest concentration in row B.

Then each plate was sealed with parafilm and incubated for 24 hrs at 25°C prior to the addition of methyl [^{3}H] thymidine.

3.2.3 Preparation of Isotope and labelling of parasites.

Uptake of methyl [^{3}H] thymidine (Amersham international p.c Amersham, U.K.) was used as an index of growth of the parasites. The Isotope was supplied as an aqueous solution containing 10% ethanol in a bottle containing 25 Ci/mM of methyl [^{3}H] thymidine. To the 10ml of Schneider's medium, supplemented with 10% FBS, 160µl of the

methyl [^{3}H] thymidine was added. Twenty five microlitres of the isotope solution (160µl of methyl [^{3}H] thymidine plus 10 ml of assay medium) was added to each well to give a final concentration of 2 µCi per well. Each plate was resealed and incubated for an additional 18 hrs prior to harvesting.

3.2.4 Harvesting parasites and Scintillation Counts.

At the end of the second incubation period each plate was harvested on a MASH II, automated cell harvester (Microbiological Associates, Bethesda Md.). This instrument aspirated and deposited the particulate contents of each of the wells onto fibre glass filter paper (Titerlek, U.K.) which was then washed with copious amounts of deionised water. Each disk was dried and placed into a scintillation vial containing 2 ml of scintillation fluid. All 96 vials corresponding to the 96 wells of the microtitre plate, were counted in a Beckman LS 1801 model liquid scintillation counter for 1 minute.

The scintillation counter measures the incorporation of methyl [^{3}H] thymidine to parasite nuclei acid, with the results expressed as counts per minute (CPM).

The calculation of the concentration of Pentostam^R causing 50% inhibition of methyl [^{3}H] thymidine uptake (IC_{50}) for each strain was done in duplicate. The parasitized control mean of CPM values were used to estimate the midpoint (Y_{50}) as shown below.

$$(\text{Midpoint } (Y_{50}) = \frac{PC - UPC}{2} + UPC$$

where PC = Parasitized Control and UPC= Unparasitized Control.

The IC_{50} values were then determined by interpolation between one data point above and below the Y_{50}. Interpolated IC_{50} values were obtained after logarithmic transformation of both concentration and CPM values, using the formula of Sixsmith *et al.* (1982, 1984) as shown below:

$$IC_{50} = \text{anti log } (\log X_1) + \frac{(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)}{(\log Y_2 - \log Y_1)}$$

where X_1 and X_2 are the drug concentrations and Y_1 and Y_2 are the means of CPM.

CHAPTER 4

4.0 Development of a Pentostam-resistant *Leishmania donovani* strain *in vitro* by continuous drug pressure.

4.1 INTRODUCTION

The *in vitro* development of *Leishmania* parasite strains resistant to drugs like Pentostam^{R}, considerably facilitate studies in the nature and the molecular basis of drug resistance and the mode of action of a given drug (Grogl *et al.*, 1989). The advantages of using an *in vitro* system for the induction of drug resistance include the development of parasite lines with levels of drug resistance comparable to those found *in vivo*, as well as the development of parasite lines with a higher level of drug resistance than can possibly be induced in animal models, due to the toxicity of the drug to the host (Grogl *et al.*, 1989).

The development of Pentostam-resistant strains of *Leishmania* has been achieved *in vitro* by exposing the susceptible *Leishmania* to continuous drug presence (Ullman *et al.*, 1989; Berman *et al.*, 1989) or exposing the susceptible *Leishmania* to discontinuous drug pressure (Grogl *et al.*, 1989). These two methods have been used to generate genetically stable, resistant *Leishmania* parasites *in vitro*.

4.2 MATERIALS AND METHODS

Leishmania donovani NLB 065R that was made resistant to Pentostam^R (100mg Pentostam^R Sb/ml, the Wellcome Foundation Ltd, London) was achieved by using continuous exposure to drug pressure as described by Ullman *et al.*, (1989). The susceptible *L. donovani* promastigotes (NLB 065) at their logarithmic phase were counted and diluted to 2.5 x 10^6 parasites/ml in complete RPMI 1640, supplemented with 20% FBS. The promastigote suspension was exposed to predetermined concentrations of Pentostam^R ranging from 1.0 mg/ml to 0.0156 mg/ml. *Leishmania donovani* promastigotes survived and multiplied in drug concentrations ranging from 0.0625 mg/ml to 0.0156 mg/ml. The highest concentration of Pentostam in which the parent susceptible *L. donovani* promastigotes survived and multiplied was 0.0625 mg/ml. The susceptible *L. donovani* promastigotes were killed with concentrations of Pentostam higher than 0.0625 mg/ml after 1 day. The promastigotes multiplying in 0.0625 mg /ml of Pentostam were sub-cultured after 2 to 3 days into 4 culture flasks containing culture medium plus Pentostam in the following way.

Flask A - Concentration of Pentostam similar to the one the Susceptible *L. donovani* were multiplying in (0.0625 mg/ml)

Flask B - Contains the concentration of Pentostam twice the concentration of Pentostam in flask A.

Flask C - Contains the concentration of Pentostam 5 times the concentration in flask A.

Flask D - Contains the concentration of Pentostam 10 times the concentration in flask A.

The promastigotes surviving and multiplying in the highest concentration of Pentostam among the 4 flasks were exposed to higher concentrations of Pentostam using the same procedure. This was done repeatedly until the *L. donovani* promastigotes were multiplying in 10.0 mg/ml of Pentostam. The control cultures of the parent susceptible *L. donovani* promastigotes were maintained in culture in the absence of drug pressure. These cultures were kept exactly under the conditions as the experimental flasks and for the same period of time. The period taken to develop the Pentostam-resistant *L. donovani* promastigotes and the highest concentrations of Pentostam to which the promastigotes were exposed to depended on the availability of Pentostam. The Pentostam-resistant *L. donovani*, NLB 065R was maintained continuously in growth medium containing 10.0mg/ml of Pentostam^{R} for 6 months. An aliquot of 065R promastigotes were removed from drug pressure, centrifuged (3600 RPM for 10 minutes), washed three times in incomplete medium to remove any traces of Pentostam and then resuspended in fresh complete medium. These promastigotes (NLB 065R$_1$) were propagated for 4 months in the absence of drug pressure.

4.3 Testing for the stability of the *in vitro* Induced Pentostam-resistant, *Leishmania donovani* (NLB 065R)

The stability of the Pentostam-resistant *L. donovani* promastigotes was studies *in vitro* as described by Grogl *et al* (1989). The Pentostam-resistant *L. donovani* promastigotes were maintained in culture in the absence of pentostam for 4 months. During this time period, changes in the inhibitory concentration (IC_{50}) of Pentostam^R to the parasites were determined using a modification of the SAMT at I month interval. These Pentostam-resistant *L. donovani* strains maintained in Pentostam free medium were named NLB 065R1. The IC_{50} of the Pentostam-resistant *L. donovani*, cultures in 10.0 mg/ml of Pentostam^R was also monitored. Cultures were maintained in complete RPMI 1640 supplemented with 20% FBS at 25°C and the promastigotes were subcultured every after 4-5 days to keep them alive.

4.4 RESULTS

4.4.1 Development of the parent susceptible *L. donovani* (NLB 065) promastigotes to a Pentostam-resistant *L. donovani* (NLB 065R) promastigotes *in vitro* by continuous drug pressure.

The Pentostam-resistant *L. donovani* promastigotes were developed by continually exposing the parent susceptible *L. donovani* promastigotes to increasing concentrations of Pentostam^R. The highest concentration of Pentostam in which the parent susceptible *L. donovani* promastigotes survived and multiplied was 0.0625 mg/ml. After continuous drug pressure for 3 months, these promastigotes were multiplying in 10.0 mg/l of Pentostam^R.

The determination of the susceptibilities of the parent susceptible *L. donovani* and the Pentostam-resistant *L. donovani* promastigotes to Pentostam^R was done by using a modification of SAMT. The assay of this technique is based upon the uptake of radiolabelled nuclei acid precursor methyl [^{3}H] Thymidine as an index of parasite growth. The suppression of the uptake of methyl [^{3}H] thymidine indicated the parasite response to Pentostam^R. This is illustrated in figures 3 and 4 showing the concentration response profile of the parent susceptible and Pentostam-resistant *L. donovani* promastigotes respectively.

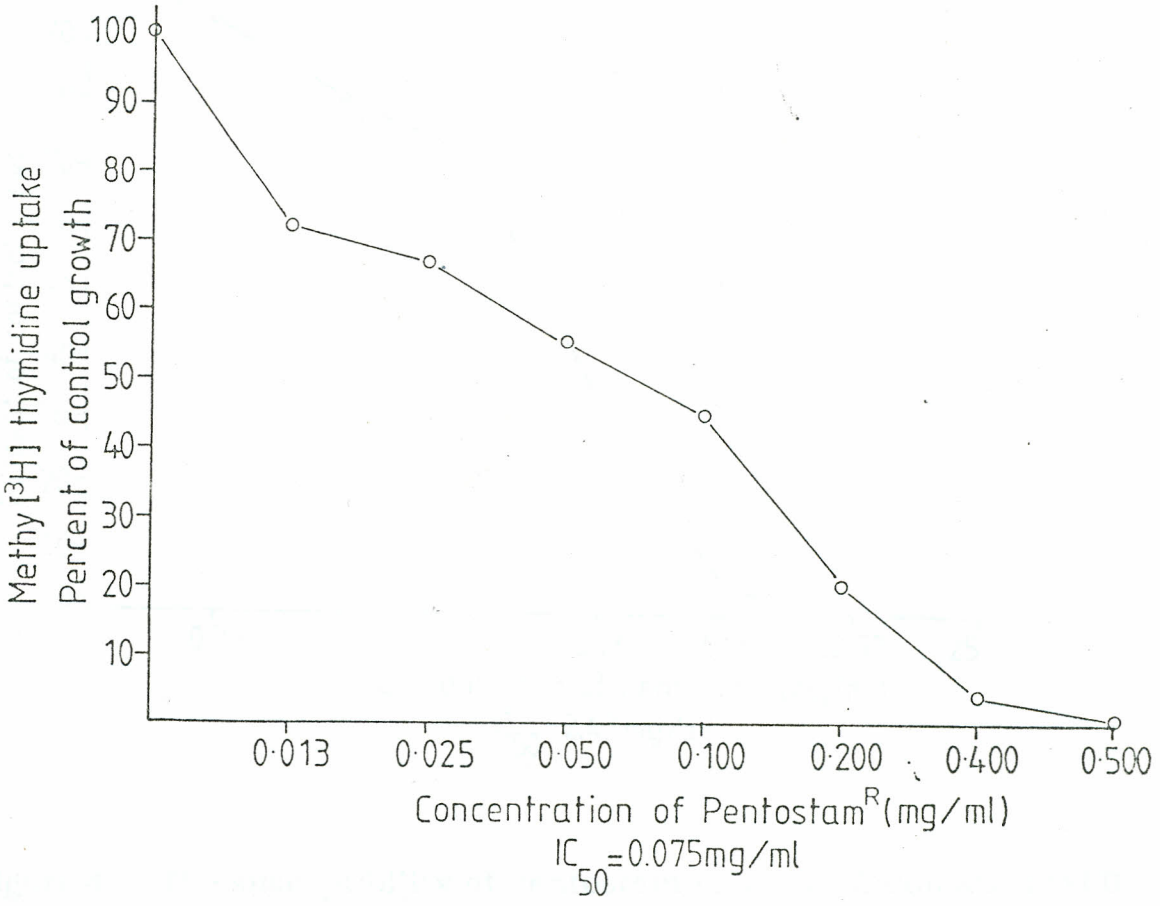

Figure 3. **The susceptibility of Parent susceptible *L. donovani* (NLB 065) promastigotes to Pentostam^R as determined using a modification of SAMT. The effects of Pentostam^R were determined on NLB 065 promastigotes maintained at 25°C in RPMI 1640 supplemented with 20% heat-inactivated FBS.**

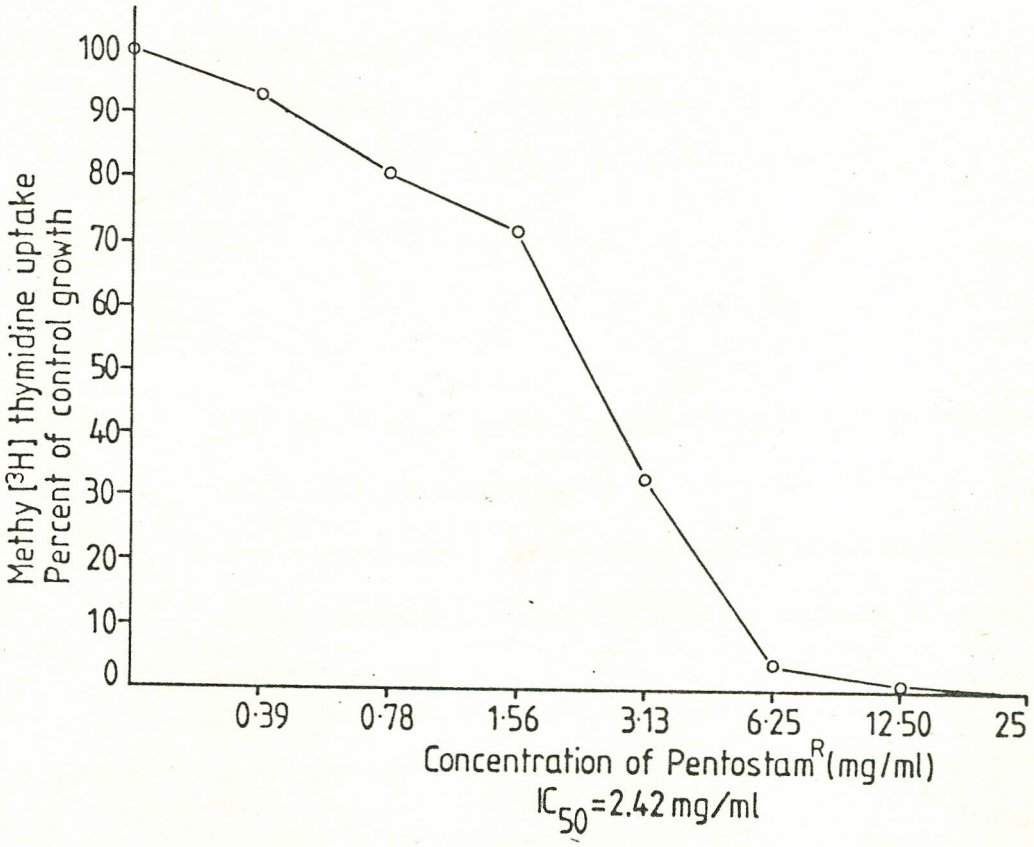

Figure 4. **The susceptibility of Pentostam-resistant *L.donovani* (NLB 065R) promastigotes to Pentostam^R, as determined using a modification of SAMT. The effects of Pentostam^R were determined on NLB 065R promastigotes maintained at 25°C and continuously grown in RPMI 1640, supplemented with 20% of heat-inactivated FBS and 10.0 mg/ml of Pentostam^R.**

The concentration of Pentostam which inhibited the growth of the control cultures of the parent susceptible *L. donovani* promastigotes by 50% (IC_{50}) was 0.084 mg/ml (Table 1). This is a mean of 5 determinations of IC_{50} of the parent susceptible *L. donovani* done at the start of the experiment using a modification of SAMT. After maintaining the parent susceptible *L. donovani* in culture medium in the absence of Pentostam for 9 months, under the same conditions as cultures exposed to continuous drug pressure, the mean of the IC_{50} of Pentostam^{R} against the parent susceptible *L. donovani* was 0.070 mg/ml (Table 2).

Table 1. The susceptibility of the parent susceptible *L. donovani* (NLB 065) promastigotes to Pentostam^{R} at the start of the experiment. As determined by semi automated microdilution technique (SAMT). standard error of the mean (S.E.M).

Number of determinations	SAMT IC_{50} mg/ml (± S.E.M)	SAMT IC_{99} mg/ml (± S.E.M)
1	0.08	0.50
2	0.07	0.55
3	0.09	0.60
4	0.08	0.56
5	0.10	0.59
	$\bar{x}$=0.084 (±0.004)	$\bar{x}$=0.56 (±0.018)

Table 2. The susceptibility of the parent susceptible *L. donovani* (NLB 065) promastigotes to Pentostam^R after being maintained in culture in the absence of Pentostam^R for 9 months. As determined by SAMT, standard error of the mean (S.E.M).

Number of determinations	SAMT IC_{50} mg/ml (± S.E.M)	SAMT IC_{99} mg/ml (±S.E.M)
1	0.06	0.60
2	0.07	0.55
3	0.08	0.65
	$\bar{x}$=0.07 (±0.005)	$\bar{x}$=0.6 (±0.029)

There was no significant difference ($t_{0.05}$ 6=2.4469: p>0.05) from the means of the IC_{50} for Pentostam^R against the parent susceptible *L. donovani* promastigotes at the start of the experiment and after 9 months of culture. These results indicate that the susceptibility of the parent susceptible *L. donovani* promastigotes showed no significant increase or decrease in their IC_{50} to Pentostam^R after having been maintained in culture in the absence of Pentostam and kept under exactly the same conditions as cultures exposed to drug pressure.

The Pentostam-resistant *L. donovani* (NLB 065R) promastigotes were developed by exposing the parent susceptible *L. donovani* to increasing concentrations of Pentostam^R and were multiplying in 10.0 mg/ml of Pentostam^R after 3 months of continuous drug pressure. These Pentostam-resistant *L. donovani* promastigotes were further maintained in culture medium containing 10.0 mg/ml of Pentostam^R for 6 months. The mean of the IC_{50} of Pentostam^R against the

Pentostam-resistant *L. donovani* promastigotes was 2.90 mg/ml (Table 3). This was significantly different ($t_{0.05}$ 6=2.447; $p<0.01$) from the mean IC_{50} of Pentostam^R against the parent susceptible *L. donovani* promastigotes, the control cultures maintained in culture for 9 months in the absence of Pentostam, but under same experiment conditions as the culture undergoing drug pressure.

These results show that the parent susceptible *L. donovani* promastigotes acquired resistance to Pentostam^R through continuous culture with increasing concentrations of Pentostam. The resistance index (IC_{50} after drug pressure / IC_{50} before drug pressure) of the *in vitro* induced Pentostam-resistant *L. donovani* promastigotes was 35. Meaning that the Pentostam-resistant promastigotes were 35 times more resistant to Pentostam killing than the parent susceptible *L. donovani.*

Table 3. The *in vitro* susceptibility of the Pentostam-resistant *L. donovani* promastigotes developed by continuous drug pressure and maintained in 10.0 mg/ml of Pentostam^R for 9 months. As determined by SAMT, standard error of the mean (S.E.M).

Number of determinations	SAMT IC_{50} mg/ml (±S.E.M)	SAMT IC_{99} mg/ml (±S.E.M)
1	2.38	15.30
2	2.31	17.17
3	3.13	15.41
4	4.25	12.33
5	2.41	12.21
	$\bar{x}$=2.90 (±0.36)	$\bar{x}$=14.47 (±0.018)

4.4.2 The stability of the *in vitro* induced Pentostam resistant in *L. donovani* promastigotes

The stability of acquired resistance to Pentostam *in vitro* was determined by culturing the Pentostam-resistant *L. donovani* promastigotes in the absence of Pentostam for 4 months. After 4 months of continuous culture without drug pressure, the mean IC_{50} for Pentostam against Pentostam-resistant *L. donovani* (NLB 065R_1) promastigotes was 2.34 mg/ml (Table 4). This was not significantly different ($t_{0.05}$ 6=2.4469; $p>0.05$) from the mean of IC_{50} of Pentostam against the Pentostam-resistant *L. donovani* (NLB 065R) promastigotes maintained under drug pressure for the same period of time (Table 4).

Table 4. The susceptibility of Pentostam-resistant *L. donovani* (NLB 065R1) promastigotes cultured in the absence of Pentostam for 4 months and susceptibility of Pentostam-resistant *L. donovani* (NLB 065R) promastigotes cultured in 10.0mg/ml of Pentostam^R for the same period of time and under the same conditions. As determined by semiautomated microdilution technique (SAMT). standard error of mean (S.E.M).

	NLB 065R$_1$		NLB 065R	
CONSECUTIVE MONTHS	SAMT IC50mg/ml (±S.E.M)	SAMT IC99 mg/ml (±S.E.M)	SAMT IC50 mg/ ml (±S.E.M)	SAMT IC99 mg/ml (±S.E.M)
1	2.75	11.15	2.38	15.30
2	2.13	12.60	2.31	17.12
3	2.14	14.60	3.13	15.41
4	2.35	13.80	2.41	12.21
	$\bar{x}$2.34 (±0.145)	$\bar{x}$13.0 (±0.75)	$\bar{x}$2.56 (±0.19)	$\bar{x}$15.01 (±1.02)

These results show that the concentration of Pentostam^R which inhibited the growth of 50% of Pentostam-resistant *L. donovani* promastigotes cultured in the absence of Pentostam for 4 months were essentially similar to the concentration of Pentostam that inhibited 50% of the Pentostam-resistant *L. donovani* cultured in 10.0 mg/ml of Pentostam for the same period of time.

4.5. DISCUSSION

The uptake and incorporation of radiolabelled nucleic acid precursor methyl [^{3}H] thymidine has been shown to be an accurate indication of parasite multiplication *in vitro* and its inhibition as response of parasite to drug or culture conditions (Desjardins *et al.*, 1980; Chulay *et al.*, 1983). In this study modification of SAMT of Desjardins *et al.* (1979; 1980) by Grogl *et al.* (1989) was used to determine the sensitivity of *L. donovani* promastigotes to Pentostam^{R} *in vitro* and to compare the sensitivities of the susceptible *L. donovani* promastigotes and the *in vitro* induced Pentostam-resistant *L. donovani* promastigotes to Pentostam^{R}.

Promastigotes of the Pentostam-resistant *L. donovani* were distinguished from the promastigotes of the parent susceptible *L. donovani* by their resistance to the killing effects of Pentostam^{R}. The results of these experiments show that the stable resistance to sodium stibogluconate in the *in vitro* induced Pentostam-resistant *L. donovani* promastigotes was by continuous culturing of the parent susceptible promastigotes in the presence of increasing concentrations of Pentostam^{R}. Pentostam^{R} resistance in NLB 065R was 35 times greater than that determined initially on the parent susceptible *L. donovani* (NLB 065). Similar findings have been reported by Grogl *et al.* (1989), when they developed clones of *Leishmania* promastigotes *in vitro* which were resistant to Pentostam^{R} 33 to 212 times greater than that determined initially on the parent susceptible clones. Thus *Leishmania* readily acquires resistance to Pentostam^{R} under drug pressure *in vitro*. These results are also consistent with the study of Ullman *et al.* (1989), when they induced Pentostam^{R} resistance to *L. donovani* promastigotes *in vitro* by continuous drug pressure. They

generated 2 strains of cells, PENTO400 and PENTO3200 with IC_{50} values of 1 and 4 mg/ml respectively, which were both resistant to Pentostam^R killing.

The *in vitro* induced Pentostam resistance in NLB 065R promastigotes remained stable genetically even after the promastigotes were removed from drug pressure. Thus the parent susceptible *L. donovani* acquired resistance to Pentostam^R through continuous drug pressure *in vitro*. These results are consistent with the studies of Grogl *et al.* (1989) and Ullman *et al.* (1989) who have developed *in vitro*, Pentostam-resistant leishmania cell lines or clones by using drug pressure. Ullman *et al.*, 1989 developed 2 resistant strains of *L. donovani*, which after being maintained continuously for 6 months in the absence of drug pressure were still genetically stable.

The question has often been raised whether pentavalent antimony (Sb) unresponsiveness is a result of sub curative therapy or if some strains of *Leishmania* are inherently less sensitive to antimonials than others (Grogl *et al.*, 1989). It is possible that both factors may contribute to the development of parasite resistance. Observations made by Bryceson (1985) on Kenyan patients suggests that *Leishmania* acquires resistance to Sb as a result of drug pressure. The development of Sb resistance experimentally, both *in vivo* and *in vitro* appears to emulate the potential clinical outcome of suboptimal treatment conditions (Grogl *et al.*, 1989).

The mechanism through which the Pentostam-resistant *L. donovani* promastigotes resisted the killing effects of Pentostam^R is not known. The genetical changes that took place as the susceptible *L. donovani* promastigotes were undergoing drug pressure could have been as a result a permanent alteration of a protein within the *L. donovani* promastigotes, resulting into the ineffectiveness of the drug

against the parasites. Ullman *et al.* (1989) have reported that *Leishmania* parasites have been known to mutate as a result of drug pressure. They have suggested that a common mechanism by which *Leishmania* becomes a resistant to drug *in vitro* is by genetic lesions in their transport system. For example, mutant *L. donovani* cell lines have been characterized with biochemically defined lesions in nucleoside (Ionvannisci *et al.*, 1984) and folate (Kaur *et al.*, 1988) transport systems. Grogl *et al.*, 1989 have also suggested that mutations leading to drug resistance may be through one step or multiple steps. Presumably, parasites have the potential of mounting several different mechanisms of resistance (Ellenberger and Beverly, 1987). Methotrexate resistance in *Leishmania* is known to occur by at least 3 distinct and independent mechanisms. (Coderre *et al.*, 1983; Ellenberger and Beverly, 1987). Thus *Leishmania* apparently has the potential to respond to drug pressure in multiple ways, all of them resulting in drug resistance (Grogl *et al.*, 1989).

The study demonstrates that the susceptible *L. donovani* promastigotes acquired resistance to Sb under continuous drug pressure *in vitro*. The fact that Pentostam-resistant *L. donovani* promastigotes can be developed *in vitro* and that drug resistant strains have been isolated from patients (Mebrahtu *et al.*, 1989) suggests that parasites do become resistant under drug pressure and this may serve as a possible explanation for reported drug failures. Although the implication of Pentostam resistance in promastigotes is not clear, drug resistant promastigotes, like the Pentostam-resistant *L. donovani*, provide a useful tool in experimental chemotherapy and potential application in the Primary Screening of candidate anti-leishmanial drugs.

CHAPTER 5

5.0 ANALYSIS OF CRUDE PROTEINS OF LOGARITHMIC AND STATIONARY PHASE CULTURES OF BOTH THE PARENT SUSCEPTIBLE *L. DONOVANI* AND THE PENTOSTAM-RESISTANT *L. DONOVANI* PROMASTIGOTES BY SDS-PAGE.

5.1 INTRODUCTION

Protein electrophoresis in polyacrylamide gel is one of the most useful analytic and preparative techniques (Andrew, 1986). A gel is formed by polymerizing acrylamide ($CH_2CHCO\text{-}NH_2$) and a suitable bifunctional cross-linking reagent, normally NN - methylenebisacrylamide. The polymerization occurs rapidly at room temperature in the presence of ammonium persulphate (APS) as the initiator and $N_1N_1N_1N_1$ - tetramethylethylene diamine (TEMED) as the catalyst. The porosity of the gel can be adjusted by varying the acrylamide concentration and degree of cross-linking. Normally gels contain between 3% and 30% weight/volume ($^W/_V$) acrylamide. A gel containing less than this disintegrate easily whereas those containing more are excessively brittle. Polyacrylamide gels have relatively low porosity and most large molecules experience sieving effects. Hence proteins are separated on the basis of both size and charge.

Polyacrylamide gel electrophoretic techniques have the advantages of high resolution and sensitivity and they are simple to carry out (Andrew, 1986).

5.1.1 The use of Sodium dodecyl sulphate for molecular weight measurements.

When proteins are heated in the presence of the anionic detergent, sodium dodecyl sulphate and reducing agents, they unfold and are almost totally denatured. It has been found that under such conditions, almost all proteins bind approximately 1.4 gram SDS per gram of protein (Pitt-Rivers and Impiombata, 1968). The denatured proteins combine with a constant ratio ($^{W}/_{W}$) of SDS. The overwhelming negative charge provided by the SDS coating makes any charge contributed by the protein negligible and so separation of such protein - SDS complexes by polyacrylamide gel electrophoresis is almost entirely due to sieving and therefore dependent on the molecular weight of the protein (Andrew, 1986). Thus a reasonably accurate value for the molecular weight of most proteins can be obtained by comparison of their electrophoretic mobility with those of standard proteins of known molecular weight. (Goding and Handman, 1984; Andrew, 1986).

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Crude protein for SDS-PAGE

Crude antigens were prepared from logarithmic phase and stationary phase cultures of NLB 065 and NLB 065R promastigotes. The logarithmic phase cultures were harvested in the 3rd day of incubation and the stationary phase cultures were harvested on the 5th day of incubation. The promastigotes suspension was centrifuged at 10,000 g for 30 minutes at 4°C, the supernatant discarded and the pellet washed 4 times with Phosphate Buffered Saline (PBS) (pH 7.2), in order to remove exogenous materials present in culture medium after harvesting. Whole cell lysate was prepared according the methods of Lepay *et al.* (1983). The pellet was solubilized with lysis buffer containing 0.5% Nonidet P-40 [BDH England], 100µl aprotinin 1mM, phenylmethyl sulfonyl fluoride [Sigma, St. Louis Mo. USA]). The lysate was kept at 4°C for 30 minutes thereafter aliquoted into eppendorf tubes and stored at -70°C. A sufficient amount was reserved for protein estimation.

5.2.2 Lowry *et al* Method of Protein estimation.

The estimation of the protein content of each antigen was carried out as described by Lowry *et al.* (1951). Protein standards were prepared containing between 20 and 160 µg/ml of Bovine albumin Fraction V (BDH, England) and made upto 1 ml with 0.5 N sodium hydroxide (May and Baker).

For the antigens to be tested, 50µl and 100µl of the antigen sample was diluted with 0.5N sodium hydroxide (NaOH) to a total of 1 ml. For the control, 1ml of saline was used.

To each tube, including the control 5 ml of a solution containing 2% sodium bicarbonate (BDH, England), 1% copper sulphate (E.T Monks, Kenya) and 2.7% potassium sodium tartrate (BDH, England) at a ratio of 100 : 1 : 1 was added. The tubes were vortex-mixed and allowed to stand at room temperature for 15 minutes after which 0.5 ml of 1N Phenol reagent (Sigma, USA) was added to each tube, mixed and allowed to stand at room temperature for 45 minutes. Absorbency values were then measured at 675 nm using a Beckman DU-50 spectrophotometer. Absorbency values of the standards were plotted against corresponding protein concentration and the protein contents of the crude antigen read from the graph. The actual protein content of the crude antigen was calculated as follows:-

Dilution factor	x	Protein concentration at that dilution	=	Actual Protein content of the antigen

5.2.3 Preparation of SDS - Polyacrylamide gels:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (1970). Glass plates (plexiglass) spacers were cleaned with absolute alcohol. Two clean dried glass plates were placed together with 2 plexiglass spacers between them and then clamped and placed vertically on a vacuum-greased rubber surface. The resolving gel solution was made as follows:-

	7.5%	10.0%	12.5%	15.0%
solution C (ml)	10.0	13.3	16.63	19.93
Solution A (ml)	10.0	10.0	10.0	10.0
Distilled deionised water (ml)	20.0	15.9	12.59	9.25

The resolving gel was degassed using negative pressure until bubbles stopped forming. To the degassed resolving gel, 300µl of 10% SDS and 300µl of 10% Ammonium persulphate (APS) was added and mixed gently. To the space between the glass plates, 28 ml of the degassed resolving gel was poured and 1 ml of 0.1% SDS was gently added with a pasteur pipette to ensure a good interface between the resolving and stacking gel. The gel was allowed to set for 20-30 minutes and the excess 0.1% SDS was poured off. The stacking gel solution was made as follows:-

Solution C (ml)	2.50
Solution B (ml)	1.875
Distilled deionised water (ml)	10.0

The stacking gel was degassed before adding 150µl of 10% SDS and 300µl of APS. The stacking gel was poured onto set resolving gel. The comb was inserted care being taken to avoid air bubbles and left to stand for 20-30 minutes. After the stacking gel had polymerized, the comb was removed carefully and the wells flushed with electrophoresis buffer using a syringe.

While the stacking gel was polymerizing, the antigen samples and molecular weight markers were prepared for loading by adding an equal amount of sample buffer (0.0625 M Tris-HCl (pH 6.8) 2% SDS, 10% glycerol, 5%, 2-mercaptoethanol and 0.001% bromophenol blue)

to each sample and boiling for 5 minutes in a water bath. They were then loaded onto the gel using a pipette man.

The upper electrophoretic chamber was fixed onto the plate with clamps and then transferred into the electrophoresis tank. Approximately 1500 ml of electrophoresis buffer (0.025 M Tris, 0.192 M glycine and 0.1% SDS [pH 8.3]) was poured into the electrophoresis tank and 600 ml of electrophoresis buffer poured on to the upper electrophoresis chamber. Any gas bubbles on the bottom of the gel was removed using a modified syringe.

The gel apparatus was then connected to the power pack (Bio Rad Model 250/2.5) and run at 100v for 5 hours or at 25v overnight. At the end of the run, the Bromophenol blue (BDH, England) reached within 1 cm of the bottom of the gel.

The gel was removed from between the glass plates and gently transferred to a sandwich box containing a fixative solution (40% methanol; 7% glacial active acid; 53% distilled deionised water [DDW]) for at least 10 hours. The gel was then stained with Coomassie blue R250 (0.25% Coomassie blue, 40% methanol, 7% glacial acetic acid, 53% DDW) for 3 hours. The staining solution was then removed, the gel rinsed quickly in distilled water and placed in destaining solution (53% DDW, 40% methanol, 7% glacial acetic acid) overnight on a rocker platform (Bellco, USA). The gel was then transferred to 7% glacial acetic acid for 3 hours.

The gel to be dried was placed on a wet piece of filter paper (Whatmann 3MM) then overlayed with cling film (Fay, London, England), ensuring that no air bubbles were present and placed on a gel drier (Bio Rad model 443) for 2 hours.

5.2.4 Determination of molecular weights

The molecular weights of the samples analysed were determine by the use of electrophoresing marker proteins of known molecular weights. The marker proteins used and their respective molecular weights are shown in table 1 below.

Table 5. Standard proteins used as marker proteins with their respective molecular weights.

Marker Proteins	Molecular weights in daltons
Myosin	205,000
ß-Galactosidase	116,000
Phosphorylase B	97,400
Albumin Bovine	66,000
Albumin Egg	45,000
Glyceraldehyde-3-phosphate dehydrogenase	36,000
Carbonic anhydrase	29,000
Trypsin inhibitor Bovine pancreas PMSF treated	24,000
Trypsin inhibitor, Soybean	20,100
- lactalbumin	14,200

5.2.5 Standardization tests

Crude antigens of the logarithmic and stationary phase promastigotes of both NLB 065 and NLB 065R were titrated on both 12.5% gels and run for 5 hours at 100V in order to standardise the optional concentration of the gel to used in electrophoresis 12.5% gel appeared to be the better concentration to use since there was a clearer spread of the bands than in 15% gels. Titrations in 12.5% gels demonstrated that the lowest dilution of antigen showing the bands most clearly was 50µl per well.

5.3 RESULTS:

5.3.1 Crude antigen analysis of logarithmic and stationary phase culture promastigotes of two strains of *L. donovani* (NLB 065 and NLB 065R)

Molecular weights were determined by electrophoresing marker proteins of known molecular weights in parallel tracks and measuring the mobilities of the proteins. All molecular weights were determined from the mobilities of standard markers.

Crude antigen of the logarithmic and stationary phase promastigotes of 2 strains of *L. donovani*, NLB 065 and NLB 065R were analysed by SDS-PAGE under reducing conditions. On staining with coomassie blue, a complex pattern of polypeptide chains was revealed. The pattern of bands observed for logarithmic and stationary phase promastigotes of NLB 065 and NLB 065 were consistent in experiments spread over a period of 3 months. All the values of the molecular weights of polypeptides reported in plates 1, 2, 3 and 4, are means ± standard deviation of 4 determinations.

5.3.2 Comparing the polypeptide composition of logarithmic and stationary phase culture promastigotes of the parent susceptible *L. donovani* (NLB 065)

The polypeptide profiles of logarithmic and stationary phase promastigotes of *L. donovani* (NLB 065), when analysed by SDS-PAGE

and coomassie blue are shown in plate 1. The polypeptide bands that were observed in logarithmic phase promastigotes of NLB 065, had molecular weights ranging from 127 to 14 kilodaltons (KD). Up to 16 polypeptides bands could be clearly distinguished in the logarithmic phase promastigotes of NLB 065. The polypeptide chains that were identified in stationary phase promastigotes of NLB 065, had molecular weights ranging from 205 to 14 KD and a total of 22 polypeptides bands were identified in the stationary phase promastigotes.

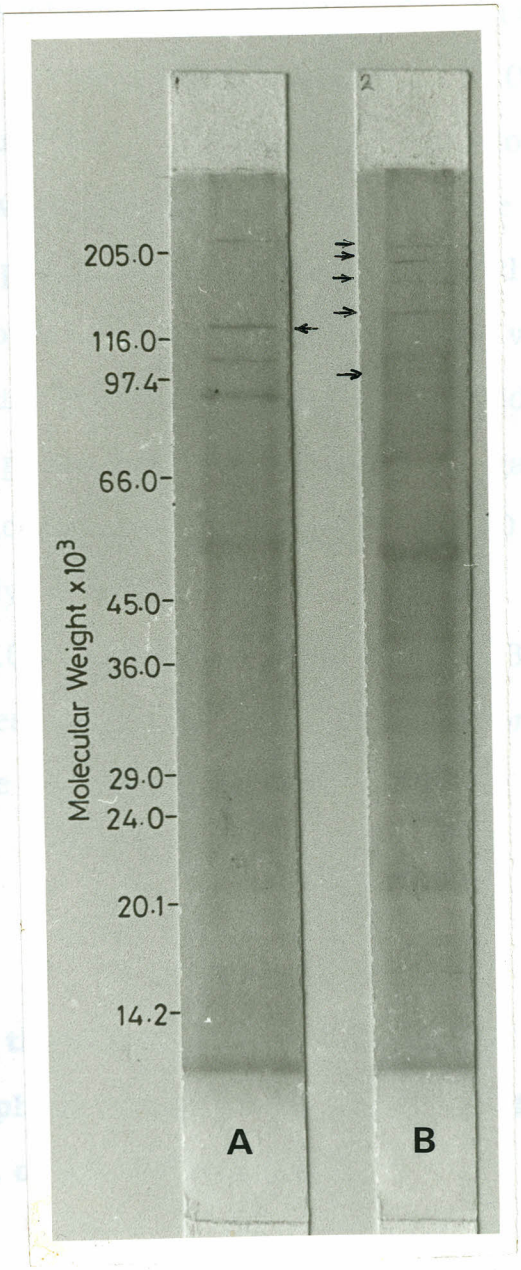

Plate 1. **Polypeptide composition of crude protein of the parent susceptible *L. donovani*, strain NLB 065 from logarithmic phase culture promastigotes (A) and stationary phase culture promastigotes (B).**

There were distinct differences between logarithmic and stationary phase promatigotes of NLB 065. The unique polypeptide to logarithmic phase promastigotes had a molecular weight of 127,000 ± 1000 Daltons (D) whereas polypeptides of molecular weights 205,000 ± 2000, 194,000 ± 1000, 172,000 ± 1500, 139,000 ± 900 and 97,000 ± 1100 D (all values are mean ± standard deviation of 4 determinations) were unique to stationary phase promastigotes of NLB 065. The major polypeptides common to logarithmic and stationary phase promastigotes of NLB 065 had molecular weights of 90,000 ± 900 and 54,000 ± 1000 D. The major polypeptide bands identified in stationary phase promastigotes but absent in logarithmic phase promastigotes had molecular weights of 172,000 ± 1500 and 97,000 ± 1100 D. The polypeptides of molecular weights 71,000 ± 2000 40,000 ± 300 35,000 ± 400, 34,000 ± 200 and 32,000 ± 300 D were major polypeptides in stationary phase but minor polypeptides in logarithmic phase promastigotes of NLB 065.

5.3.3 Comparing the polypeptide composition of the logarithmic and stationary phase culture promastigotes of the Pentostam-resistant *L. donovani* (NLB 065R)

The polypeptide profiles of logarithmic and stationary phase promastigotes of the Pentostam-resistant *L. donovani* are shown in plate 2. The range of the molecular weights of the polypeptide bands observed in logarithmic and stationary phase promastigotes were 127 to 14 KD and 205 to 14 KD respectively. These ranges are similar to

those observed in the polypeptide profile of logarithmic and stationary phase promastigotes of the parent susceptible *L. donovani*.

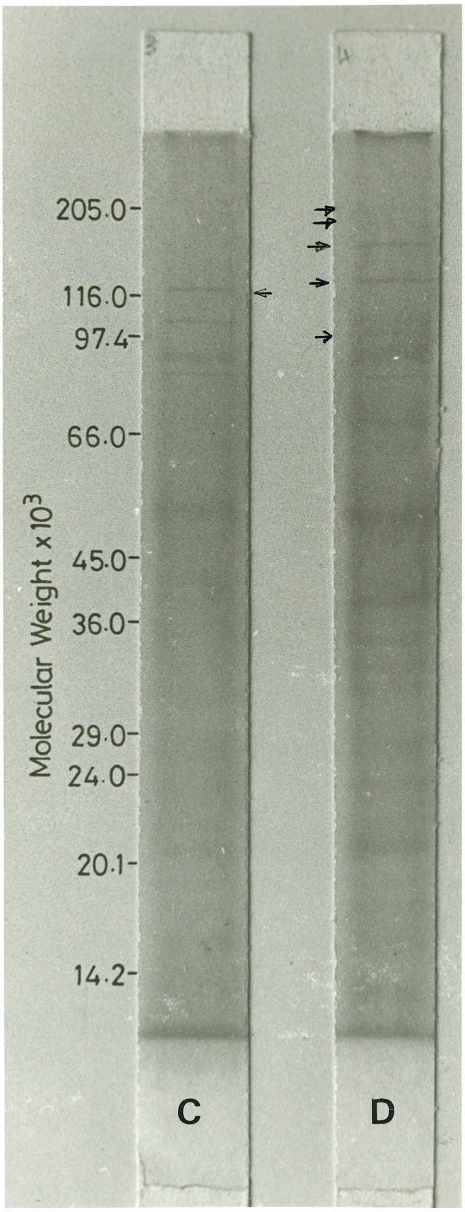

Plate 2. **Polypeptide composition of the crude proteins of the Pentostam - resistant *L. donovani*, strain NLB 065R from logarithmic phase culture promastigotes (C) and stationary phase culture promastigotes (D).**

Up to 17 polypeptide bands were observed in the logarithmic phase promastigotes of NLB 065R and 23 polypeptide bands were observed in stationary phase promastigotes of NLB 065R. The polypeptide bands of molecular weight 127,000 ± 1000 D was identified only on the polypeptide profiles of logarithmic phase promastigotes but absent in stationary phase promastigotes of NLB 065R. This same polypeptide band was found to be unique only to logarithmic phase promastigotes of NLB 065. The unique polypeptide bands to stationary phase promastigotes of the Pentostam-resistant *L. donovani*, had molecular weights of 205,000 ± 2000, 194,000 ± 1000, 172,000 ± 1500, 139,000 ± 900 and 97,000 ± 1100 D. These same polypeptides were also identified to be unique to stationary phase promastigotes of the parent susceptible *L. donovani*.

The polypeptides band of molecular weights 90,000 ± 900 and 54,000 ± 1000 D were major polypeptides of the logarithmic phase promastigotes of NLB 065R. The polypeptides bands of molecular weights 106,000 ± 1000, 71,000 ± 2000, 40,000 ± 300, 35,000 ± 400, 34,000 ± 200 and 32,000 ± 300 D were major polypeptides in stationary phase promastigotes but minor polypeptides in logarithmic phase promastigotes of NLB 065R.

In the polypeptide profiles of logarithmic and stationary phase culture of the Pentostam-resistant *L. donovani*, a minor polypeptides band of molecular weight 85,000± 500 D was identified. This polypeptide band was observed in the logarithmic and stationary phase promastigotes of the Pentostam-resistant *L. donovani* but absent in the logarithmic and stationary phase promastigotes of the parent susceptible *L. donovani*.

5.3.4 Comparing the polypeptide composition of the logarithmic phase culture promastigotes of the parent susceptible *L. donovani* and the logarithmic phase culture promastigotes of the Pentostam-resistant *L. donovani*.

The polypeptide profiles of logarithmic phase promastigotes of both the parent susceptible *L. donovani* and the Pentostam-resistant *L. donovani* were similar with the exception of the polypeptide band of molecular weight 85 KD which was present in the Pentostam-resistant *L. donovani* but absent in the parent susceptible *L. donovani* (plate 3).

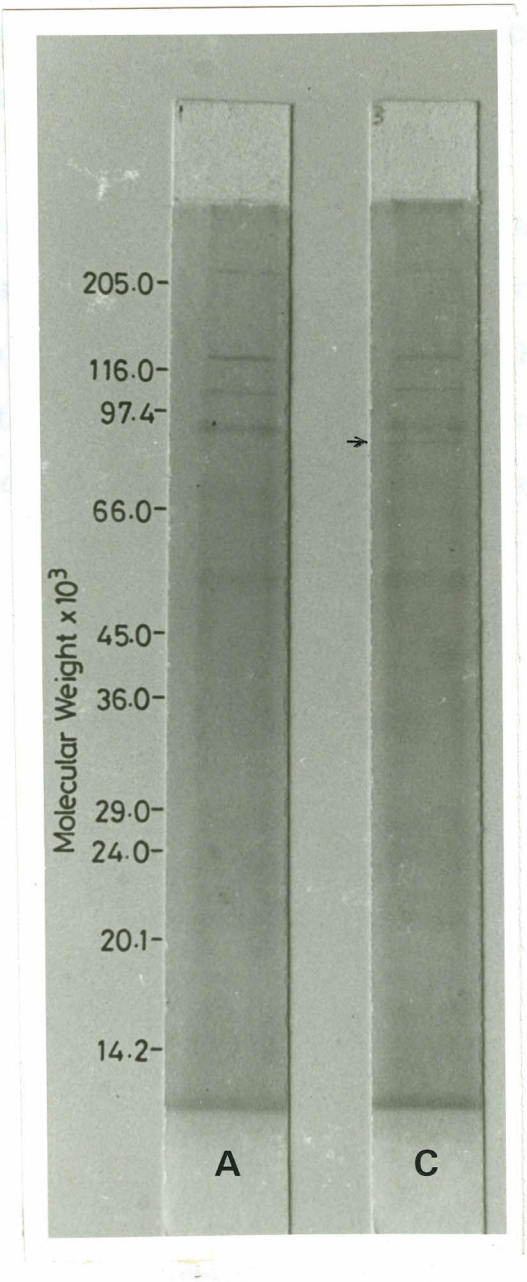

Plate 3. **Polypeptide composition of crude protein of the parent susceptible *L. donovani*, strain NLB 065 from logarithmic phase culture promatigotes (A) and polypeptide composition of the Pentostam-resistant *L. donovani*, strain of NLB 065R from logarithmic phase culture promastigotes (C).**

5.3.5 Comparing the polypeptide composition of the stationary phase culture promastigotes of both the parent susceptible *L. donovani* and the Pentostam-resistant *L. donovani*.

The polypeptide profiles of the stationary phase promastigotes of both the parent susceptible *L. donovani* and the Pentostam-resistant *L. donovani* were also similar, with the exception of the polypeptide band of molecular weight 85,000 ± 500 D observed in the Pentostam-resistant - *L. donovani* and absent in the parent susceptible *L. donovani* (Plate 4).

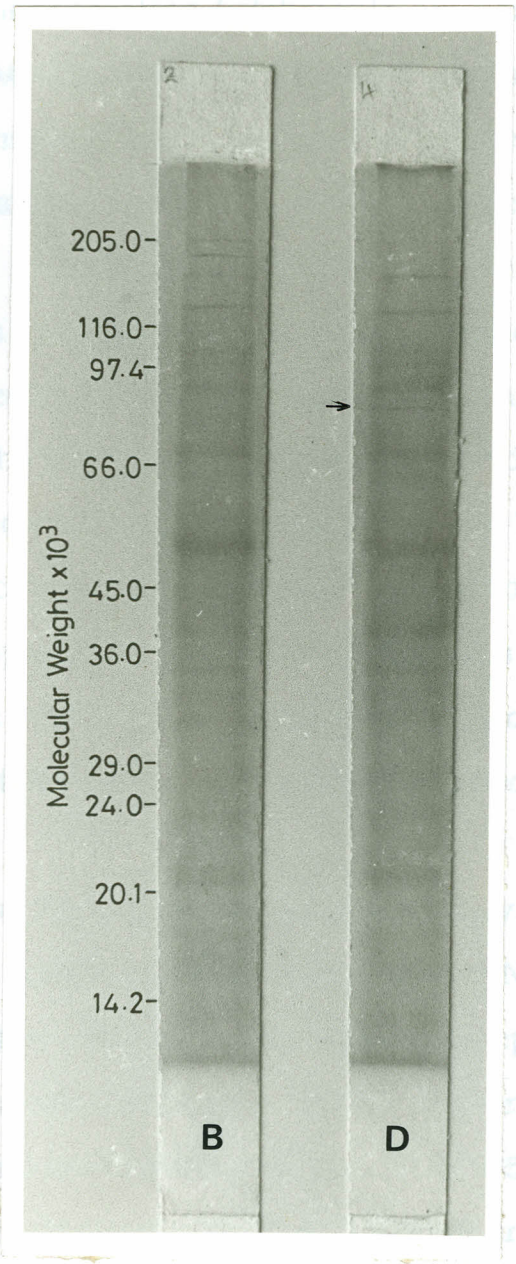

Plate 4. **Polypeptide composition of crude protein of the parent susceptible *L. donovani* strain NLB 065, from stationary phase culture promastigotes (B) and the Pentostam-resistant *L. donovani* strain NLB 065R, from stationary phase culture promastigotes (D).**

5.4. DISCUSSION

The method of separating *Leishmania* promastigote lysates proteins by SDS-PAGE has served to identify similarities and differences in organisms which have been suggested to belong to different leishmanial species or subspecies by other criteria (Jamnadas, 1983). In the presence of SDS, the native conformation of membrane proteins is completely disrupted. The disulphide bridges are broken by 2-Mercaptoethanol, a strong reducing agent (Jamnadas, 1983). Therefore the patterns of proteins observed in plates 1, 2, 3, and 4 are those of denatured polypeptide chains. The polypeptides profile patterns of components revealed by electrophoresis are usually very complex. Despite the complexity, differences between the polypeptide profiles of the Pentostam-resistant *L. donovani* promastigotes and the parent susceptible *L. donovani* promastigotes could be detected.

Comparison of the logarithmic and stationary phase promastigotes of *L. donovani* strain, NLB 065 and NLB 065R promastigotes revealed differences in their polypeptide composition. The polypeptide of molecular weight 127KD was unique to only the logarithmic phase promastigotes of strains NLB 065 and NLB 065R. The polypeptides unique to the stationary phase promastigotes of strains NLB 065 and NLB 065R were of molecular weights 205, 197, 172, 139 and 97KD. A similar study done by Grogl *et al.* (1987) on two strains of *Leishmania braziliensis panamensis*, detected polypeptides unique to logarithmic phase promastigotes to have a molecular weights of 102, 66 and 31KD, while a polypeptide of 79KD was detected only on the stationary phase promastigotes. The differences between the results of the present study and those of

Grogl *et al.* (1989) could be attributed to the differences of the species of *Leishmania* studied. These results also show that different species of *Leishmania* may exhibit different polypeptides in their logarithmic and stationary phase promastigotes.

The polypeptide bands were also more distinct in stationary phase as compared to logarithmic phase. These results indicate that the stationary phase of *L. donovani* promastigote of NLB 065 and NLB 065R possess more proteins than the logarithmic phase. Grogl *et al.* (1987) showed that promastigotes of *L. braziliensis panamensis* from infective stationary phase culture possess more concanavalin A (Con A) and lentil lectin binding molecules on their surfaces than promastigotes from logarithmic phase cultures which indicated a greater number of exposed mannose moieties. The association of increased mannose moieties with infective stationary phase promastigotes has also been observed in *L. tropica* (Ebrahimzadeh and Jones, 1983). Since no further tests to determine the differences in antigenicity and lectin binding characteristics of both NLB 065 and NLB 065R promastigotes was done, it was not possible to relate the differences in the polypeptide profiles of stationary and logarithmic phase cultures and the number of mannose moieties on the surface of both NLB 065 and NLB 065R. However it is believed that the biochemical changes that occur during the switch from logarithmic phase promastigotes to stationary phase promastigotes may be pre adaptation that increase the infectivity of *Leishmania* species for its vertebrate host (Grogl *et al.*, 1987).

The difference between the Pentostam-resistant *L. donovani* promastigotes and the parent susceptible *L. donovani* promastigotes was the presence of a polypeptide band of molecular weight 85 KD in the Pentostam-resistant *L. donovani* promastigotes but absent in the

parent susceptible *L. donovani* promastigotes. From these results, this polypeptide of molecular weight 85 KD is unique only for the Pentostam-resistant *L. donovani* promastigotes. These results only reveal that the polypeptide of molecular weight 85 KD is a protein which may have been formed in the Pentostam-resistant *L. donovani* as they were undergoing drug pressure. Drug pressure has been known to induce mutations in *Leishmania* parasites (Ullman *et al.*, 1989) and these genetical variations in species of living organisms are easily detected by electrophoresis technique (Hopkinson, 1982).

CHAPTER 6

6.0 GENERAL DISCUSSION AND CONCLUSIONS

Pentavalent antimonials in the form of sodium stibogluconate or Meglumine antimonate are the treatment of choice for all forms of leishmaniasis in human (Steck, 1981). The efficacy of these drugs require a lengthy treatment and is complicated by considerable toxicity (Bryceson *et al.*, 1985; Marsden *et al.*, 1985; Campbell and Rew, 1986). Moreover, the incidence of leishmaniasis resistance to antimonial drug therapy is becoming an increasing important problem in the treatment of leishmaniasis (Napier, 1942; Marsden, 1949; Bryceson *et al.*, 1985) with unknown specific reasons to explain why leishmaniasis is insensitive to Pentavalent antimonials.

Among the biological phenomena of the *Leishmania* parasites, resistance to Sb is probably the one which is causing the most important interference with the control of the disease. It helps the parasite to overcome the challenge posed by the Sb. Knowledge of biological basis of drug resistance and the mode of action and the pharmacokinetics of Sb has become a matter of practical concern (Peters, 1974). Further research in this area may make it possible to limit the spread and to eliminate or at least reduce the impact of drug resistant *Leishmania* in exposed population.

The experimental use of the *in vitro* developed Pentostam-resistant *L. donovani* promastigotes may help in understanding the mechanism through which *Leishmania* parasites develop resistance to anti-leishmania drugs, suggest chemotherapeutic strategies to

overcome drug resistance and may make it possible to include Pentostam-resistant strains in drug testing programmes.

This study demonstrates that *Leishmania* readily acquires resistance to Sb under drug pressure *in vitro* and this acquired resistance was genetically stable during the period of the study. The development of Sb resistance experimentally both *in vivo* and *in vitro* appears to emulate the potential clinical outcome of suboptimal treatment conditions (Grogl *et al.*, 1989). Sb unresponsiveness is a problem of clinical and economic importance affecting a considerable number of people in the developing countries, where the disease is endemic. The socio-economic conditions in these countries seem to favour the development of Sb unresponsiveness in the field in response to minimal dose therapy. Availability, price and quality of the drug coupled with the need for prolonged injection schedules of the pentavalent antimonials are important contributing factors to suboptimal treatment (Grogl *et al.*, 1989). It is therefore essential that the initial treatment be adequate in terms of dose and frequency to eliminate the parasite and diminish the possibility that Sb resistance could develop in the field in response to subcurative dose therapy (Grogl *et al.*, 1989).

The mechanism through which the *Leishmania* resist the killing effect of Pentostam^{R} is not known, but has been speculated to be through genetic lesions in the transport systems (Ullman *et al.*, 1989). It is possible that when *Leishmania* parasites are exposed to drug pressure, some metabolic functions of the parasites are altered as a results of drug pressure. The altered protein in the resistant parasites could be what is observed as a polypeptide or molecular weight 85KD in the logarithmic and stationary phase promastigotes of NLB 065R.

The question of whether the changes induced in the susceptible *L. donovani* promastigotes, *in vitro*, resulting in drug resistant parasites corresponds to the changes believed to precede the expression of drug resistance in nature or to the events leading to unresponsiveness in patients has not yet been established. Further studies with the parent susceptible and the Pentostam-resistant *L. donovani* may provide information on potential biochemical and genetical changes accompanying acquisition of resistance to Pentostam^{R}. Thus there is a great need not only to study drug resistant *Leishmania* species *in vitro*, but to continue to isolate parasites from patients who do not respond to Sb treatment.

Further work needs to be done, like purification of the polypeptide band of molecular weight 85KD, which was found to be unique only to the Pentostam-resistant *L. donovani* promastigotes. The purified 85KD polypeptide band could be used as a tool for diagnosis of Pentostam-resistant cases.

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APPENDIX I

RESULTS OF THE PROTEIN CONTENTS DETERMINATION BY LOWRY METHOD

L. donovani Strain	Mean concentration (µg/ml) of crude antigen (logarithmic phase)	Mean concentration (µg/ml) of crude antigen (stationary phase)
NLB 065	4974.0	4128.0
NLB 065R	2694.0	2545.8

Table 1 **Mean concentration of crude antigen of strains of *L. donovani* at their logarithmic and stationary phases of development.**

APPENDIX II

BUFFERS AND SOLUTIONS

Solution A

HCl - 240ml
Tris Base - 181.5gm
Temed - 1.15ml
adjust pH to 8.9

Solution B

HCl - 240ml
Tris Base - 29.9gm
Temed - 2.3ml
adjust pH to 6.8

Solution C

acrylamide - 150gm
Bis acrylamide - 2.0gm
distilled water - 500ml

Electrode Buffers stock solution

Tris - 60.5g
Glycine - 288.0g
SDS - 20.0g

Dissolved and diluted with water to a final volume of 2.0 litres final pH should be about 8.3.

2 x sample buffer

Prepare solution by combining:

Tris - 1.51g

Glycerol - 20.0ml

Dissolve with 35ml of water

Adjust to pH 6.75 with concentrated HCl,

then add:

SDS - 4.0g

2-mercaptoethanol 10.0ml

Bromophenol blue - 0.002g

Dilute with water to a final volume of 100ml.

LYSIS BUFFER

Nonidet P-40 - 0.5%

Aprotinin - 100µl

Phenylmethyl sulfonyl fluoride - 2mM

Fixative solution

methanol - 400ml

Glacial acetic acid - 70ml

distilled water - 500ml

Staining Reagent

Prepared by dissolving 1.25gm of Coomassie brilliant blue into 500ml of the fixative solution

Destaining solution

Same as fixative solution

Composition of Schneider's Medium

L. Glutamine	-	42g
L. Cystine	-	0.2g
L. Tyrosine	-	1.0g
L. Yeastolate	-	4.0g
Sodium hydrogen Carbonate	-	0.8g

All these are dissolved to 2 litres of distilled water.