Characterization of transplasma membrane electron transport system and Plasma membrane bound P-type ATPase in *Leishmania donovani* promastigote

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DECLARATION

The research work entitled **"Characterization of transplasma membrane electron transport system and Plasma membrane bound P-type ATPase in *Leishmania donovani* promastigote"** has been carried out by me in Himalayan Pharmacy Institute, Majhitar, East Sikkim, under the supervision of **Prof. (Dr.) Bishnu Pada Shah** and in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, under the co-supervision of **Prof. (Dr.) Tanmoy Bera.** I hereby declare that the work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

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DEDICATED TO
MY FAMILY

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Nihar Ranjan Bhuyan.

NIHAR RANJAN BHUYAN

CONTENTS

ADP	:	Adenosine diphosphate
AIDS	:	Acquired immuno deficiency syndrome
ALA	:	α – lipoic acid
AMP	:	Adenosine monophosphate
APAOS	:	4-aminophenyl arseneoxide-N-sulphonic acid
ATP	:	Adenosine triphosphate
ATPase	:	Adenosine triphosphatase
BPS	:	Bathophenanthroline disulphonate
BSA	:	Bovine serum albumin
cAMP	:	Cyclic adenosine monophosphate
cDNA	:	Complementary DNA
CL	:	Cutaneous Leishmaniasis
CoA	:	Coenzyme A
CoQ_{10}	:	Coenzyme Q_{10}
Da	:	Dalton
DAT	:	Direct agglutination test
DCCD	:	N, N-Dicyclohexyl carbodimide
DDT	:	Dichloro-diphenyl trichloroethane
DIDS	:	4,4′-diisothiocyanatostilbene-2, 2′-disulfonic acid
DMF	:	Dimethyl formamide
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DNAase	:	Deoxyribonuclease
DPA	:	Diphenylamine
DPC	:	Digitonin permeabilized cell
DTNS	:	5,5′-dithiobis (2-nitroaniline – N-sulfonic acid)
DTT	:	Dithiothreitol
ECG	:	Electro cardiograph
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal growth factor
ELISA	:	Enzyme-linked immunosorbent assay
FAD	:	Flavin adeninedinucleotide

FCCP	:	P-trifluromethoxychlorophenyl hydrozone
FMN	:	Flavin mononucleotide
G	:	Guanine residue
Gal/GalNAc	:	Galactose/N/acetyl galactosamine
GTP	:	Guanidine triphosphate
HCl	:	Hydrochloric acid
HCS	:	Hepatocyte cell suspension
HEPES	:	N- (2-hydroxyethyl piperazine) –N′-(2-ethane sulfonic acid)
HIV	:	Human immuno deficiency virus
HLA	:	Human leukocyte antigen
HQNO	:	2-heptyl-4-hydroxyquinoline-N-oxide
h	:	Hour
ICMR	:	Indian Council of Medical Research
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
$K_3Fe(CN)_6$	:	Potassium ferricyanide
kb	:	Kilobase
kDa	:	Kilodalton
LDC	:	*Leishmania donovani* cell
M	:	Molar
MA	:	Meglutamine antimoniate
Mb	:	Megabase
MCL	:	Mucocutaneous Leishmaniasis
MIC	:	Minimum inhibitory concentration
mM	:	Millimol
MOON	:	2-methyl-3- (1′-oxooctadecyl)-1,4-napthoquinone
mRNA	:	Messenger RNA
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide hydrogenase
NADP	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Nicotinamide adenine dinucleotide phosphate hydrogenase

NEM	:	N- Ethylmalimide
nM	:	Nanomolar
NQSA	:	1,2 – Naphthoquinone -4-sulfonic acid
NSAID	:	Non steroidal anti inflammatory drug
PBS	:	Phosphate buffer saline
PCMBS	:	p-chloromercuribenzenesulphonate
Pet. Ether	:	Petroleum ether
PGPA	:	p-glycoprotein A
Pi	:	Inorganic phosphate
PKDL	:	Post kala-azar dermal leishmaniasis
PPase	:	Pyrophosphatase
PPi	:	Inorganic pyrophosphate
PVAs	:	Pentavalent antimonides
rDNA	:	Ribosomal DNA
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	Ribosomal RNA
T&W	:	Treated and Washed
TCA	:	Tricarboxylic acid
TDR	:	Tropical Disease Research
TransPMET	:	Transplasma membrane electron transport system
Tris	:	Tris (hydroxymethyl) aminomethane
TTFA	:	2-Thenoyl trifluro acetone
UDP	:	Uridine diphosphate
UV	:	Ultra violet
VL	:	Visceral Leishmaniasis
WHO	:	World Health Oraganisation
µg	:	Microgram
µl	:	Microliter
µm	:	Micrometer
µM	:	Micromolar

Chapter- 1

Introduction

1. INTRODUCTION

1.1. Protozoan Parasitic Diseases: General Concept

Protozoa are considered to be the simplest organisms in the animal kingdom. They are all single-celled and considered being a subkingdom of the kingdom Protista, although in the classical system they were placed in the kingdom Animalia. Some protozoa are more closely related to animals, others to plants, and still others are relatively unique. Although it is not appropriate to group them together into a single taxonomic category, the research tools used to study any unicellular organism are usually the same, and the field of protozoology has been created to carry out this research. Protozoans are found in all moist habitats, but we know little about their specific geographic distribution. Because of their small size, production of resistant cysts, and ease of distribution from one place to another, many species appear to be cosmopolitan and may be collected in similar microhabitats worldwide [1]. Other species may have relatively narrow limits to their distribution. More than 50,000 species have been described, most of which are free-living organisms; protozoa are found in almost every possible habitat. The fossil record in the form of shells in sedimentary rocks shows that protozoa were present in the Pre-Cambrian era. Antonvan Leeuwenhoek was the first person to see protozoa, using microscopes he constructed with simple lenses. Between 1674 and 1716, he described, in addition to free-living protozoa, several parasitic species from animals, and *Giardia lamblia* from his own stools. Virtually all humans have protozoa living in or on their body at some time, and many persons are infected with one or more species throughout their life. Some species are considered commensally, i.e., normally not harmful, whereas others are pathogens and usually produce disease. Protozoan diseases range from very mild to life- threatening. Individuals whose defenses are able to control but not eliminate a parasitic infection become carriers and constitute a source of infection for others. In geographic areas of high prevalence, well-tolerated infections are often not treated to eradicate the parasite because eradication would lower the individual's immunity to the parasite and result in a high likelihood of re-infection [2- 4].

Many protozoan infections that are mild in normal individuals can be life- threatening in immuno-suppressed patients, particularly patients with acquired immune deficiency syndrome (AIDS). Evidence suggests that many healthy persons harbor low numbers of

Pneumocystis carinii in their lungs. However, this parasite produces a frequently fatal pneumonia in immuno-suppressed patients such as those with AIDS. *Toxoplasma gondii,* a very common protozoan parasite, usually causes a rather mild initial illness followed by a long-lasting latent infection. AIDS patients, however, can develop fatal toxoplasmic encephalitis. *Cryptosporidium* was described in the 19th century, but widespread human infection has only recently been recognized. *Cryptosporidium* is another protozoan that can produce serious complications in patients with AIDS. Microsporidiosis in humans was reported in only a few instances prior to the appearance of AIDS. It has now become a more common infection in AIDS patients. As more thorough studies of patients with AIDS are made, it is likely that other rare or unusual protozoan infections will be diagnosed [5].

1.2. Structure [6,7]

Most parasitic protozoa in humans are less than 50μm in size. The smallest (mainly intracellular forms) are 1 to 10 μm long, but *Balantidium coli* may measure 150 μm. Protozoa are unicellular eukaryotes. As in all eukaryotes, the nucleus is enclosed in a membrane. In protozoa other than ciliates, the nucleus is vesicular, with scattered chromatin giving a diffuse appearance to the nucleus; all nuclei in the individual organism appear alike. One type of vesicular nucleus contains a more or less central body, called an endosome or karyosome. The endosome lacks DNA in the parasitic amebas and trypanosomes. In the phylum Apicomplexa, on the other hand, the vesicular nucleus has one or more nucleoli that contain DNA. The ciliates have both a micronucleus and macronucleus, which appear quite homogeneous in composition.

The organelles of protozoa have functions similar to the organs of higher animals. The plasma membrane enclosing the cytoplasm also covers the projecting locomotors structures such as pseudopodia, cilia, and flagella. The outer surface layer of some protozoa, termed a pellicle, is sufficiently rigid to maintain a distinctive shape, as in the trypanosomes and *Giardia*. However, these organisms can readily twist and bend when moving through their environment. In most protozoa the cytoplasm is differentiated into ectoplasm (the outer, transparent layer) and endoplasm (the inner layer containing organelles); the structure of the cytoplasm is most easily seen in species with projecting pseudopodia, such as the amebas. Some protozoa have a cytosome or cell "mouth" for

ingesting fluids or solid particles. Contractile vacuoles for osmoregulation occur in some, such as *Naegleria* and *Balantidium*. Many protozoa have sub-pellicular microtubules; in the Apicomplexa, which have no external organelles for locomotion, these provide a means for slow movement. The trichomonads and trypanosomes have a distinctive undulating membrane between the body wall and a flagellum. Many other structures occur in parasitic protozoa, including the Golgi apparatus, mitochondria, lysosomes, food vacuoles, conoids in the Apicomplexa, and other specialized structures. Electron microscopy is essential to visualize the details of protozoal structure. From the point of view of functional and physiologic complexity, a protozoan is more like an animal than like a single cell.

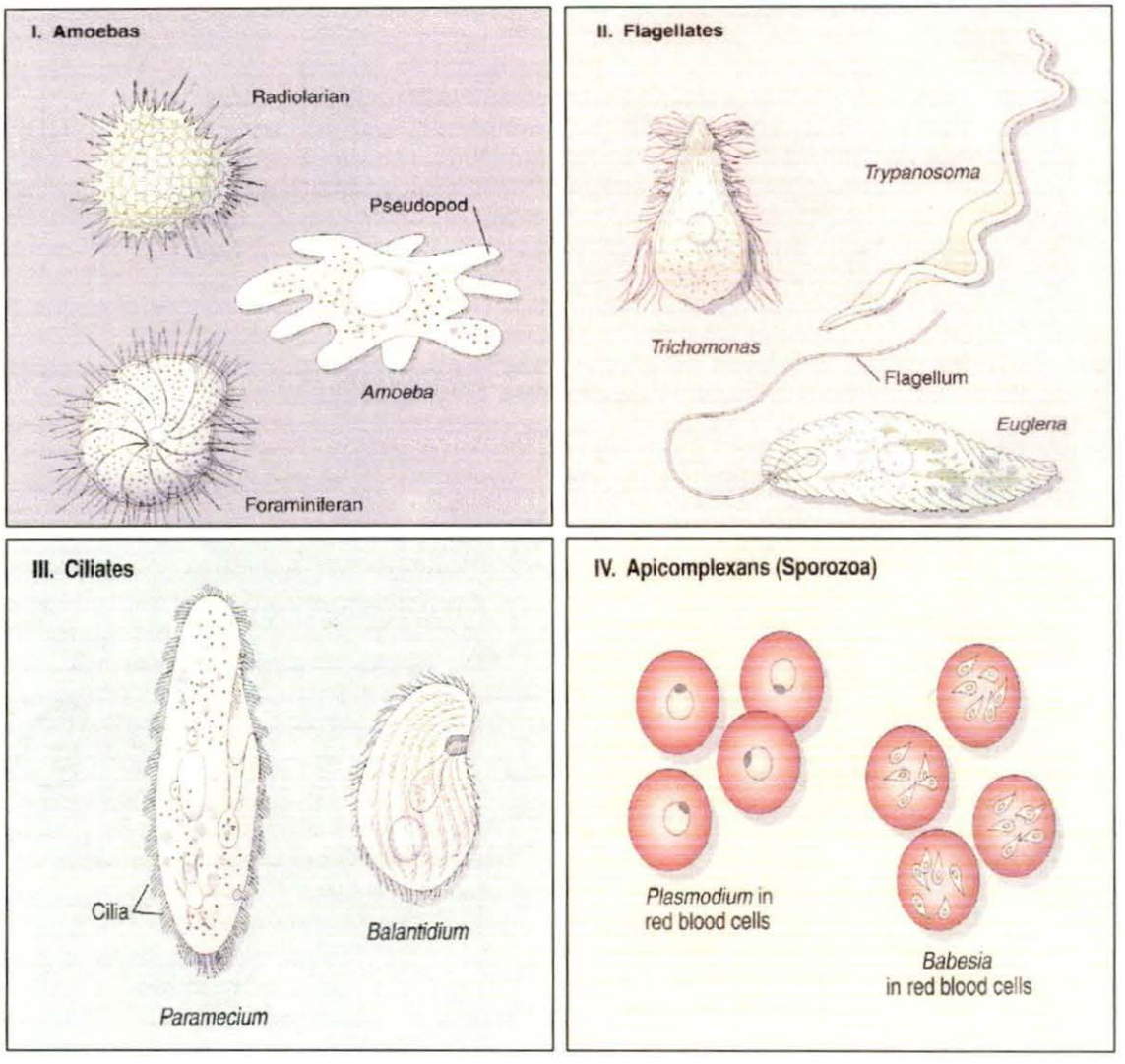


Fig. 1. Fine structure of different protozoan parasite

1.3. Characteristics of Protozoa

- Largest organisms included in microbial world.
- Except a few, they lack chlorophyll or other photosynthetic pigments.
- Though unicellular, but able to perform all functions characteristic of multi-cellular organisms.
- Mostly live in water, damp soil or mud, in drainage ditches or puddles, in ponds or ocean.
- A great diversity in form.
- Cells enclosed by a membrane, which in some cases surrounded by a pellicle containing chitin like material for rigidity. Cell wall, however, is absent. True nucleus, flagella, cilia are present. Freshwater forms take in water by osmosis and eliminate it via organelles called contractile vacuoles.
- Nutrition by ingestion i.e. engulfs food particles by phagocytosis or through special organs of ingestion. A membrane encloses the particle, forming a food vacuole. The latter commonly joins with a submicroscopic organelle, lysosome that contains digestive enzymes.
- Generally heterotrophic, saprobic, some pathogenic. The feeding form is commonly known as the trophozite.
- Aerobic in Whittaker's system, they along with certain algae are placed in the kingdom Protista.

1.4. Classification

In 1985 the Society of Protozoologists published a taxonomic scheme that distributed the Protozoa into six phyla. Two of these phyla the Sarcomastigophora and the Apicomplexa contain the most important species causing human disease. This scheme is based on morphology as revealed by light, electron, and scanning microscopy. *Dientamoeba fragilis*, for example, had been thought to be an ameba and placed in the family Entamoebidae. However, internal structures seen by electron microscopy showed that it is properly placed in the order Trichomonadida of flagellate protozoa. In some instances,

organisms that appear identical under the microscope have been assigned different species names on the basis of such criteria as geographic distribution and clinical manifestations; a good example is the genus *Leishmania*, for which subspecies names are often used. Biochemical methods have been employed on strains and species to determine isoenzyme patterns or to identify relevant nucleotide sequences in RNA, DNA, or both. Extensive studies have been made on the kinetoplast, a unique mitochondrion found in the hemoflagellates and other members of the order Kinetoplastida. The DNA associated with this organelle is of great interest. Cloning is widely used in taxonomic studies, for example to study differences in virulence or disease manifestations in isolates of a single species obtained from different hosts or geographic regions. Antibodies (particularly monoclonal antibodies) to known species or to specific antigens from a species are being employed to identify unknown isolates. Eventually, molecular taxonomy may prove to be a more reliable basis than morphology for protozoan taxonomy, but the microscope is still the most practical tool for identifying a protozoan parasite.

Table: 1.1. Classification of parasitic protozoa and associated diseases

Phylum	Subphylum	Representative genera	Major diseases produced in human beings
Sarcomastigophora (With flagella, Pseudopodia, or both)	Mastigophora	Leishmania	Visceral, cutaneous and mucocutaneous infection
		Trypanosoma	Sleeping sickness Chagas' disease
		Giardia	Diarrhea
		Trichomonas	vaginitis
	Sarcodina (Pseudopodia)	Entamoeba	Dysentery, liver abcess
		Dientamoeba	Colitis
		Naegleria and Acanthamoeba	CNS and corneal ulcer
		Babesia	Babesiosis
Apicomplexa (Apical complex)		Plasmodium	Malaria
		Isospora	Diarrhoea
		Sarcocystis	Diarrhoea
		Cryptosporidium	Diarrhoea
		Toxoplasma	Toxoplasmosis
Microspora		Enterocytozoon	Diarrhoea
Ciliophora (with cillia)		Balantidium	Dysentery

1.5. Life cycle

During its life cycle, a protozoan generally passes through several stages that differ in structure and activity. Trophozoite (Greek for "animal that feeds") is a general term for the active, feeding, multiplying stage of most protozoa. In parasitic species this is the stage usually associated with pathogenesis. In the hemoflagellates the terms amastigote, promastigote, epimastigote, and trypomastigote designate trophozoite stages that differ in the absence or presence of a flagellum and in the position of the kinetoplast associated with the flagellum. A variety of terms are employed for stages in the Apicomplexa, such as tachyzoite and bradyzoite for *Toxoplasma gondii*. Other stages in the complex asexual and sexual life cycles seen in this phylum are the merozoite (the form resulting from fission of a multinucleate schizont) and sexual stages such as gametocytes and gametes. Some protozoa form cysts that contain one or more infective forms. Multiplication occurs in the cysts of some species so that excystation releases more than one organism. For example, when the trophozoite of *Entamoeba histolytica* first forms a cyst, it has a single nucleus. As the cyst matures nuclear division produces four nuclei and during excystation four uninucleate metacystic amebas appear. Similarly, a freshly encysted *Giardia lamblia* has the same number of internal structures (organelles) as the trophozoite. However, as the cyst matures the organelles double and two trophozoites are formed. Cysts passed in stools have a protective wall, enabling the parasite to survive in the outside environment for a period ranging from days to a year, depending on the species and environmental conditions. Cysts formed in tissues do not usually have a heavy protective wall and rely upon carnivorism for transmission. Oocysts are stages resulting from sexual reproduction in the Apicomplexa. Some apicomplexan oocysts are passed in the feces of the host, but the oocysts of *Plasmodium*, the agent of malaria, develop in the body cavity of the mosquito vector.

1.6. Reproduction

Reproduction in the protozoa may be asexual, as in the amebas and flagellates that infect humans, or both asexual and sexual, as in the apicomplexa of medical importance. The most common type of asexual multiplication is binary fission, in which the organelles are duplicated and the protozoan then divides into two complete organisms. Division is longitudinal in the flagellates and transverse in the ciliates; amebas have no apparent

anterior-posterior axis. Endodyogeny is a form of asexual division seen in *Toxoplasma* and some related organisms. Two daughter cells form within the parent cell, which then ruptures, releasing the smaller progeny, which grow to full size before repeating the process. In schizogony, a common form of asexual division in the Apicomplexa, the nucleus divides a number of times, and then the cytoplasm divides into smaller uninucleate merozoites. In *Plasmodium, Toxoplasma*, and other apicomplexans, the sexual cycle involves the production of gametes (gamogony), fertilization to form the zygote, encystation of the zygote to form an oocyst, and the formation of infective sporozoites (sporogony) within the oocyst.

Some protozoa have complex life cycles requiring two different host species; others require only a single host to complete the life cycle. A single infective protozoan entering a susceptible host has the potential to produce an immense population. However, reproduction is limited by events such as death of the host or by the host's defense mechanisms, which may either eliminate the parasite or balance parasite reproduction to yield a chronic infection. For example: leishmania, plasmodium species.

Most free-living protozoa reproduce by cell division (exchange of genetic material is a separate process and is not involved in reproduction in protozoa). The relative importance for population growth of biotic versus chemical-physical components of the environment is difficult to ascertain from the existing survey data.

1.7. Nutrition

The nutrition of all protozoa is holozoic; that is, they require organic materials, which may be particulate or in solution. Amebas engulf particulate food or droplets through a sort of temporary mouth, perform digestion and absorption in a food vacuole, and eject the waste substances. Many protozoa have a permanent mouth, the cytosome or micropore, through which ingested food passes to become enclosed in food vacuoles. Pinocytosis is a method of ingesting nutrient materials whereby fluid is drawn through small, temporary openings in the body wall. The ingested material becomes enclosed within a membrane to form a food vacuole.

Protozoa are found living actively in nutrient-poor to organically rich waters and in fresh water varying between 0- 50°C . Nonetheless, it appears that rates of population growth increase when food is not constrained and temperature is increased [8-10]. Comparisons

of oxygen consumption in various taxonomic groups show wide variation [11], with some aerobic forms able to function at extremely low oxygen tensions and to thereby avoid competition and predation. Many parasitic and a few free-living species are obligatory anaerobes (grow without atmospheric oxygen). Of the free-living forms, the best known is the plagiopylid ciliates that live in the anaerobic sulfide-rich sediments of marine wetlands. The importance of plagiopylids in recycling nutrients to aerobic zones of wetlands is potentially great [12].

Protozoa have metabolic pathways similar to those of higher animals and require the same types of organic and inorganic compounds. In recent years, significant advances have been made in devising chemically defined media for the in vitro cultivation of parasitic protozoa. The resulting organisms are free of various substances that are present in organisms grown in complex media or isolated from a host and which can interfere with immunologic or biochemical studies. Research on the metabolism of parasites is of immediate interest because pathways that are essential for the parasite but not the host are potential targets for antiprotozoal compounds that would block that pathway but be safe for humans. Many antiprotozoal drugs were used empirically long before their mechanism of action was known. The sulfa drugs, which block folate synthesis in malaria parasites, are one example.

The rapid multiplication rate of many parasites increases the chances for mutation; hence, changes in virulence, drug susceptibility, and other characteristics may take place. Chloroquine resistances in *Plasmodium falciparum* and arsenic resistance in *Trypanosoma rhodesiense* are two examples.

Competition for nutrients is not usually an important factor in pathogenesis because the amounts utilized by parasitic protozoa are relatively small. Some parasites that inhabit the small intestine can significantly interfere with digestion and absorption and affect the nutritional status of the host; *Giardia* and *Cryptosporidium* are examples. The destruction of the host's cells and tissues as a result of the parasites' metabolic activities increases the host's nutritional needs. This may be a major factor in the outcome of an infection in a malnourished individual. Finally, extracellular or intracellular parasites that destroy cells while feeding can lead to organ dysfunction and serious or life-threatening consequences.

1.8. Protozoa: As parasites

Protozoa generally exist in two basic forms: the active, growing form called the "trophozoite;" and the dormant, resistant form called the "cyst." The trophozoite form proliferates tissues, causing damage that result in clinical diseases. The cyst is able to survive in an external environment and is usually the form that is transmitted from host to host. Some protozoa go through an intermediate stage in blood-sucking insects.

The four groups of protozoa that are mainly responsible for human disease include the following: sarcodina, ciliophora, mastigophora and sporozoa. All grouped according to their form of locomotion.

- **Sarcodina**, commonly known as amoebas, move by extending a section of their cytoplasm (called a pseudopodium or false foot) in one direction, causing the remainder to follow. They are usually found in marine and fresh water. Members include eight species (see Endoparasites). Three are parasitic to humans, with one causing more of a problem than the others. (*Entamoeba histolytica* causes the disease amebiasis.)
- **Ciliophora or Ciliates**, move by using the many fine cilia that beat in rhythmic patterns to propel the organism. Members include and Paramecium, but only one species causes disease in humans; and that is of a dysentery nature. *Balantidium coli* are a large oval-shaped cell that is the largest intestinal protozoa found in humans. Increasingly, it is showing up in the human intestinal tract, where it can invade and destroy the intestinal lining. Its normal habitat is the intestinal tract of hogs, but it can also be found in marine and fresh water worldwide, causing the disease known as balantidiasis. The life cycle is similar to that of the amoeba *E. histolytica* and has been associated with chronic fatigue syndrome.
- **Mastigophora** is a subphylum of protozoa that has one or more whip like flagella that propel the organism like swimmers. They are commonly known as Flagellates and are normally found in fresh water. Two relatively mild diseases, trichomoniasis and giardiasis, are produced from them, as well as the more serious diseases of trypanosomiasis and leishmaniasis.

- **Sporozoa** (singular sporozoon) is a class of parasitic protozoa that include Plasmodium and Toxoplasma. These two are commonly known as the parasites, found in vectors responsible for malaria and toxoplasmosis. They have both a sexual and asexual phase. They mainly target the epithelial cells of the intestinal tract, but can also be found in the liver and other organs

Protozoa are infamous for their role in causing disease, and parasitic species are among the best-known protozoa. Nevertheless, our knowledge has large gaps, especially of normally free-living protozoa that may become pathogenic in immuno-compromised individuals. For example, microsporidia comprise a unique group of obligate, intracellular parasitic protozoa. Microsporidia are amazingly diverse organisms with more than 700 species and 80 genera that are capable of infecting a variety of plant, animal, and even other protist hosts. They are found worldwide and have the ability to thrive in many ecological conditions. Until the past few years, their ubiquity did not cause a threat to human health, and few systematists worked to describe and classify the species. Since 1985, however, physicians have documented an unusual rise in worldwide infections in AIDS patients caused by four different genera of microsporidia (*Encephalitozoon*, *Nosema*, *Pleistophora*, and *Enterocytozoon).* According to the Centers for Disease Control in the United States, difficulties in identifying microsporidian species are impeding diagnosis and effective treatment of AIDS patients. There are over 50,000 species of protozoa, of which a fifth are parasitic, some 10,000 species. They infect vertebrates and invertebrates and some are even parasitic in plants. Parasitic protozoa are, in general, small, have short generation times, high rates of reproduction and a tendency to induce immunity to reinfection in those hosts that survive. Structurally a protozoan is equivalent to a single eukaryotic cell. Among the unique features in protozoa are the mega- and micronucleus found in Ciliates and the *kinetoplast*, a DNA containing structure in the mitochondrion of kinetoplastid flagellates. Parasitic protozoa are in no way simple or degenerate and adaptations to parasitism frequently include complex life cycles and specialized ways of entering and maintaining themselves in their hosts. It is, therefore, surprisingly humans and their domesticated animals should act as hosts to protozoa, but the diseases thus caused are out of all proportion to the number of species involved. The protozoa that infect humans range from forms that are never pathogenic to those that cause malaria, sleeping sickness, Chagas' disease and leishmaniasis, now

regarded as being among the major diseases of tropical countries, and which together threaten over one quarter of the population of the world [13].

Table: 1. 2. List of protozoan diseases in human

Disease	Causative agent	Motion by	Transmission
Amoebiasis	*Entamoeba histolytica* (Sarcodina)	Pseudopodia	Water, Food
Giardiasis	*Giardia lambila* (Mastigophora)	Flagella	Water, Contact
Trichomoniasis	*Trichomonas vaginalis* (Mastigophora)	Flagella	Sexual,Contact
African Sleeping Sickness	*Trypanosoma brucei* (Mastigophora)	Flagella	Tsetse fly (Glossina)
American Sleeping Sickness	*Trypanosoma cruzi* (Mastigophora)	Flagella	Triatomid bug (Triatoma)
Leishmaniasis (Kala - azar)	*Leishmania donovani* (Mastigophora)	Flagella	Sandfly (Phlemotomus)
Balantidiasis	*Balantidium coli* (Ciliophora)	Cilia	Food, Water
Toxoplasmosis	*Toxoplasma gondii* (Sporozoa)	NA	Cats, Food
Malaria	Plasmodium Spp. (Sporozoa)	NA	Mosquito (Anopheles)
Babesiosis	*Babesia microti* (Sporozoa)	NA	Tick (Ixodes)

Chapter- 2

Review of literature

2. REVIEW OF LITERATURE

2.1. Leishmaniasis: A Brief History

Leishmaniasis is a parasitic disease caused by the protozoa belonging to the genus, Leishmania. This common zoonotic infection is transmitted by Phlebotemus and Lutzomyia sandflies to humans and other vertebrate hosts. Leishmaniasis is a public health problem in at least 88 countries, of which 67 are in the old world and 21 in the new world [14]. Over 23 different species of Leishmania exist and manifest into one of the three common forms: cutaneous, mucocutaneous, and visceral leishmaniasis.

Visceral (VIS-er-al) leishmaniasis, the name it means that, the organism affects the internal organs and invades liver, spleen and bone marrow. If this disease remains untreated, mortality rate remains between 90–98%. Leishmaniasis has been found on pre-Inca pottery from Peru and Ecuador dating back to the first century AD. They are evidence that some forms of leishmaniasis prevailed as early as this period. The discovery of parasites in lesions of cutaneous or visceral leishmaniasis was reported in the late 1800s and early 1900s. Incan text from the 15th and 16th century and accounts from Spanish conquistadors noted the presence of skin lesions on agricultural workers returning from the Andes. These ulcers resembled leprosy lesions and were labeled, "white leprosy," "Andean sickness," or "valley sickness." In Africa and India, reports in the mid-18th century describe the disease now known as visceral leishmaniasis, as "kal-azar" or "black fever". In 1756, Alexander Russell made an important advance in the discovery of Leishmaniasis after examining a Turkish patient. According to Russell, "after it is cicatrised, it leaves an ugly scar, which remains through life, and for many months has a livid colour. When they are not irritated, they seldom give much pain. Russell called this disease "Aleppo boil" [15, 16].

Visceral leishmaniasis or kala–azar was first recognised in 19th century hilly regions of Assam and later it was proved to be a disease distinct from malaria. The disease broke out in the epidemic form every few years and caused havoc and devastation in part of the Bengal, Bihar and Assam. Vast regions became uninhabitable and part of Bengal became 'a valley of the shadow of death' [17]. Phlebotomus argentips was considered to be the vector for the organism in 1924 by Knowles *et al.* [18] in Calcutta by direct

demonstration of the parasite in these sandflies after a suitable blood meal. The first species of phlebotominae were found in Lower Cretaceous in the Lebanon, South of the Tethys Sea. A phlebotomus was found in amber in the Baltic area in the Upper Eocene belonging to about 30 million years ago. From then on, various phlebotomine sandflies have been found in East Africa. The first description of kala–azar that is acceptable is that of Twining [19]. He found cancrum oris, anemia and the characteristic skin pallor. In the 1860s it began to become obvious that a considerable infectious fever was rife in Garo hills of Assam, and then progressed steadily up the Brahmaputra valley over a 10–year period. More local synonyms include kala–jwar, kala–dukh, Burdwan fever, Sahib's disease and Shirkari disease in India, Ponos in Greece, and Semieh in Sudan. The Civil Surgeon of Burdwan, Dr. J. Eliot, traced the origins and spread of the disease. He was able to trace the disease back to 1824–25 to a village called Mahomedpore east of Jessore, infamous as the starting point of the first great pandemic of Cholera in 1817. Eliot mentions the inefficacy of Quinine and the splenomegaly. It was a disease of fearsome mortality and seems to have been a disease of swifter mortality than at present, but then the apparent celerity would depend on how soon patients sought the infective help. The disease travelled slowly westward, totally depopulating some villages, and reached Burdwan in the 1860s. The government of Bengal wrung its hands and reported that as many as 30% of the areas population may have died of the disease. According to Baker [20], a vector–borne infectious disease of humans is frequently one, which commences as an infection of blood sucking invertebrates and progresses to an infection of a vertebrate animal. From this state it may infect humans via the invertebrate, eventually dispensing with the animal reservoir zoonotic, where animal reservoir hosts are involved in the transmission cycle; and becoming a human disease transmitted human to human (anthroponotic) anthroponotic, where man is the sole reservoir and sole source of infection by the blood sucking invertebrate. As the disease builds up in humans it way finds a more direct form of transmission between humans, such as a droplet infection or via an ectoparasite of humans. This stage will normally be the most virulent form of the disease in humans. It may then fall away and become only a mild disease of humans.

The first investigation led to a conclusion that the disease was beriberi and caused by Ankylostoma, but this view was soon discarded. A second investigation by Surgeon Captain (later Sir) Leonard Rogers made the link between the Assamese disease and

Burdwan fever. Rogers concluded that the disease was a highly virulent form of malaria. *Leishmania* parasites are named after W.B. Leishman. In 1903 Leishman [21] noted that soldiers invalided home to Britain from the cantonment of Dum–Dum (the place of the present Calcutta airport) had a characteristic illness, "an extreme degree of cachexia", irregularly intermittent fever, anaemia, muscular atrophy and great enlargement of the spleen. He referred to these patients to as cases of Dum–Dum fever. He had no immediate explanation for these bodies of spleen but 3 years later he found similar bodies in the internal organs of a rat that had died from experimental trypanosomiasis, he proposed that Dum–Dum fever might be a form of Indian trypanosomiasis. This possibility was published in the British Medical Journal of 30 May 1903. In the same year, Professor Donovan from Madras Medical College, on reading *Leishman's* article, immediately realized the significance of the bodies, which he had found similar bodies in a post mortem spleen smears. He first thought that they might be a resting form of a malaria parasite, but had then decided they were probably post mortem artifacts. He later demonstrated that the bodies were neither post–mortem artifact nor an Indian form of trypanosomiasis [22].

U. N. Brahmachari, who nominated for the Nobel Prize, a young chemist-cum-physician in Calcutta inspired by the work of Erlich, synthesized a large number of pentavalent antimonials of which urea-stibamine, synthesized in 1920, and was found to be most effective against kala-azar in Assam and Bengal. Several tens of thousand of people in

W.B.Leishman

L. Donovan

U. N. Brahmachari

these area were saved during epidemic periods with the help of this drug, even though it had not undergone any systematic preclinical trial. This compound was found to have considerable toxicity and was of an undefined chemical composition [23]. At this early stage of drug development against various forms of leishmaniasis, the progress was greatly retarded because of the no availability of a good animal model against both visceral and cutaneous form of leishmaniasis in many parts of the world including Brazil, Italy and India. Leishmanicidal activity was tested on animal model of *T. equiperdum*. Smyly and Young [24] finally succeeded in infecting the Chinese hamster where liver puncture showing parasites was used as a safe criterion for infection. Fulton and Joyner introduced a convenient cotton rat model in 1948 [25].

By the end of fifties, improved treatment (pentavalent antimonial compounds) and house-to-house DDT spraying campaign under Indian National Malaria Eradication Programme, contributed to a steady decline in the number of kala-azar cases. It is generally thought that the reduction or cessation of these control measures has permitted the buildup of the *P. argentips* population to a sufficient level to spark off the recent Bihar epidemic. World Health Organization (WHO) has established a special research programme into their most important parasitic diseases like malaria, trypanosomiasis, schistosomiasis and leishmaniasis. Some of the main objectives of the scientific working group of leishmaniasis are epidemiology, vaccine studies and development of novel compounds in addition to the existing drugs [26, 27]. Due to wide spread resurgence of the above-mentioned diseases, the fresh interest have regained during the last decade, and have placed the subject of parasitology in a new phase. The table 2.1 represents the important events in leishmaniasis [28].

Table: 2.1. The Important events in leishmaniasis

9th Century	:	Razi, Zakarya (also known as Al–Rhazi) described cutaneous leishmaniasis; later known as 'Balkh Sore'.
10th Century	:	Avicenna and independently Abu Mansour Bokharai described cutaneous leishmaniasis. Bokharai called it 'Pasheh gazidegi' meaning mosquito bite in the Persian language.
1885	:	Cunningham saw infected macrophages from an Oriental Sore.
1898	:	Borowsky recognized the amastigotes of *Leishmania* in an Oriental Sore.
1903	:	Leishman and Donovan discovered the amastigotes in kala–azar and Ross named the parasite *L. donovani.*
1903	:	Wright found amastigotes in a case of Oriental Sore and named them *L. tropica.*
1908	:	Nicolle grew promastigotes in cutaneous culture.
1912	:	Vianna introduced antimonials for treatment.
1921	:	The Sergent brothers infected humans with flagellates from infected sandflies.
1923	:	Shortt and Sen introduced Brahmachari's antimonial for the cure of kala azar.
1937	:	Lawrow and Dubowokaj used live vaccine in USSR as a means of control, later called leishmanization.
1941	:	Alder and Colleuges established that Phlebotomus papatasi could transmit cutaneous leishmaniasis.

1942	:	Swaminath, Short and Anderson transmitted kala-azar from human to human via the sandfly.
1948	:	Alder introduced the Syrian hamster as an experimental host for leishmaniasis.
1953	:	Latyshev showed that the great gerbil was the reservoir of *L. major*.
1970	:	Bryceson and Colleugues showed that immunity to leishmaniasis was largely cellular.
1984	:	Sacks and Perkins identified the infective form of Leishmania in the sandfly.
1986	:	Badaro and Colleugues showed self–curing and asymptomatic visceral leishmaniasis in humans.
1987	:	Convit and Colleuges showed the efficacy of killed Leishmania Plus BCG in treatment of cutaneous leishmaniasis.
1988	:	Simon Croft reported meltifosine as an antileishmanial agent.
1988	:	Prof. S. Sundar and Prof. Henry Murray of Cornell University conducted successful clinical development programme under the supervision of TDR Task force.
1991	:	Beverley and Cruz constructed *Leishmania* recombinants by depleting selected genes.
1992	:	Effective oral treatment of visceral leishmaniasis in mice with Hexadecylphosphocholine (novel phospholipid derivative) by A Kuhlencord
1998	:	First vaccine tried for CL, comprised of whole killed *L. major* promastigotes together with BCG as adjuvant by TDR scientists.

2001	:	Vaccine Against Sand Fly Saliva Prevents Leishmaniasis in Mice, reported by Jos Ribeiro of the National Institute of Allergy and Infectious Disease
2005	:	Leishmania vaccine was successfully applied in dogs, developed by researchers at the French Institute for Research and Development in Montpelier.
2006	:	A new family of antimicrotubule drugs named (3-haloacetamido benzoyl) ureas and ethyl 3-haloacetamidobenzoates were found to be cytotoxic to the Leishmania parasite protozoa
2007	:	Leishmaniasis drug (antimonials) resistance mechanism exposed, published in American Journal of Tropical Medicine and Hygeine, 2007.
2008	:	Pujals Naranjo from University of Barcelona targeted the macrophages of Leishmania parasites by means of different technological strategies like micro-nanoparticles by spray drying produced by Nanotechnology.

2.2. Leishmaniasis: In general

The trypanosomatid parasite of the genus *leishmania* is the etiological agent of a variety of disease manifestations, collectively known as Leishmaniasis. There are a number of types of protozoa that can cause leishmaniasis. Each type exists in specific locations, and there are different patterns to the kind of disease each cause. The overall species name is Leishmania (commonly abbreviated L.). The specific types include: *L. donovani, L. infantum, L. chagasi, L. mexicana, L. amazonensis, L. tropica, L. major, L. aethiopica, L. brasiliensis, L.guyaensis, L. panamensis, L. peruviana.* Some of the names are reflective of the locale in which the specific protozoa are most commonly found, or in which it was first discovered.

2.2.1. Types of leishmaniasis

- **Visceral leishmaniasis** (VL), also known as kala-azar, is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the

spleen and liver, and anaemia. In this disease, the protozoa use the bloodstream to travel to the liver, spleen, lymph nodes, and bone marrow. Fever may last for as long as eight weeks, disappear, and then reappear again. The lymph nodes, spleen, and liver are often quite enlarged. Weaknesses, fatigue, loss of appetite, diarrhea, and weight loss are common. Kala-azar translates to mean "black fever." The name kala-azar comes from a characteristic of this form of leishmaniasis. Individuals with light-colored skin take on a darker, grayish skin tone, particularly of their face and hands. A variety of lesions appear on the skin. This type of leishmaniasis occurs in India, China, the southern region of Russia, and throughout Africa, the Mediterranean, and South and Central America.

- **Localized cutaneous leishmaniasis**

This is perhaps the least drastic type of disease caused by any of the Leishmania. Several weeks or months after being bitten by an infected sandfly, the host may notice an itchy bump (lesion) on an arm, leg, or face. Lymph nodes in the area of this bump may be swollen. Within several months, the bump develops a crater (ulceration) in the center, with a raised, reddened ridge around it. There may be several of these lesions near each other, and they may spread into each other to form one large lesion. Although localized cutaneous leishmaniasis usually heals on its own, it may take as long as one year. A depressed, light-colored scar usually remains behind. Some lesions never heal, and may invade and destroy the tissue below. For example, lesions on the ears may slowly, but surely, invade and destroy the cartilage that supports the outer ear. This type of disease occurs most commonly in China, India, Asia Minor, Africa, the Mediterranean Basin, and Central America. It has occurred in an area ranging from northern Argentina all the way up to southern Texas. It is called different names in different locations, including chiclero ulcer, bush yaws, oriental sore, Aleppo boil, and Baghdad sore.

- **Cutaneous leishmaniasis (CL)**

Cutaneous leishmaniasis also known as oriental sore. Cutaneous forms of the disease normally produce skin ulcers on the exposed parts of the body such as the face, arms and legs. The disease can produce a large number of lesions sometimes up to 200 - causing serious disability and invariably leaving the patient permanently scarred, a

stigma, which can cause serious social prejudice. This type of disease is widely distributed in the Mediterranean, Middle East, India, and Africa. This form of the disease produces one or a small number of sores primarily on the face and limbs. Initially, the sores may appear as small red bumps that may itch and grow to a sore that is flat in the center and raised on the edges. These skin lesions usually heal spontaneously within a few months.

- **Diffuse cutaneous leishmaniasis**

This type of disease occurs most often in Ethiopia, Brazil, Dominican Republic, and Venezuela. The lesions of diffuse cutaneous leishmaniasis are very similar to those of localized cutaneous leishmaniasis, except they are spread all over the body. The body's immune system apparently fails to battle the protozoa, which are free to spread throughout. The characteristic lesions resemble those of the dread biblical disease, leprosy.

- **Mucocutaneous leishmaniasis (MCL)**

MCL or *espundia* produces lesions that can lead to extensive and disfiguring destruction of mucous membranes. The lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues of the nose, mouth and throat cavities. This form of leishmaniasis occurs primarily in the tropics of South America particularly Peru, Bolivia, Paraguay, Ecuador, Colombia, and Venezuela. The disease begins with the same sores noted in localized cutaneous leishmaniasis. Sometimes these primary lesions heal, other times they spread and become larger. Some years after the first lesion is noted (and sometimes several years after that lesion has totally healed), new lesions appear in the mouth and nose, and occasionally in the area between the genitalia and the anus (the perineum). These new lesions are particularly destructive and painful. They erode underlying tissue and cartilage, frequently eating through the septum (the cartilage that separates the two nostrils). If the lesions spread to the roof of the mouth and the larynx (the part of the wind pipe which contains the vocal cords), they may prevent speech. Other symptoms include fever, weight loss, and anemia (low red blood cell count). There is always a large danger of bacteria infecting the already open sores.

2.2.2. The disease manifestation and symptoms

Leishmania are tiny protozoa (a simple living organism). Their parasitic life cycle includes the sandfly and the right host. Humans are one such host. Leishmania infection can cause skin disease (called cutaneous leishmaniasis), which can also affect the mucous membrane. The infection can also cause systemic (throughout the body) disease. Various terms have been used to describe leishmania systemic disease including visceral leishmaniasis, Dum-dum fever, Sikari disease, Burdwan fever, Shahib's disease and tropical splenomegaly. However, the most commonly used term is kala-azar, which in Hindi means black sickness or black fever. The terms originally referred to Indian VL due to its characteristic symptoms, blackening or darkening of the skin of the hands, feet, face and the abdomen.

Visceral leishmaniasis is caused by the parasites *Leishmania donovani donovani*, *Leishmania donovani infantum* and *Leishmania donovani archibaldi* in the old world and by *Leishmania donovani chagasi* in the new world. In endemic cases of VL, the disease is chronic and onset is gradual. Although people of all ages are susceptible in the old world, children below the age of 15 are more commonly affected with *L.d infantum* being largely responsible [29]. In sporadic and epidemic cases of VL the disease is usually acute and symptoms appear suddenly with people of all ages being at risk except those who have conferred immunity due to a past infection. The symptoms of VL vary between individuals and according to geographical foci. Visceral leishmaniasis can have fatal complications. When introduced into the body by the bite of a sandfly, the parasite migrates to the bone marrow, spleen, and lymph nodes. The parasites damage the immune system by decreasing the numbers of disease-fighting cells.

However, some of the common symptoms include high undulating fever often with two or even three peaks in 24 hours and drenching sweats, which can easily be misdiagnosed as malaria. The incubation period is highly variable; the disease can appear anything between ten days to over one year. Even longer incubation periods have been documented [30]. The duration of the disease can be 1-20 weeks, in endemic areas of Western Sudan the illness usually lasts about 12-16 weeks with and average of about 6 weeks. Systemic infection in children usually begins suddenly with vomiting, diarrhoea, fever, and cough. In adults, fever for 2 weeks to 2 months is accompanied by nonspecific

symptoms, such as fatigue, weakness, and loss of appetite. Weakness increases as the disease progresses. Clinical sings include splenomegaly, hepatomegaly and lymphadenopathy.

Affected mucous membranes can have a wide range of appearances, most frequently ulcers. Leishmaniasis may cause skin lesions that resemble those of other diseases including cutaneous tuberculosis, syphilis, leprosy, skin cancer, and fungal infections. The skin may become grayish, dark, dry, and flaky. Death usually results from complications (such as other infections) rather than from the disease itself. Death often occurs within 2 years.

2.2.3. Diagnostic tests for leishmaniasis

In visceral leishmaniasis parasites may be found in a splenic aspirate, liver biopsy or bone marrow biopsy. These techniques, especially splenic aspirate and liver biopsy can be hazardous and require previous expertise in the procedure.

VL produces large amounts of specific IgG which can be used for diagnosis. Several antibody-detection tests have been developed for field diagnosis of VL. Conventional methods such as gel-diffusion immunoelectrophoresis, a complement-fixation test, indirect haemagglutination test and counter-current immunoelectrophoresis have limited diagnostic accuracy and/or feasibility for field use. Indirect fluorescence antibody (IFA) tests showed acceptable estimates for sensitivity (87–100%) and specificity (77–100%). The most used sero diagnostic tests are Indirect-immuno Fluorescent Antibody Test (IFAT), Enzyme Linked Immunosorbent Assay (ELISA) and Direct Agglutination Test (DAT).

Recently, Sarkari et al. described a urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate that was detected in the urine of VL patients. An agglutination test to detect this antigen has been evaluated in laboratory trials, using urine collected from well-defined cases and controls from endemic and non-endemic regions. This test showed 100% specificity and sensitivity between 64% and 100%.

In cutaneous and mucocutaneous leishmaniasis the margin of the lesion contains leishmanial bodies whereas the centre contains debris and dead skin material. The margin of the lesion is aseptically punctured with a hypodermic needle and syringe

containing a small amount of saline. The aspirate which is drawn up into the needle is examined microscopically.

Gene amplification technique like polymerase chain reaction is powerful and sensitive method and is useful in diagnosis of cutaneous leishmaniasis particularly when organisms cannot be detected microscopically. It is also very useful for the speciation of *Leishmania* parasites thus the correct treatment can be administered.

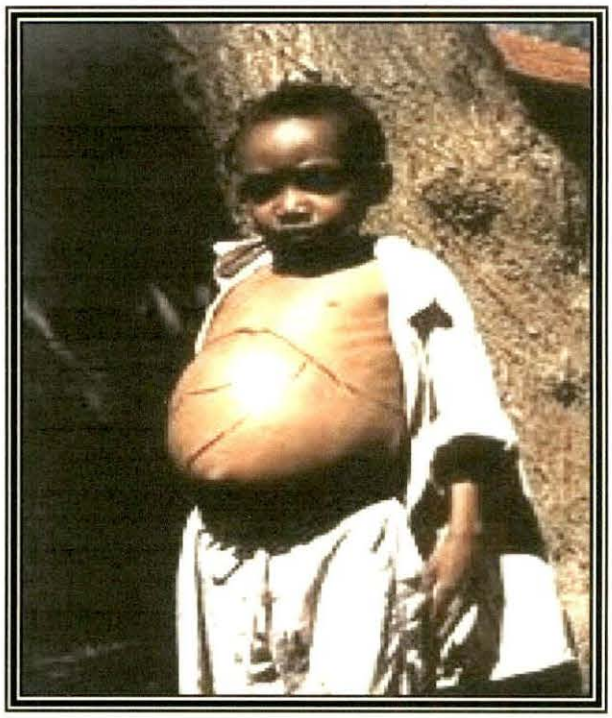


Fig.2.2.1. Person suffering from visceral leishmaniasis

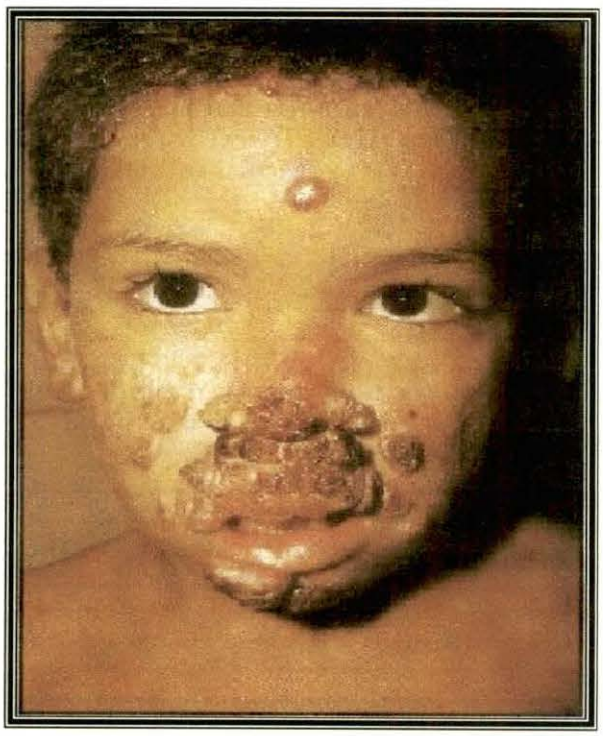


Fig.2.2.2. Mucoceutaneous leishmaniasis resulting in a total disfigurement of the face

Table 2.2.1. Symptoms of various forms of leishmaniasis

Visceral leishmaniasis	Cutaneous leishmaniasis	Mucocutaneous leishmaniasis
Persistent fever	Macule or papule, erythematous	Nasal stuffiness
Night sweats	Skin ulcer, forms at site of original lesion	Runny nose
Fatigue	Ulcer heals very slowly over a matter of months	Nosebleed
Weakness	Smaller lesions may form around the ulcer (satellite lesions)	Ulcers and erosion of tissue (mouth, tongue, gums, lips, nose, nasal septum)
Appetite loss	-	Swallowing difficulty (dysphagia) with esophageal involvement
Weight loss	-	Breathing difficulty, with tracheal involvement
Abdominal discomfort, vague	-	-
Vomiting, Diarrhoea and cough in children	-	-
Thinning hair	-	-
Gray, dark, ashen & scaly skin	-	-

2.2.4. Different forms of kala-azar

The different types of kala-azar exist, which vary considerably in clinical symptoms, severity and response to antimony treatment [31]. It can be suggested that these variations have come about as the disease has developed from its primitive state as a zoonosis. They are mentioned in the Table. 2.2.

(a) Indian kala-azar

(b) Acute toxic kala-azar

(c) Infanite or Mediterranean kala-azar

(d) Chinese kala-azar

(e) Russian kala-azar

(f) Sudanese kala-azar

(g) East African kala-azar

Table: 2.2.3. Main difference between important forms of kala-azar [32]

		Indian KA	**Sudanese KA**	**E. Africana KA**
1.	Skin lesions with visceral diseases	Do not occur	Fairly common on legs and head	Sometimes seen on legs
2.	Frequency of *leishmania* in blood	Often seen	Rarely seen	Rarely seen
3.	Response to pentavalent antimony treatment	Good	Little or none	Little or none
4.	Incidence of relapse	Not common	Common	Common
5.	Post kala-azar dermal leishmaniasis (PKDL)	Latent period 1–2 years. Duration long. Found in 5–10% of cases.	Little or no latent period. Duration long. Found in 30% of cases.	Latent period 5–9 months. Duration long. Found in small proportion of cases

2.2.5. Geographical distribution and epidemiology

Visceral leishmaniasis is endemic in the tropical and sub-tropical regions of Africa, Asia, the Mediterranean, Southern Europe, South and Central America [Fig-2.1]. The distribution of VL in these areas however is not uniform; it is patchy and often associated with areas of drought, famine and densely populated villages with little or no sanitation. In endemic areas children below the age of 15 are commonly affected. In sporadic and epidemic cases of VL people of all ages are susceptible with males at least twice as likely to contract the disease as females, except those who have conferred immunity due to past infection [30, 33].

In Pakistan 239 cases of VL due *L. infantum* were reported between 1985 and 1995, of these 52% were children below the age of 2 years, 86% were children below the age of 5 years, this represented an increase of ten-fold in infantile VL cases over the 10 year period from 0.2 to 2 per 100000 population and male cases out numbered female cases by three times. Visceral leishmaniasis has been known to exist in the Himalayas in Pakistan for over three decades. However recently sporadic cases are beginning to appear in the North West Frontier Province (NWFP), Punjab and Azad Jammu and Kashmir (AJK). All of these areas are mountainous and contain large farming communities [30].

In India VL is endemic in the states of Bihar, Uttar Pradesh and West Bengal. One of the largest epidemics occurred in 1978 in North Bihar where over half a million people fell victim to VL. In the first eight months of 1982, 7500 cases were reported in India and in one year alone between 1987 and 1988, 22, 000 cases of VL were registered [33].

In Bangladesh cases of VL greatly declined between 1953-1970, probably as a result of mass chemotherapy with pentavalent antimonials and wide spread spraying with DDT to control malaria. Following the end of the malaria control programme in 1970, sandfly vector populations increased and so did the cases of VL and currently appear at a rate in excess of 15000 per year [34].

In Brazil, VL is distributed widely in the south, east and the central regions of the country. Visceral leishmaniasis commonly affects poor and malnourished children below the age of 15 years. The disease is highly endemic in the states of Bahia and Ceara, which together account for 70% of the total cases of VL in Brazil [33]. Up to 1989, 15000 cases of VL had been recorded in the states of Alagoas, Espirito Santo, Gias, Mato Grosso do

Sul, Minas Gerais, Para, Paraiba etc. Recently the foci of VL has shifted from rural villages to large cities probably as a result of migration of settlers from villages into these cities creating densely populated ghettos living sub-standard housing with improper sanitation and keeping farm animals in their gardens. The two cities of Teresin and Sao Luis together accounted for 40-50% of the total number of VL cases in Brazil during 1993 and 1994 that is approximately 3000 cases per year.

In Central America, where previously only isolated cases of VL were recorded, the disease is on the increase. Especially in Costa Rica, Honduras and Nicaragua. This is most probably due to an increase in the human population and their movements in and out of these areas [35].

Since the first reported case of VL in Sudan in 1938, the disease has become wide spread and is endemic in south and eastern parts of the White Nile and Upper Nile states. Other areas affected include the provinces of Kasala, Jonglei and Kapoeta in the south, El Fasher and El Nahud in the west and also north of Khartoum. As in most countries males are almost twice as likely to be affected by VL as females, with young children being at the highest risk. In the village of Um-Salala in eastern Sudan, the average age of VL patients was found to be 6.6 years with a male to female ratio of 1.8:1 and an annual incidence rate of 38.4 per 1000 population between 1991 and 1992, and 38.5 per 1000 during the period 1992 and 1993 [36].

The first case of VL in Ethiopia was documented in 1942 in the southern parts of the country. Since then the disease has spread to become endemic in the Segen, Woito and Gelana river valleys. The highest incidence has been recorded in the Aba Roba area [20]. During an 8-year study leading upto 1990, 142 cases of VL were reported in the villages close to the Segen river valley. It was found that 58% of the people affected were children below the age of 15 years with the lowest risk groups being males above the age of 39 years and females above 24 years, also surprisingly, children below the age of 5 years. The reason for this reduced risk is not clear; however it is probably due to acquired immunity in the adult population [37]. However this does not explain the reduced risk in the children younger than 5 years of age.

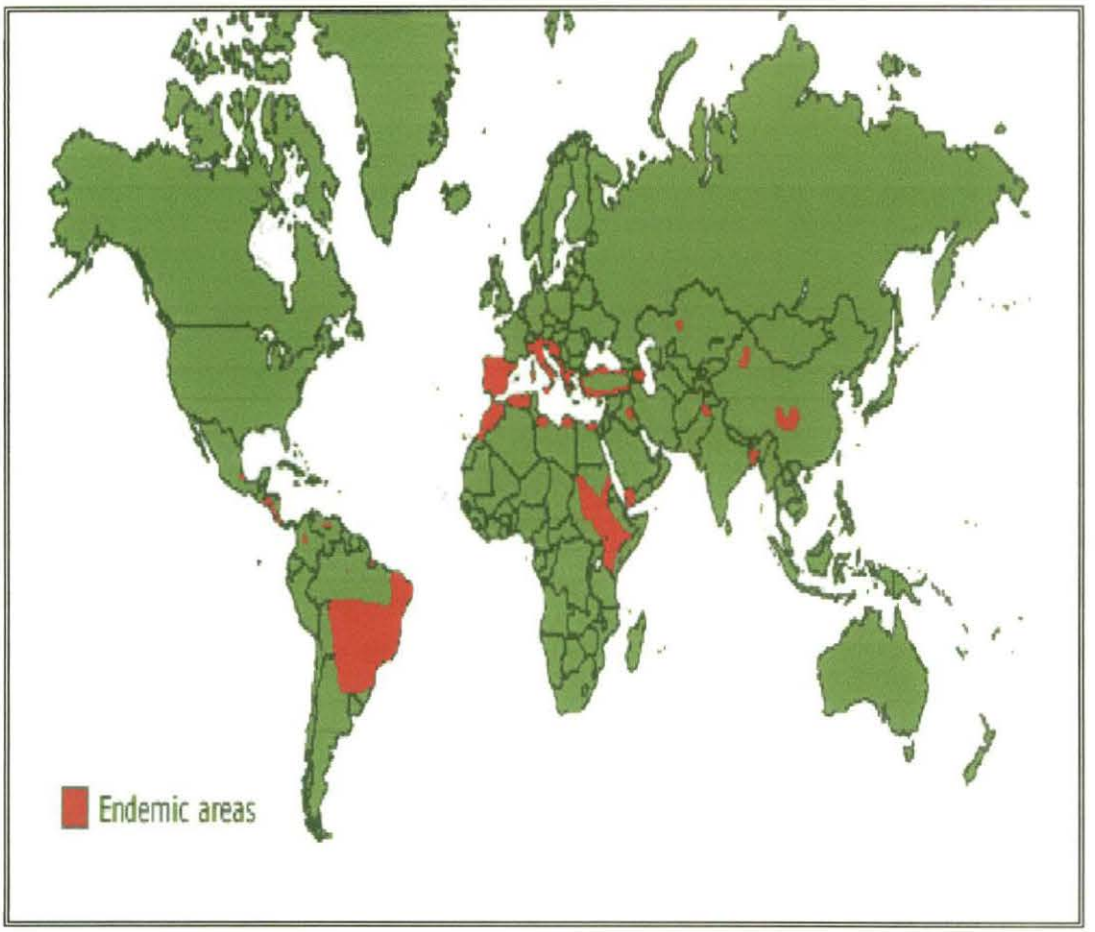


Fig 2.3. Geographycal distribution of Leishmaniasis World wide

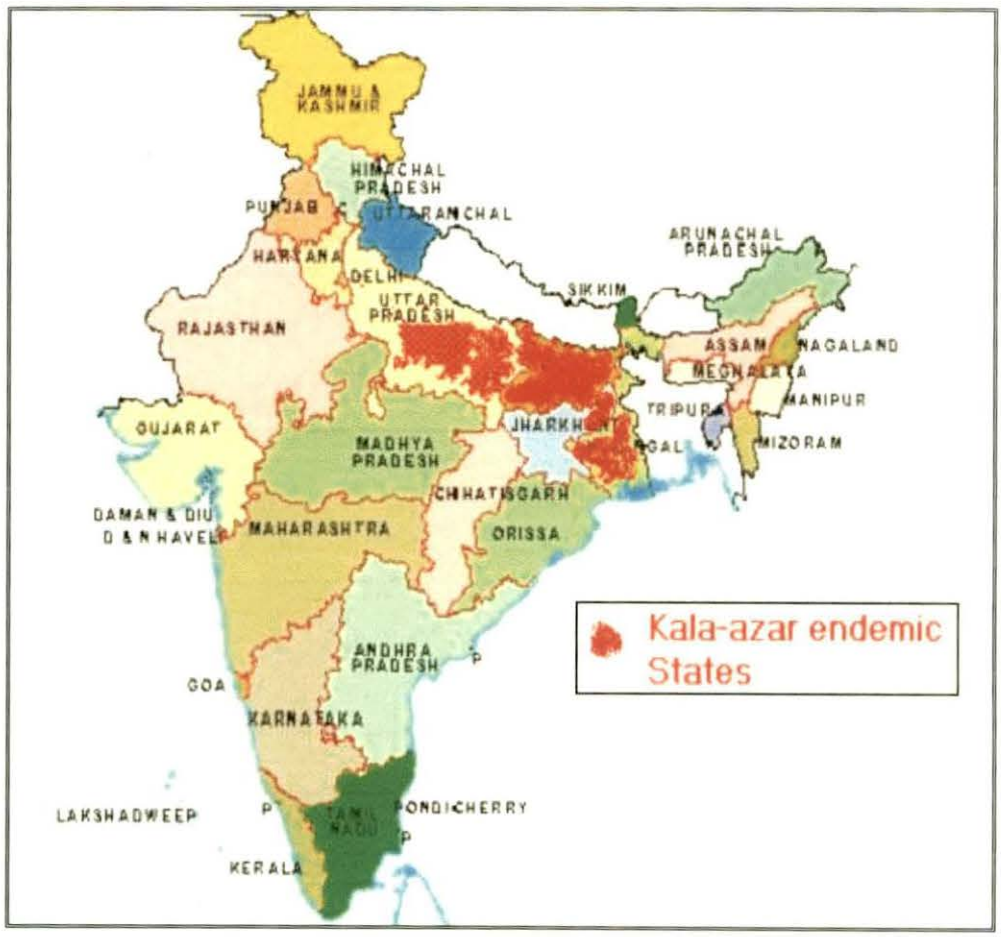


Fig.2.4. Geographycal distribution of Leishmaniasis in Indian region

In Somalia sporadic cases of VL first appeared in 1934, mainly in the Middle Shabelle and Lower Juba areas. A recent retrospective study has shown that VL is endemic in these areas. Children below the age of 15 years were at the highest risk and males were over three times more susceptible than females [38].

In Israel VL is rare and the few cases that have been reported are largely confined to the run down Arab villages in western Galilee, proving that the disease is linked to poverty, poor sanitation and sub-standard housing. Between 1960 and 1989, 62 cases of VL were recorded with only 18 cases in the past 13 years and 6 cases of infantile VL between 1992 and 1994. This fall in the incidence rate is most probably due to improved standards of living, diet and the use of insecticides [39].

2.3. Protozoology of genus *Leishmania*

In the animal kingdom protozoa may be regarded either as a phylum or as a group of microorganisms within the protista having the basic characteristics of animal cells [40]. Still there is controversy over which of each pair of definitions is more correct. Eukaryotic cells are the basic cellular organization of protozoa. The cell contents are delineated into large number of membrane bound organelle such as nuclei, mitochondria, glycosomes (microbodies), golgi apparatus, lysosomes and food vacuoles. This organism is quite distinct from that of prokaryotic microorganisms, which lack membrane bound organelles, but is similar to other lower eukaryotes such as algae and fungi [41]. Absence of mechanically rigid cell wall, external to the plasma membrane distinguishes protozoan cells from that of algae, fungi and higher plants and underlies their similarity to those of multicellular animals.

2.3.1. Morphology and ultrastructure of *Leishmania*

The outcome of electronic microscopic studies on *Leishmania* has revealed many differences between amastigote form and promastigote form (Fig.2.5). The amastigote stage is a round or oval body about 2-6 μm in diameter, containing a nucleus, a kinetoplast and an internal flagellum seen clearly in electron micrographs. The amastigotes multiply within the parasitophorous vacuoles of macrophages. The promastigote stage has a long and slender body (about 15-30 μm by 2-3 μm), with a central nucleus, a kinetoplast and a long free anterior flagellum. Many scientists have confirmed that the mitochondrion was extended during amastigote to promastigote

transformation [42,43]. It has been observed that during the transformation of amastigote to promastigote, there was the lengthening and elaboration of the mitochondrion [44], except for some exceptions where a long and tortuous mitochondrion have been observed in amastigotes [45,46]. The distance between the microtubules differs between mammalian and reptilian species [47]. The subpellicular microtubules have been used as a potential means of separating *Leishmania* species.

The number of subpellicular microtubules in amastigotes is not same in all the species in *Leishmania*. In case of *L. donovani*, 80-120 subpellicular microtubules have been found [48], whereas in case of *L. mexicana* their number is 180-200 [49]. The microtubules of the promastigotes radiate in all directions from a point near the flagellar base, unlike in other trypanosomatids in which they are spiralled [50]. The nucleus of *Leishmania* is covered with two nuclear membranes of 7nm thickness, and having a prominent nucleolus (endosomes), situated centrally with 0.6-1μm in diameter. The nuclear membrane remains intact during division. Nuclear membrane may contain extensions, which penetrate deep into cytoplasm to form dilated vesicle [42]. The kinetoplastid DNA is in the form of a coiled filament (20-50 Å wide) in *Leishmania*. On division this coil elongates then split transversely inside the kinetoplastic membrane.

Kinetoplast has been found to be connected to the basal body by a band of amorphous material [51]. Promastigotes may sometimes contain pigment granules and lipid bodies [52]. Both amastigotes and promastigotes contain peroxisomes, which contain all the glycolytic enzymes in their vesicle [53]. Lysosomes were absent in promastigotes, but present in amastigotes [54]. All species of *Leishmania,* except *L. tropica*, contain rough endoplasmic reticulum.

Four isolated tubules were observed in the reservoir region that may serve to anchor the subpellicular tubules to the flagellar apparatus. The amastigote–promastigote transformation in *Leishmania* (Fig.2.6) is associated with an increase in the number of mitochondrial profiles per section, the relative mitochondrial volume was decreased, and the concentration of the DNA fibrils to the centre of the kinetoplast with a wider disposition of the cytoplasmic RNA granules have been observed [42].

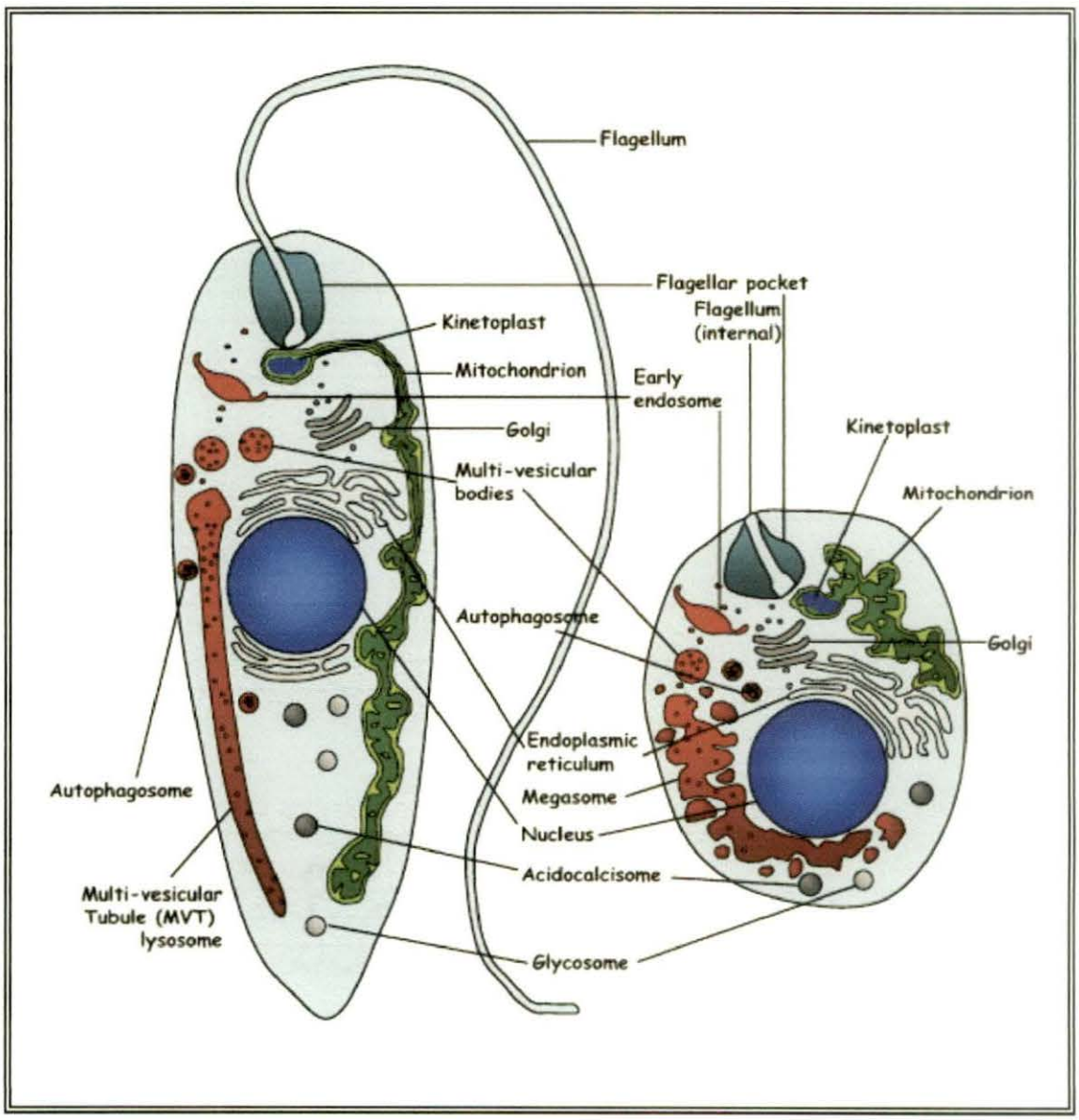


Fig. 2.5. A schematic representation of the fine structure of a promastigote (left) and an amastigote (right)

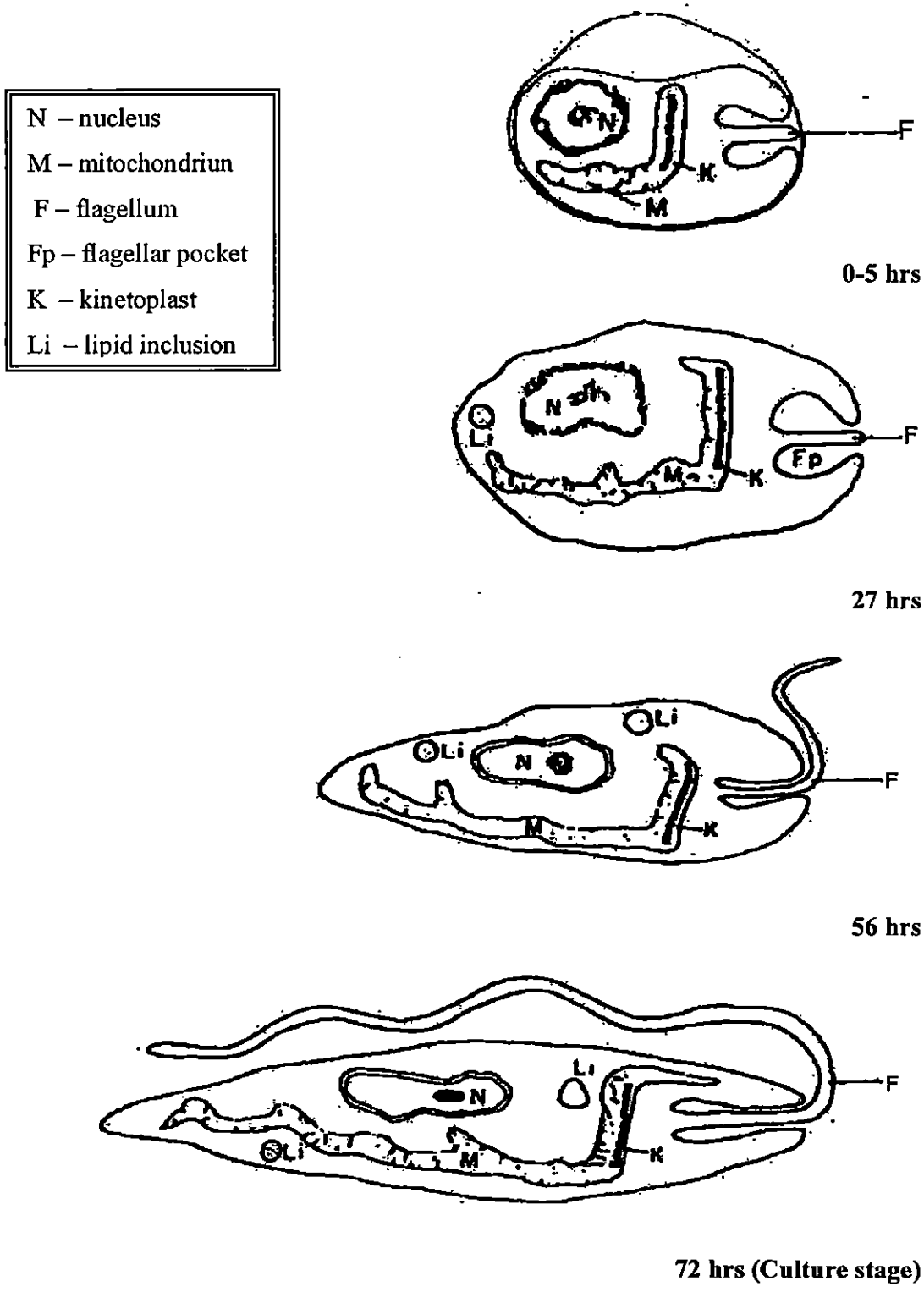


Fig. 2.6. Schematic diagram of the fine structural changes of *L. donovani* in the course of amastigote to promastigote transformation

Some studies have been done on the transformation of the intracellular amastigote form to the motile promastigote form and vice versa. Promastigote to amastigote transformation has been claimed to trigger off by withdrawing riboflavin from defined medium without affecting the cell growth at 25°C [55]. Amastigote forms of mammalian *Leishmania* have been produced *in vitro* by adopting the organisms to grow at 34°C [56]. However *L. torentolae* does not respond to elevated temperature in this way; growth of this organism in a defined medium is inhibited at 33°C. Addition of red blood cell extract permits growth at this temperature, but the formation of amastigote form has not been observed [57].

The promastigote stage of *Leishmania* can be grown readily on a variety of complex media at temperatures ranging from 16°C to 32°C. Inoculation of *Leishmania donovani* bodies in the media containing red blood cell extract and human or hamster serum can produce morphologically intermediate forms at 37°C [58, 59]. At 37°C culture of spleen from hamsters infected with *L. donovani* contained initially only the amastigote form of the organism [60]. In older cultures, the parasites after having escaped from destroyed cells, multiplies as promastigotes. In the system of Lamy *et al.* the intracellular stages of *L. donovani* in presence of carcinosarcoma cells could be maintained several months by serial transfers [61].

The effect of temperature upon conversion of culture to blood stream forms has been observed by many workers and seems to play an important part also in reciprocal transformation [62-66]. A change from amastigote to promastigote in the body is accompanied by the highly developed chondriome structures [67]. Sensitivity of the cultural form of *L. donovani* to higher temperature has been shown to be mainly due to increased template RNA degradation [68]. The crithidia flagellates develop lipid requirements when kept at elevated temperature (32.5°C – 33.5°C) to maintain normal growth [69]. An additional nutritional requirement due to elevated temperature has been attributed to be due to inactivation of certain enzymes [70].

Tubulin had been previously shown to be a component of functional microtubules that are present in axonemal, subpellicular and nuclear structure of trypanosomatid protozoa [71]. *L. donovani* surface membrane was specifically shown to have tubulin by Dwyer

[72] in 1980. Fong and Chang [73] have shown that tubulin biosynthesis is severely restricted during this transformation to the amastigote form.

Amastigote to promastigote transformation has been found to require amino acids and glucose [74]. It is accompanied by an increase in polyamine levels [75] in mitochondrial volume [42] with substantial proliferation [Fig.2.5], respiratory rate [76] and cyclic AMP level [77]. Actinomycin D, puromycin [78], cycloheximide, antileishmanial drugs [79] and lymphocyte factors [80, 81] have been reported to inhibit or to perturb this transformation. Further, different authors have shown that the amastigotes and promastigotes differ in their surface coat [82], antigenic properties [83] and possibly in the levels of cAMP catabolising enzymes [77] Wallach [84] has shown that the effect on tubulin biosynthesis during this transformation is controlled at the post transcriptional level.

2.3.2. Classification of the genus *Leishmania*

The animal kingdom has been divided into two groups, metazoa and protozoa. The metazoans are multicellular; different group of cells perform different biochemical reactions to fulfill the diverse physiological requirements of life process. The protozoa's are unicellular performing all function of life within the campus of a cell. Some important characteristics of protozoa are given below.

(a) Protozoa's are generally larger than bacteria and yeast.

(b) Protozoa has well defined nucleus with nuclear membrane.

(c) Protozoa's are generally motile throughout the life cycle or at least during certain periods of life cycle. The phylum protozoa were classified by Doflin [41] into two subphyla - plasmodroma and ciliophora. The classification in short can be enumerated as below.

Various types of classification have been successively applied to the genus *Leishmania.* Those proposed between 1916 and 1987 were monothetic Linnean classifications based on few hierarchical characters. Lainson and Shaw are the authors who worked the most on these types of classification and who made them evaluative. Their last classification (1987) divided the genus *Leishmania* into two sub-genera: *Leishmania* sensu stricto

(Table.2.3) present in both Old and New World, and Viannia (Table.2.4), restricted to New World. Within these two sub-genera various species complexes were individualized.

Haemoflagellates infect the vascular system and various tissues of the body. These groups are responsible for various types of diseases like kala-azar, sleeping sickness, and oriental sore in man. The *Leishmania* parasites are classified into two subgenera according their life cycle in the sandfly and it is important to understand this classification since the biochemical characteristics, treatment and epidemiology-all depend on it. The two subgenera are as follows [85].

a) *Leishmania* – *L.* (L.) development in the foregut of sandfly (suprapylaria).

b) *Viannia* – *L.* (V.) developed in the legs and midgut of the sandfly (peripylaria).

The sandfly are under phlebotomus species and scientifically it is known as *Phlebotomus argentips* to be the vector of *Leishmania*.

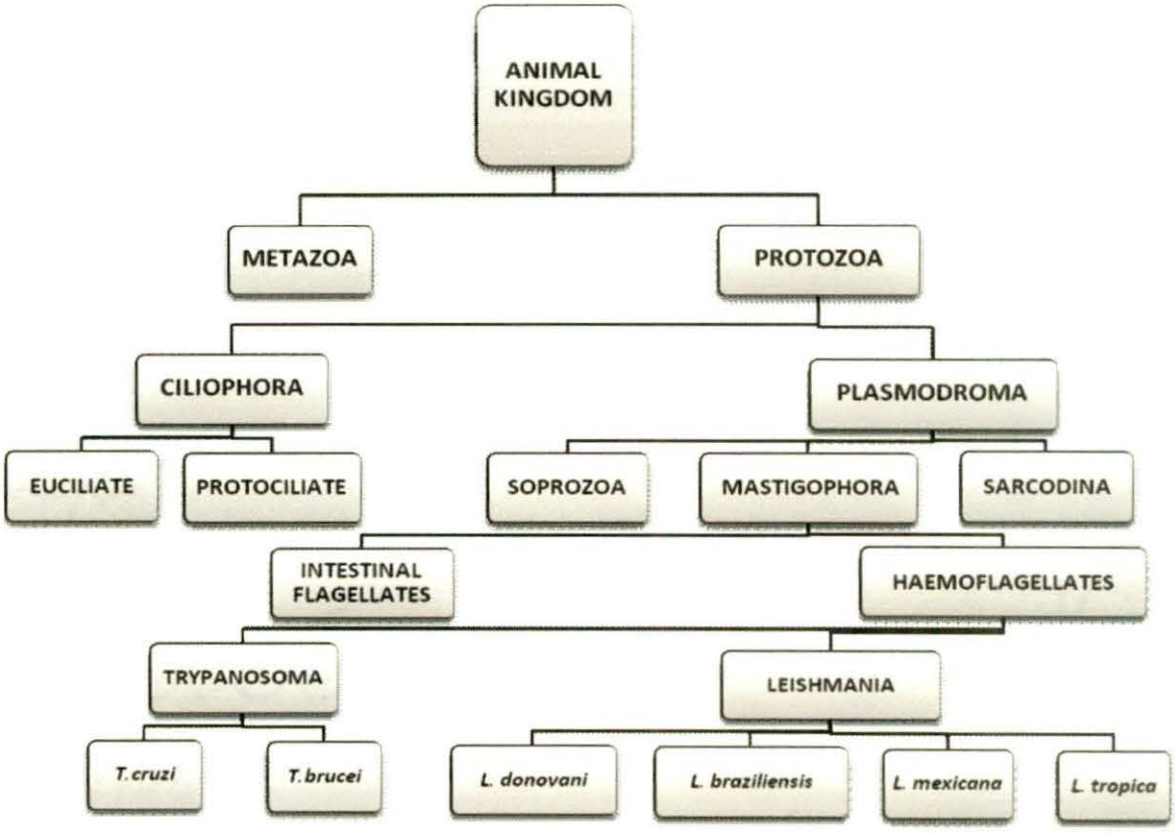


Fig. 2.7. Classification of protozoa

Table. 2.3. Sub-genus *Leishmania* Ross, 1903 [86]

L. donovani complex	*L. donovani* (Laveran & Mesnil, 1903) *L. archibaldi* Castellani & Chalmers , 1919
L. infantum complex	*L. infantum* Nicolle, 1908 (syn. *L. chagasi* Cunha & Chagas, 1937)
L. tropica complex	*L. tropica* (Wright, 1903)
L. killicki complex	*L. killicki* Rioux, Lanotte & Pratlong, 1986
L. aethiopica complex	*L. aethiopica* Bray, Ashford & Bray, 1973
L. major complex	*L. major* Yakimoff & Schokhor, 1914
L. turanica complex	*L. turanica* Strelkova, Peters & Evans, 1990
L. gerbilli complex	*L. gerbilli* Wang, Qu & Guan, 1964
L. arabica complex	*L. arabica* Peters, Elbihari & Evans, 1986
L. mexicana complex	*L. mexicana* Biagi, 1953 (syn. *L. pifanoi* Medina & Romero, 1959)
L. amazonensis complex	*L. amazonensis* Lainson & Shaw, 1972 (syn. *L. garnhami* Scorza *et al.*, 1979) *L. aristidesi* Lainson & Shaw, 1979
L. enriettii complex	*L. enriettii* Muniz & Medina, 1948
L. hertigi complex	*L. hertigi* Herrer, 1971 *L. deanei* Lainson & Shaw, 1977

Table.2.4. Sub-genus *Viannia* Lainson and Shaw, 1987 [87]

L. braziliensis complex	*L. braziliensis* Vianna, 1911 *L. peruviana* Velez, 1913
L. guyanensis complex	*L. guyanensis* Floch, 1954 *L. panamensis* Lainson & Shaw, 1972 *L. shawi* Lainson *et al.*, 1989
L. naiffi complex	*L. naiffi* Lainson & Shaw, 1989
L. lainsoni complex	*L. lainsoni* Silveira *et al.*, 1987

2.3.3. Reservoir Host

Leishmaniasis is primarily a zoonotic (transmitted to humans from animals) disease in which wild and domestic animals such as the fox, jackal, rodents and wolves serve as reservoir hosts (Table. 2.5). Other animals in the surrounding areas can become infected and these are referred to as secondary or incidental hosts. Of all the potential animal hosts, domestic dogs by far play the most important role in harboring and transmitting the disease to humans due to the close association between humans and dogs as pets (WHO, 1991).

In anthroponotic visceral leishmaniasis due to *Leishmania donovani* such as in India and Sudan, human beings are the principal reservoir host. Asymptomatic carriers and PKDL patients are a particular source of infection for sandflies (WHO, 1991).

Table: 2.5. Important *Leishmania* spp. and its effects

Species	Type of disease	Reservoir hosts	Geographic distribution	Vector
Cutaneous Leishmaniasis				
L. tropica minor	Dry cutaneous	Rodents, dogs	Southern Europe, Middle East	Phlebotomus spp.
L. tropica major	Oriental sore, wet cutaneous	Rodents, dogs	Southern Europe, Africa, Middle East	Phlebotamus spp.
L. braziliensis braziliensis	Espundia, mucocutaneous	Rodents	Mexico, Brazil	Lutozomyia spp. Psychodopypus spp.
L. mexicana mexicana	Cutaneous, chilcero ulcer	Rodents	Central America	Lutzomyia spp.
L. mexicana amazonensis	Diffuse, cutaneous	Rodents	Amazons region	Lutozomyia spp.
L. peruviana	Uta, cutaneous	Dogs	Peru	Lutzomyia spp.
Visceral Leishmaniasis				
L. donovani	Kala–azar, dum–dum fever, visceral	Dogs, Foxes	Africa, Asia, Middle East, South America	Phlebotomus spp.
L. donovani chargasi	Visceral	Foxes, cats, dogs	South America	Lutzomyia spp.
L. donovani infantum	Visceral infantile	Dogs	Mediterranean countries	Phlebotomus spp.

2.3.4. Insect Vector

The only proven vector of the *Leishmania* parasite is the blood-sucking female of the genus Phlebotomus in the old world and Lutzomyia in the new world. The insects are 2-3 mm long and their small size allows them to pass through ordinary mesh screens and mosquito netting. They are generally found throughout the tropical and temperate parts of the world. The sandfly larvae require organic matter, heat and humidity for development and so are commonly found in house-hold rubbish, bark of old trees, burrows of old trees and in cracks in house walls. The sandflies usually feed at night while the host is asleep. Only 30 or so of the over 500 species of Phlebotomine sandflies are known to transmit *Leishmania* parasites, these include *P. argentipes* on the Indian sub-continent, *P. martini* and *P. orientalis* in Africa and the Mediterranean basin, *P. chinensis* and *P. alexandri* in china. In the new world Lutzomyia logipalpis is the only known vector of *Leishmania donovani* Chagasi (WHO,1997).

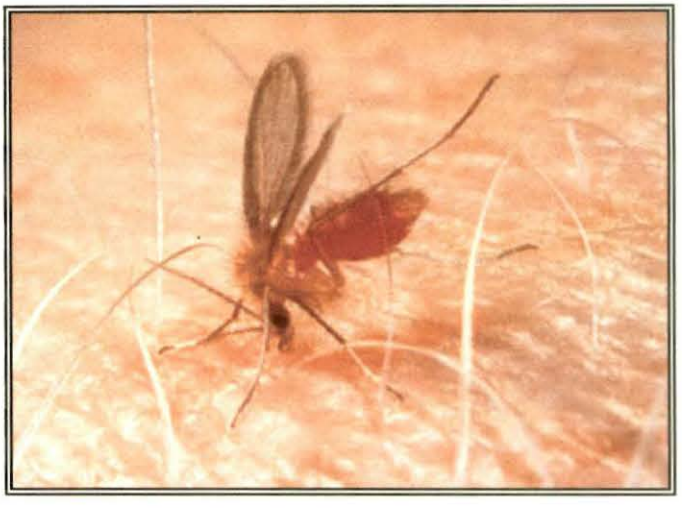


Fig. 2.8. Phebotomus sand fly

The manner in which the transmission takes place was for a long time a mystery. The first hint of an answer was found in 1904 by Leonard Rodgers. He put some spleen tissue from a patient into a flask with simple culture medium. The parasite appeared to multiply *in vitro* without much difficulty. In this culture medium the form of the parasite was, however, totally different. Instead of the spherical Leishman-Donovan bodies, such as were observed in man, elongated organisms (promastigotes) that had a flagellum were now seen. This implied that the Leishman-Donovan bodies that were found in man were but one of several stages in the life cycle of the parasite. The promastigote stage would thus occur somewhere in nature, outside of man [85]. The intestine of the sandfly consists of three major sections: an anterior intestine (cibarium, pharynx, oesophagus or gullet and oesophageal crop or gizzard), a middle intestine (stomach; the cranial part is called the cardia) and a posterior or terminal intestine (ileum, rectum). The transition from anterior

to middle intestine is formed by a small valve (stomodeum valve). The transition from middle intestine to terminal intestine is formed by the pylorus. The anterior and posterior intestines are coated with chitin. The middle intestine is not coated with chitin. *Leishmania* parasites that develop only in the stomach [the *Leishmania* (*Leishmania*) group] are sometimes known as the Suprapylaria. Those that develop on both sides of the pylorus are the Peripylaria [the *Leishmania* (Viannia) group] (WHO, 1997).

2.3.5. Life cycle of Leishmania species

Leishmania species have a dimorphic life cycle (Fig.2.9); one is the nonflagellated intracellular amastigotes living in the mononuclear phagocytic system of mammals. Other is extracellular flagellated promastigotes that live in the intestinal tract of insect vector such as sandfly i.e., female phlebotomus spp and in culture medium.

The intracellular amastigote form proliferates in the acid pH of secondary lysosomes of human macrophages [85, 88]. When *Leishmania* invades the sand fly vector, the promastigote form settles in the midgut and reproduces asexually. The extracellular promastigote stage is introduced into subcutaneous tissue in the human host during the bite of an infected sandfly vector. It is phagositosed by a mononuclear phagocyte after which it converts into the obligate intracellular amastigote form.

The amastigote form (non-flagellated form, a-without; mastix-whip), this unicellular parasite penetrates and residing inside the cells of reticuloendothelial system multiply by binary fission. Before division the parasites increases in size and becomes spherical. In the reticuloendothelial system is contained within a parasitophorus vacuole within a macrophage. There is a prominent nucleus and kinetoplast, and the vacuolated cytoplasm contains lysosomes. The outer membrane has a polysaccharide component but there is no surface coat. Once inside the host cell, *Leishmania* is able to protect itself from powerful host immunities by using several unique defense mechanisms, including its rapid cell division. *Leishmania* is a heterotophic organism whose prominent flagella may allow it to puncture host cells, allowing the promastigote to consume nutrients and obtain energy from the cell sap. The multiplication goes on continuously till the cells become packed with the parasites.

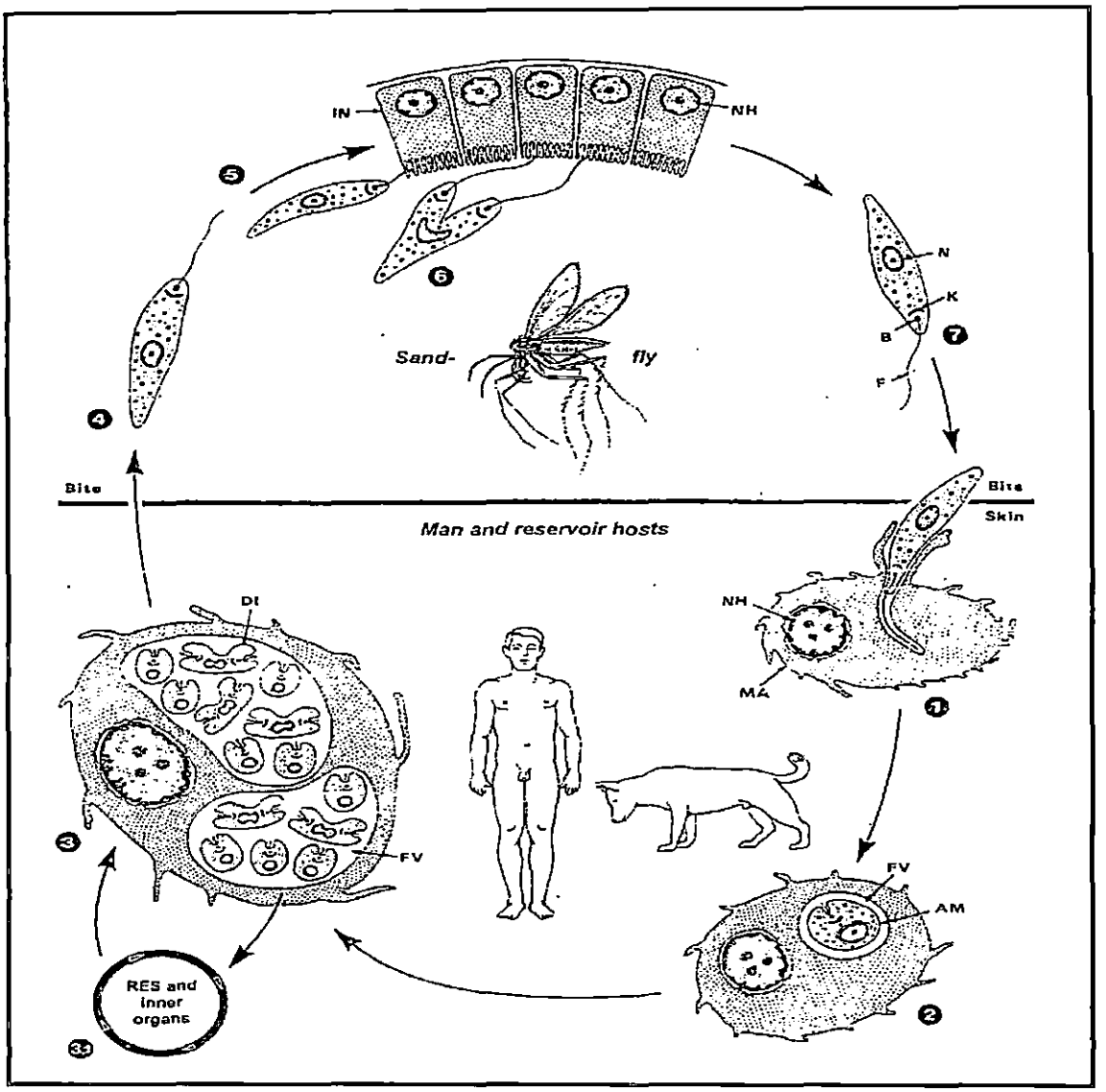


Fig. 2.9. Life cycle of *Leishmania* spp.

(1) After bite of the sandfly vector the injected promastigote state is engulfed by macrophages in the skin of the vertebrate host. (2). Transformation of promastigotes into amastigote states (2-4μm in diameter) requires 1-4hrs: reproduction proceeds as binary fission inside a parasitophorous vacuole, which later breaks down. (3). When macrophages are closely filled with amastigotes (after 48hrs), they finally burst and set free the parasites, which may enter other macrophages in the skin, leading to a cutaneous leishmaniasis. (3.1). Amastigotes of the L.donovani group are carried to inner organs and may enter various host cells, where they are reproduced by repeated binary fissions and lead to a visceral leishmaniasis within 4-6 months. (4-7). When a sandfly ingests amastigotes along with its blood meal, (5) the latter are transformed into slender promastigotes (10-20μm in length) in the midgut, where they multiply by repeated binary fission. (6). Quickly they block up the gut of the vector and move to the pharynx and buccal cavity, where they are injected to a new host with the fly's next bite. (7). All stages have a slight surface coat. AM-amastigote stage; B-basal body of flagellum; DI-dividing stage; F-free flagellum; FV-food vacuole; INB-intestinal cell; K-kinetoplast; MA-macrophages; N-nucleus; NH-nucleus of host cells.

Host cell is thereby enlarged and when it is unable to hold any further parasites, eventually ruptures. As many as 50 to 200 or even more may be found embedded in the cytoplasm of the enlarged host cell. The parasites are liberated into circulation and are again taken up by or invade fresh cells and the cycle is repeated again and again. In this way the entire reticulo-endothelial system becomes progressively infected. While in the blood stream, some of the free *Leishmania* cells are phagocytosed by the neutrophillic granulocytes and monocytes (macrophages). A blood seeking insect draws these free amastigote forms as well as those within the monocytes during the blood meal and these amastigotes forms converted into promastigote forms in midgut of certain species of sandfly. The promastigote forms again multiply by binary fission and an enormous number of flagellates appear. In the mid gut of the sandfly species the surface membrane has binding site molecules such as glycoproteins, and manose receptors have also been detected. These are important in the uptake of the promastigotes by macrophages in the host cell. Antibodies in the host serum bind to the promastigotes and facilitate uptake and entry into the macrophage. The macrophages have Fc receptors on their surface. The blood meal in the stomach is completely surrounded by a peritrophic membrane. The parasite transforms into a different form (promastigote with flagellum) in the insect and then multiplies. After 2-3 days the peritrophic membrane is digested and the parasites are released into the lumen of the stomach and intestine. They then attach to the microvilli of the intestine by means of their flagellae. They produce a chitinase, which damages the chitin coating of oesophageal-gastric junction, so that the valve between stomach and oesophagus no longer functions adequately and leaks, resulting in a backflow of parasites to the mouthparts. The parasites accumulate 7 to 10 days later in the insect's proboscis. Haemoglobin degradation products inhibit the secretion of chitinase and/or inhibit the enzyme itself making backflow of parasites to the mouthparts more difficult. Certain plant sugars do not have this effect. The insects also feed on plant juices. A balance between plant and animal feeding is required for successful transmission. The process of division is similar to that of amastigote forms but the flagellum does not split. The second flagellum is, however, produced from the daughter blepharoplast. Multiplication proceeds in the midgut of sandflies and the flagellates tend to spread forward to the anterior part of the alimentary canal. A heavy pharyngeal infection of the sandfly is usually observed between the sixth and ninth day of its infective blood meal. If this infected sandfly bite

any man, the promastigote will enter into the blood stream and the above cycle will be repeated and cause the potentially fatal disease leishmaniasis.

Mitotic cycle of *L. donovani* has been found to have a sequence of 15.2-hour resting phase, 1.1hour prophase, 3.9hour metaphase, 0.9hour anaphase and 1.8hour telophase and binucleate phase to make a total turnover of 24hour.

The receptor-mediated ingestion of promastigotes into the mononuclear phagocyte is accompanied by an oxidative burst of the phagocyte, during which oxidants such as superoxide and hydrogen peroxide are formed [88- 90]. Hydrogen peroxide can be converted to hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction in the presence of a source of iron: $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}$.

2.4. Biochemical status of the genus *Leishmania*

2.4.1. Cultural Requirements

Since the haemoflagellates need very complex media for their growth, a systematic evaluation of their growth requirements is a difficult problem. Only a few of the *Leishmania* species can be cultivated in a well defined media outside their hosts [91- 93]. The earliest observation regarding the requirement of blood in haemoflagellates was done by Novy and McNeal [94] who first used blood agar medium for their growth. Nicolle grew *L. donovani* and *L. tropica* in the same media [95]. Later workers confirmed that no *Leishmania* or *Trypanosoma* can grow in a completely haemoglobin free medium [96, 97]. Further, it was observed that the requirement of hemin in the haemoflagellates was due to their inability to synthesize it [98, 99]. Subsequent experiments proved that other components of blood, other than hemin, were also responsible for the growth of haemoflagellates [100]. Some vitamins were found to have growth promoting activity. Some strains of *Leishmania* and *Trypanosoma* require ascorbic acid as growth factor [101, 102]. *L. torentolae* requires choline for growth; but it could be omitted if methionine was present in the medium.

Flagellates essentially need some amino acids *L. torentolae* requires at least 10 amino acids for their growth [103]. However, the requirements of various amino acids in media vary in different class and species of protozoa. As far as *L. donovani* promastigote is concerned, Steiger and Black [93] had clearly shown that glucose could be completely

replaced by high concentration of L-proline. L-proline may be assumed to be the major energy substrate of *L. donovani*. This also confirmed the earlier biochemical and nutritional work by Krassner and Flory [104] who showed rapid catabolism of L–proline for the promastigote form. Studies by Steiger and co–workers [91-93] have further showed that *L. donovani* promastigotes have no specific and absolute requirement for any lipid material at lower growth temperatures and the organism probably possess de novo synthetic and disaturase pathways. This conclusion was consistent with the earlier works of Beach *et al.* on lipid metabolism of the organism [105].

Requirement for at least one Purine derivative hold good for most of the organisms [106]. The lower *trypanosomatids* inter–convert purine and their derivatives [107-109]. Uracil can alone supplement all their pyrimidine requirements in some flagellates and ciliates [110-114].

In protozoa, the need for additional nutrient at elevated environmental temperature had been observed. Krassner obtained stimulation at 28°C and also a good growth at 33°C for *L. torentolae* when the media was fortified with red blood cell-extract [98]. It was found that *T. cruzi* and *L. donovani* required chick embryo extract into the media for proper growth at higher environmental temperature [115].

2.4.2. Utilization of substrates

Chang [116] and Von Brand [117, 118] studied on the utilization of substrate for *L. donovani*. Chang working with four haemoflagellates of *L. donovani, L. braziliensis, L. tropica* and *L. cruzi* showed that, they could oxidize glucose and fructose; but not maltose and lactose. But Mukherjee showed that *L. donovani* promastigotes could effectively use mono and disaccharides [119]. They are glucose, fructose, mannose, maltose, glycerol, sucrose, ribose, erythritol, arabinose, galactose and erythrose [120]. Glucose only being metabolized when the culture reaches the stationary phase [104] and both proline and glutamate support the growth of promastigotes of *L. donovani*, but in some species the proline is more preferred substrate.

The breaking down of complex protinaceous substances such as peptone and gelatin by *L. tropica* is less pronounced when glucose is present. This suggests that the glucose is the preferred substrate [96].

2.4.3. Intermediary metabolism

Promastigotes of *L. donovani* possesses a full glycolytic chain [121]. They have many large mitochondria with plate like crisate, a functional TCA cycle and glyoxylate cycle [122, 123]. Chatterjee and Datta studied the formation of succinate from glucose via pathways that involve pyruvate [124]. Hexokinase, phosphofructokinase has been shown to present in *L. donovani* and *L. braziliensis*. Very less studies has been done regarding the status of the pentose phosphate pathway in this organism. Ryley showed that the cell free extract is unable to oxidize 6-phosphogluconate [125]. Ghosh had reported the formation of ketopentoses and sedoheptuloses during metabolism in case of *L. donovani* [126]. Mukherjee had showed the presence of large amount of glucose-6-phosphate dehydrogenase [119, 127].

Berens *et al.* have shown the presence of pentose phosphate shunt activity in *L. donovani* and *L. braziliensis* [128]. Cell fractionation experiments with blood stream from of *T. brucei* have shown that the enzymes of the glycolytic pathway are located in a microbody called glycosome [129]. Further kinetic work with U^{14}-C-D-glucose has revealed evidence for the existence of two pools of glycolytic intermediates or metabolites [130]. Apparently the glycerophosphate dependent oxygenase system is not located in this microbody.

Glycolytic chain is sensitive to iodoacetate, arsenite, fluoroacetate and malonate. Oxygen utilization is sensitive to cyanide, azide and antimycin A. Mukkada showed the presence of NADH dehydrogenase, succinate dehydrogenase, cytochrome b, cytochrome c_1, cytochrome c, cytochrome a, cytochrome a_3 and cytochrome O in electron transport chain of *L. donovani* promastigotes [131]. Employments of powerful biochemical techniques like carbon-13 NMR [132] and advanced enzymology [133] are revealing many complexities in glucose catabolism that were not expected earlier.

2.5. Epidemiology of Leishmaniasis

The quantitative epidemiology of leishmaniasis is still descriptive compared to malaria, which reached a fairly advanced level. The epidemiology of vector–borne disease is evolving towards quantitative epidemiology. The vast development of quantitative epidemiology requires the knowledge of all the parameters of the transmission cycle of the parasite, which may be incorporated into complex mathematical models. Study of the

mathematical model in parallel with field observations is one of the important ways of testing the adequacy of current epidemiological concepts and control strategies. The main reasons are a great heterogeneity of epidemiological or epizoological pattern and poor knowledge of many factors of the natural history of the parasites, vectors, and vertebrate hosts. From the point of view of quantitative epidemiology, clinico–epidemiological parameters may be compared to differentiate between the different leishmaniases.

2.5.1. Zoonotic form vs Anthroponotic form

In case of zoonotic form, the role of humans is usually negligible, and the force of infection does not depend on non-immunes in the community. Whereas, in case of anthroponotic the proportion of non-immunes is very important. Hence, the description of the accumulation of the infection in human beings may be simplified in zoonotic models, the movement of the pathogen from the reservoir to human beings independent from the amount of the leishmaniasis in population.

2.5.2. Acute form vs Chronic form

The infection is self–limiting and has short duration in many forms of leishmanaisis; but in others, it is chronic and may go on for years. In zoonotic cutaneous leishmaniasis of central Asia, the lesions heal in 3-4 months, but in few cases they persist upto 1 yr. The same *Leishmania* may behave differently in different hosts. For example, zoonotic cutaneous leishmanaisis is self-limiting in human beings, but chronic and life long in gerbils.

2.5.3. Visceral form vs Cutaneous form

The main difference between two forms is in the fatality rate (severity) of the disease, susceptibility and diagnosis. In visceral form, the severity or fatal rate is high and differential mortality is taken into picture in the model. The susceptibility of human varies greatly in visceral leishmaniasis. Not all the individuals exposed to Mediterranean form are susceptible [134, 135].

In case of cutaneous leishmanaisis all the individuals are susceptible. Each act of transmission result in overt disease and because each infective bite occurring over the weeks between the first bite and the development of a protective immunity produces a separate leishmanioma multiple acts of transmission in the same person may sometimes

be recognized. The diagnosis of visceral leishmaniasis is comparatively expensive than cutaneous leishmaniasis, in which it is easy, safe and more definite.

2.6. Transmission and Vectors

The probable evolutionary history of *Leishmania*, from a parasite of insects and eventually to one of mammals, implies that infected sandflies are primary hosts, which are known as vectors. Modes of transmissions are congenital or direct contact or inoculation. The parasite is mainly transmitted from infected to uninfected person through the bites of female sandfly. Rarely the parasite can transmit through placenta from mother to child, through sexual intercourse, as laboratory acquired and through blood transfusion. Leishmaniasis is transmitted by about 30 species of Phlebotomine sandflies, which is the commonest mode of transmission. It is presumed that skin lesions or peripheral parasitaemia act as reservoirs, from where the female sandfly takes up the infective form of the parasite (amastigotes) during the blood meals and transmits to new human host through another bite. Other than the insect route, transmissions through placental [136] semen [133] injection needles [137] and laboratory acquired [138] infections have also been reported, though rarely.

2.6.1. Sandfly Transmission

The activity of sandflies, which may enhance their role as vectors, is increased flight range under certain conditions [139]. Many vectors transmit leishmaniasis to people who make contact with them through agriculture, road-building, military monoeuvers, herding, charcoal burning and other activities [140, 141]. In other epidemic centers different species of sandfly are involved: Phlebotomus major in Eastern Mediterranean; *P. orientalis* and *P. clydei* in the Sudan; *P. perniciosus* in Western Mediterranean and North Africa; *P. arpaklensis* in Tajikistan and Transcaucasia; *P. chinese* and *P. sergenti* var. mongolicus in China; *P. longeroni* in Sudan; *P. garnhami* in Eastern Africa; *P. longipalpis* and *P. intermedius* in South America. Killick-Kendrick *et al.*, [142] showed that *Phlebotomus ariasi* is sufficiently mobile to spread the infection (leishmaniasis) to neighbouring areas within a radius of 1-2 km. The peri-domestic or domestic habits of species such as *P. papatasi, P. Sergenti* and *P. argentipes* and *Lutzomyia longipalpis* ensure close association with human being. The observation that there was a correlation between the distribution of *phlebotomus argentipes* in India and kala–azar it was found

that there was a rapid development of promastigote forms in *P. argentipes*, and in 1942 KA was successfully transmitted to human volunteers by the bite of *P. argentipes* [143].

Sandflies are the principal agents of transmission in nature. Sandflies are small hairy flies with long hairy legs (Fig. 2.8). The female sandfly feeds blood and transmits the infection. One or more blood meals are necessary to complete the maturation of each batch of eggs. The male sandfly does not suck blood but feeds on plant juices and does not take part in transmission. Sandflies are inactive in day light, seeking shelter in dark moist places and coming out at dusk. The normal flight (more of a hop) is usually less than a metre, but sandflies can cover more than a kilometer overnight. Female sandflies feed on a variety of both cold- and warm-blooded animals and do not specially feed on man, but a few species such as *P. argentipes* in India have become domestic and depend on man.

Breeding sites are dark damp places rich in organic matter and female flies are ready to lay eggs 3-10 days after a blood meal. The eggs are laid and larvae hatch which require high humidity to compete their development in less than three weeks; but species which live in colder climate may take up to three months. Flies emerge during the hours of darkness and mate, the female storing sufficient sperm to lay eggs at intervals throughout life, which in nature is probably rarely more than a few weeks. The life cycle from egg to adult varies from just under one to three months.

A sandfly is infective to a new host from 5 to 10 days after the infective blood meal and remains infected for the rest of its life. Infection of a new host occurs with the second blood meal after egg laying has taken place.

2.6.2. Blood Transfusion

Kala-azar is one of the protozoal diseases that can be transmitted by blood transfusion [144-146]. Amastigotes may occur in the peripheral blood in small numbers in the early stages of infection and in asymptomatic carriers who may be infective for a short period.

The *in vitro* studies have clearly shown that viscerotropic *Leishmania tropica* survived as intracellular parasites in monocytes for 30 days at 4oC and for at least five days at 24°C [147]. The first report of transfusion-transmitted kala-azar came from China in 1948 [144]. The blood was donated from infected mother to two daughters. One was four and

another six year old. Intramuscular injection of 20 ml of mother's blood was given to these daughters as a prophylaxis for measles prevention. After a few days the mother was admitted to a local hospital for paleness, fever and distension of abdomen and was diagnosed with kala-azar one month later. Both the daughters were closely observed and both developed kala-azar nine and ten month after receiving the transfusion, respectively [144]. Other reports of transfusion-transmitted kala-azar followed these two reports and have been reported from France, [148] Sweden, [149] Belgium, [150] United Kingdom, [151] India [152] and Brazil [153].

2.6.3. Congenital Transmission

The kala-azar may occasionally be a congenital infection has proved by Low, G.C, in 1925. He diagnosed this disease in a child seven months old, born in England of a mother who suffered severely from kala-azar during pregnancy [154-157].

2.6.4. Direct Contact

The amastigotes can be demonstrated in stools containing blood and mucus in a patient with dysentery; and in nasal mucosa and nasal discharges, direct transmission via these routes is possible [158, 159]. Direct transmission by the sexual route; sexual intercourse has also been described [133].

A case of accidental infection with *L. donovani* in a laboratory worker, whose fingers had been bitten on several occasions by experimentally infected animals, had been recorded by Terry *et al.,* [160].

2.7. Review of P-type ATPase

ATPases are a class of enzymes that catalyze the decomposition of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and a free phosphate ion. This dephosphorylation reaction releases energy, which is required by the enzyme to drive other chemical reactions. This process is widely used in all known forms of life.

Some such enzymes are integral membrane proteins (anchored within biological membranes), and move solutes across the membrane. (These are called transmembrane ATPases).

$$ATP \xrightarrow{\text{ATPase enzyme}} ADP + P_i$$

2.7.1. Classification of ATPase

Several families of ATPases, which can be distinguished by their ion transport mechanism, their structure (F-, V- and A-ATPases contain rotary motors) and their sensitivity towards specific inhibitors have been found in various membranes and cell compartments [161].

- F-ATPases (F1F0-ATPases) in mitochondria, chloroplasts and bacterial plasma membranes are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts).
- V-ATPases (V1V0-ATPases) are primarily found in eukaryotic vacuoles, catalysing ATP hydrolysis to transport solutes and lower pH in organelles.
- A-ATPases (A1A0-ATPases) are found in Archaea and function like F-ATPases.
- P-ATPases (E1E2-ATPases) are found in bacteria and in eukaryotic plasma membranes and organelles, and function to transport a variety of different ions across membranes.
- E-ATPases are cell-surface enzymes that hydrolyse a range of NTPs, including extracellular ATP.

P-ATPases are found in bacteria and in a number of eukaryotic plasma membranes and organelles. P-ATPases function to transport a variety of different compounds, including ions and phospholipids, across a membrane using ATP hydrolysis for energy. There are many different classes of P-ATPases, each of which transports a specific type of ion: H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ag^+ and Ag^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Cu^+ and Cu^{2+}. P-ATPases can be composed of one or two polypeptides, and can usually assume two main conformations called E1 and E2, so called E1E2-ATPases.

P-type (or E1-E2-type) ATPases constitute a superfamily of cation transport enzymes, present both in prokaryota and eukaryota, whose members mediate membrane flux of all common biologically relevant cations [162]. The ATPases, can be divided into following major groups:

- Ca^{2+}-transporting ATPases
- Na^+/K^+-transporting ATPases

- H^+/K^+-transporting ATPases
- Mg^{2+}-transporting ATPases

2.7.1.1. Plasma membrane Ca^{2+} ATPase

Calcium ATPase is a form of P-ATPase, which transfers calcium after a muscle has contracted. The calcium ATPase is of two types:

- Plasma membrane Ca^{2+} ATPase (PMCA)
- Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA)

PMCAs were first discovered in the 1960s in the membranes of red blood cells [163]. The presence of an ATPase was discovered in the membranes in 1961, and then in 1966 it was discovered that these ATPases pump Ca^{2+} out of the cytosol [164].

The plasma membrane Ca^{2+} ATPase (PMCA) is a transport protein in the plasma membrane of cells that serves to remove calcium (Ca^{2+}) from the cell. It is vital for regulating the amount of Ca^{2+} within cells [165]. In fact, the PMCA is involved in removing Ca^{2+} from all eukaryotic cells. There is a very large transmembrane electrochemical gradient of Ca^{2+} driving the entry of the ion into cells, yet it is very important for cells to maintain low concentrations of Ca^{2+} for proper cell signalling; thus it is necessary for the cell to employ ion pumps to remove the Ca^{2+}. Since it transports Ca^{2+} into the extracellular space, the PMCA is also an important regulator of the calcium concentration in the extracellular space [166]. The PMCA is expressed in a variety of tissues, including the brain [167].

- **Actions**

The pump is powered by the hydrolysis of adenosine triphosphate (ATP), with a stoichiometry of two Ca^{2+} ions removed for each molecule of ATP hydrolysed. It binds tightly to Ca^{2+} ions (has a high affinity, with a K_m of 100 to 200 nM) but does not remove Ca^{2+} at a very fast rate [168]. Thus the PMCA is effective at binding Ca^{2+} even when its concentrations within the cell are very low, so it is suited for maintaining Ca^{2+} at its normally very low levels. Calcium is an important second messenger, so its levels must be kept low in cells to keep signalling accurate [169]. In brain tissue, it has been postulated that certain types of PMCA are important for regulating synaptic activity,

since the PMCA is involved in regulating the amount of calcium within the cell at the synapse, and Ca^{2+} is involved in release of synaptic vesicles.

- **Isoforms**

There are four isoforms of PMCA [167]

1) *ATP2B1*-PMCA1, 2) *ATP2B2*-PMCA2, 3) *ATP2B3*-PMCA3, 4) *ATP2B4*-PMCA4

Each isoform is coded by a different gene and is expressed in different areas of the body. Three PMCA isoforms, PMCA1, PMCA2, and PMCA3, occur in the brain in varying distributions. PMCA1 is ubiquitous throughout all tissues in humans. PMCA4, which is also very common in many tissues, is survivable, but leads to infertility in males. PMCA2 causes inner ear problems, including hearing loss and problems with balance, PMCA4 exists in caveolae. Isoform PMCA4b interacts with nitric oxide synthase and reduces synthesis of nitric oxide by that enzyme [170].

SERCA resides in the sarcoplasmic reticulum (SR) within muscle cells. It is a Ca^{2+} ATPase, which transfers Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation.

2.7.1.2. Na^+/K^+-ATPase system

Jens Christian Skou discovered Na+/K+-ATPase in 1957 while working as assistant professor at the Department of Physiology, University of Aarhus, Denmark. He published his work in 1957. In 1997, he received one-half of the Nobel Prize in Chemistry "for the first discovery of an ion-transporting enzyme, Na^+, K^+ -ATPase [171]. Na^+/K^+-ATPase (also known as the Na^+/K^+ pump, sodium-potassium pump, or simply NAKA, is an enzyme located in the plasma membrane (specifically an electrogenic transmembrane ATPase). It is found in the plasma membrane of virtually every human cell and is common to all cellular life.

The Na^+/K^+-ATPase helps maintain resting potential, avail transport and regulate cellular volume.

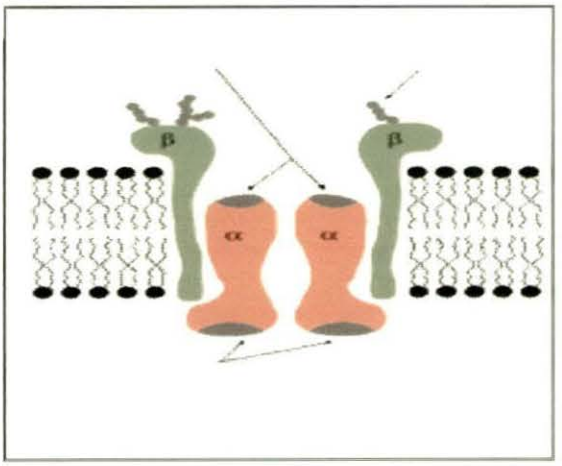


Fig. 2.10. Alpha- Beta subunits

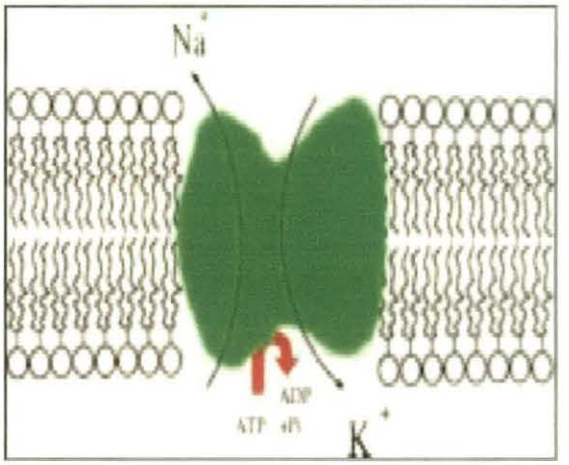


Fig. 2.11. Flow of ions

- **Resting potential**

In order to maintain the cell potential, cells must keep a low concentration of sodium ions and high levels of potassium ions within the cell (intracellular). Outside cells (extracellular), there are high concentrations of sodium and low concentrations of potassium, so diffusion occurs through ion channels in the plasma membrane. In order to keep the appropriate concentrations, the sodium-potassium pump pumps sodium out and potassium in through active transport. As the plasma membrane is far less permeable to sodium than it is to potassium ions, an electric potential (negative intracellularly) is the eventual result.

- **Transport**

Export of sodium from the cell provides the driving force for several facilitated membrane transport proteins, which import glucose, amino acids and other nutrients into the cell. Translocation of sodium from one side of an epithelium to the other side creates an osmotic gradient that drives the absorption of water.

Another important task of the Na^+-K^+ pump is to provide a Na^+ gradient that is used by certain carrier processes. In the gut, for example, sodium is transported out of the resorbing cell on the blood side via the Na^+-K^+ pump, whereas, on the resorbing side, the Na^+-Glucose symporter uses the created Na^+ gradient as a source of energy to import both Na^+ and Glucose, which is far more efficient than simple diffusion. Similar processes are located in the renal tubular system.

- **Mechanism**

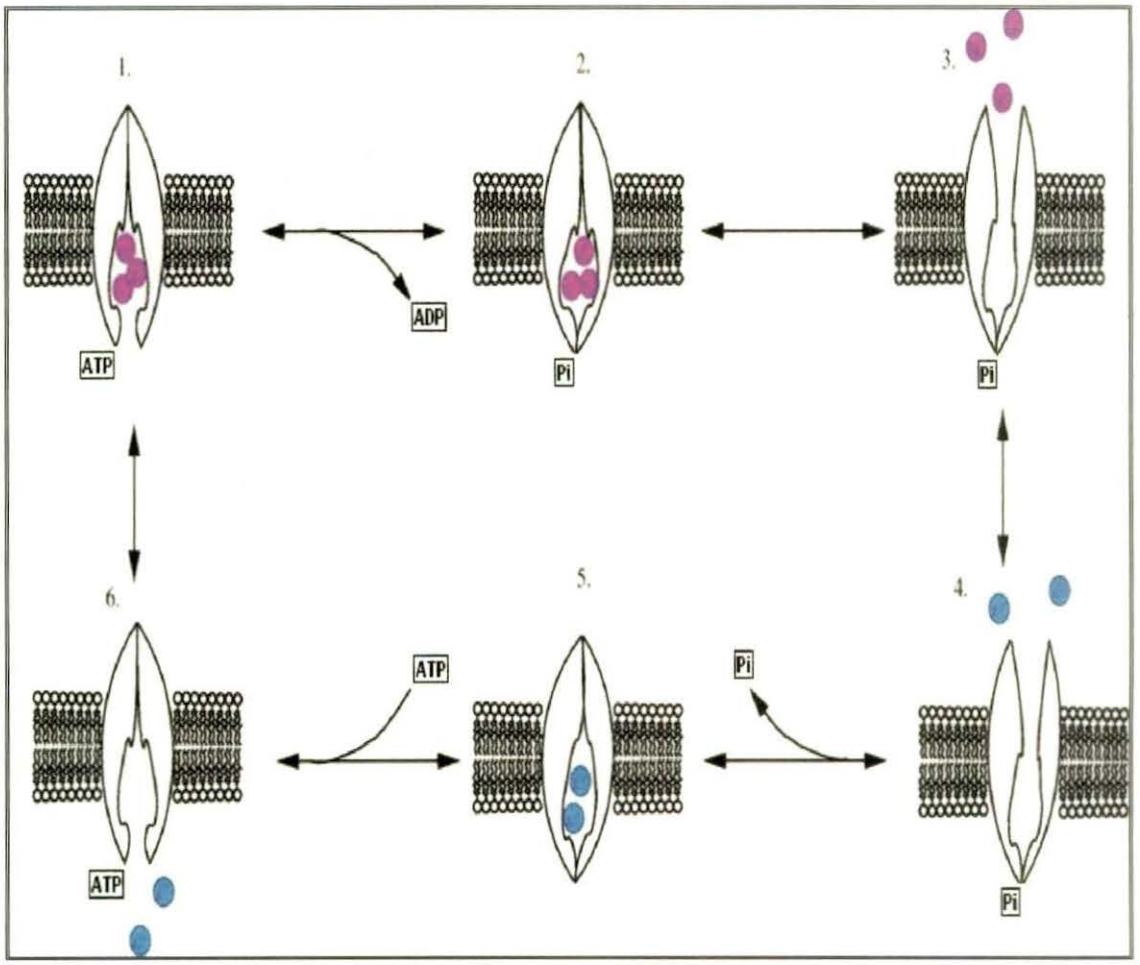


Fig. 2.12. Mechanism of Na^+/K^+ transport in ATPase system

- The pump, with bound ATP, binds 3 intracellular Na^+ ions.
- ATP is hydrolyzed, leading to phosphorylation of the pump at a highly conserved aspartate residue and subsequent release of ADP.
- A conformational change in the pump exposes the Na^+ ions to the outside. The phosphorylated form of the pump has a low affinity for Na^+ ions, so they are released.
- The pump binds 2 extracellular K^+ ions. This causes the dephosphorylation of the pump, reverting it to its previous conformational state, transporting the K^+ ions into the cell.
- The unphosphorylated form of the pump has a higher affinity for Na^+ ions than K^+ ions, so the two bound K^+ ions are released. ATP binds and the process start again.

- **Genes**
  - Alpha: *ATP1A1, ATP1A2, ATP1A3, ATP1A4.*
  - Beta: *ATP1B1, ATP1B2, ATP1B3, ATP1B4.*

2.7.1.3. Hydrogen potassium ATPase

Gastric hydrogen potassium ATPase is also known as H^+/K^+ ATPase

- **Function and location**

The gastric hydrogen potassium ATPase or H^+/K^+ ATPase is the proton pump of the stomach and as such is the enzyme primarily responsible for the acidification of the stomach contents. The H^+/K^+ ATPase is found in parietal cells, which are highly specialised, epithelial cells located in the inner cell lining of the stomach, which is called the gastric mucosa. Parietal cells possess an extensive secretory membrane system and the H^+/K^+ ATPase is the major protein constituent of these membranes.

- **Genes and Protein structure**

The H^+/K^+ ATPase is a heterodimeric protein, the product of 2 genes. The gene *ATP4A* encodes the H^+/K^+ ATPase α subunit contains and is a 1000 amino acid protein that contains the catalytic sites of the enzyme and forms the pore through the cell membrane that allows the transport of ions. The gene *ATP4B* encodes the β subunit of the H^+/K^+ ATPase, which is an 300 amino acid protein with a 36 amino acid N-terminal cytoplasmic domain, a single transmembrane domain, and a highly glycosylated extracellular domain. The H^+/K^+ ATPase β subunit stabilizes the H^+/K^+ ATPase α subunit and is required for function of the enzyme. It also appears to contain signals that direct the heterodimer to membrane destinations within the cell, although some of these signals are subordinate to signals found in H^+/K^+ ATPase α subunit.

- **Enzyme activity of the H^+/K^+ ATPase**

The H+/K+ ATPase is a member of the P-type ATPase superfamily, a large family of related proteins that transport ions, most usually cations, across biological membranes in nearly all species. The H^+/K^+ ATPase transports one hydrogen ion (H^+) from the cytoplasm of the parietal cell in exchange for one potassium ion (K^+) retrieved from the gastric lumen. As an ion pump the H^+/K^+ ATPase is able to transport ions against a concentration gradient using energy derived from the hydrolysis of ATP. Like all P-type

ATPases a phosphate group is transferred form ATP to the H^+/K^+ ATPase during the transport cycle. This phosphate transfer powers a conformational change in the enzyme that helps drive ion transport.

2.7.1.4. Magnesium-ATPase

Magnesium ATPase (Mg-ATPase) is an ATPase that pumps magnesium. It is found e.g. in erythrocytes. The antihypertensive medication guanethidine works by inhibiting it. It is encoded by the gene *ATP3*.

3.3. Review of Pyrophosphatase

Pyrophosphatase are acid anhydride hydrolases that act upon diphosphate bonds.

Examples include:

- Inorganic pyrophosphatase
- Thiamine pyrophosphatase

3.3.1. Inorganic pyrophosphatase

Inorganic pyrophosphatase is an enzyme that converts one molecule of pyrophosphate to two phosphate ions. This highly exergonic reaction (about -34KJ change in free energy) can be coupled to unfavorable biochemical transformations in order to drive these transformations to completion, as in Lipid synthesis and other biochemical transformations.

In chemistry, the anion, the salts, and the esters of pyrophosphoric acid are called pyrophosphates. The anion $P_2O_7^{4-}$ is abbreviated PP_i and is formed by the hydrolysis of ATP into AMP in cells. $ATP \rightarrow AMP + PP_i$.

The pyrophosphate anion has the structure $P_2O_7^{4-}$, and is an acid anhydride of phosphate. It is unstable in aqueous solution and rapidly hydrolyzes into inorganic phosphate:

$P_2O_7^{4-} + H_2O \rightarrow 2\ HPO_4^{2-}$ or in shorthand notation: $PP_i + H_2O \rightarrow 2\ P_i$.

This hydrolysis to inorganic phosphate effectively renders the cleavage of ATP to AMP and PP_i irreversible, and biochemical reactions coupled to this hydrolysis are irreversible.

From the standpoint of high energy phosphate accounting, the hydrolysis of ATP to AMP and PP_i will require two high energy phosphates, as to reconstitute AMP into ATP will require two phosphorylation reactions.

- $AMP + ATP \rightarrow 2\ ADP$
- $2ADP + 2P_i \rightarrow 2\ ATP$

Philip de Clermount first described the synthesis of tetraethyl pyrophosphate in 1854 at a meeting of the French Academy of Sciences.

The term pyrophosphate is also the name of esters formed by the condensation of a phosphorylated biological compound with inorganic phosphate as for dimethylallyl pyrophosphate. This bond is also referred to as a high-energy phosphate bond.

3.3.2. Thiamine pyrophosphatase

Thiamine pyrophosphatase is an enzyme, which cleaves thiamine pyrophosphate. Thiamine pyrophosphate (TPP) or thiamine diphosphate (ThDP) is a thiamine derivative, which is cleaved by thiamine pyrophosphatase. Thiamine phyrophosphate is the active form of thiamine (vitamin B1).

ThDP is a prosthetic group in many enzymes, such as: Pyruvate dehydrogenase complex, alpha-ketoglutarate dehydrogenase complex, branched-chain amino acid dehydrogenase complex, 2-hydroxyphytanoyl-CoA lyase and transketolase.

Chapter- 3

Review of TransPMET System

3. REVIEW ON TRANSPLASMA MEMBRANE ELECTRON TRANSPORT SYSTEM [transPMET system]

3.1. Introduction

There is a precedent for electron transport systems in eukaryotic plasma membranes. Transmembrane electron transport is clearly recognized and defined as to components and function in bacteria. Other prokaryotes (e.g., blue green algae) have plasma membrane electron transport, although not as well defined. For bacteria the importance is clear, since the plasma membrane has all of the energy coupling machinery for oxidative ATP synthesis. The situation is not as clear in prokaryotic algae, since they have thylakoid membranes, which have ATP synthesizing machinery [172]. In most eukaryotic cells the mitochondria handle the major high-efficiency ATP synthesis, so there is no need for an ATP-synthesizing system associated with plasma-membrane electron transport. If ATP can supply energy for all plasma membrane transport functions, and if transport is the only energy requiring function of plasma membranes, then energy–coupled redox systems would be redundant in the outer membrane. There have been proposals for direct coupling of electron transport to ion or nutrient transport activity, but these have not developed clearly [173]. The question is: do eukaryotic plasma membranes have electron transport systems coupled to proton transport or ion movement and, if not, is the electron transport related to an energy-coupling process or does it serve another function? Clearly, the presence of masses of thiol groups on receptors requires electron transfer across the membrane to maintain the thiol state [174].

Other types of redox function are found in plasma membranes for special roles. The peroxide-generating NADPH oxidase in neutrophils used for killing bacteria [175], the xanthine oxidase that may have similar functions in addition to purine rescue [176], and the cytochrome b_5–dependent fatty acyl CoA desaturase [177] are examples. Proton transfer across the plasma membrane by activation of a channel has been associated with the neutrophil transmembrane NADPH oxidase [178]. This enzyme may serve as a model for other eukaryotic plasma membrane redox systems associated with proton release. The NADH-cytochrome b_5 reductase, fatty acid desaturase, or methemoglobin reductase are

exclusively on the cytoplasmic side of the plasma membrane, so these enzymes have not been associated with any proton transport [179].

3.1.1. The evidence for trans PMET

Evidence for transplasma membrane electron transport is found in all animal cells examined, ranging from protozoa to man [180-183]. It is detected by reduction of impermeable dyes or complex ions by intact cells and by histochemistry [184, 185]. Electron transport reactions based on oxidation of NADPH by oxygen or the same impermeable compounds can be detected in isolated plasma membranes in nonvesicular form [186]. If the isolated membranes form sealed vesicles, then either the impermeable reductant or impermeable oxidant will not be available at its reaction site or the oxidoreductions observed would represent internal or external dehydrogenases, which may or may not be connected to the transmembrane enzyme. Insertion of substrate into vesicles followed by resealing has only been successful with electroporation of NADH into right side–out plant plasma membrane vesicles [187]. The NADH in these vesicles can then be oxidized by external ferricyanide. Ascorbate has been inserted successfully by other methods [188, 189]. Oxygen is permeable to membranes, so a transmembrane NADH oxidase can be measured if the plasma membrane vesicles are inside–out with the NADH dehydrogenase on the exposed face. On the other hand, impermeable hormones or other ligands for external surface sites will not be able to influence the oxidase reaction in the inside–out sealed vesicles. Fortunately, isolated liver plasma membranes do not vesiculate easily because of desmosomes [190]. If liver plasma membranes are not homogenized too vigorously, they show NADH dehydrogenase activity, which is consistent with the transmembrane electron transport. Erythrocyte membranes must be prepared in the open ghost form to show transmembrane electron transport [191].

Plasma membranes also have sites for NADH oxidation on their external surface [192]. NADH oxidase or ferricyanide reductase observed with right side–out vesicles will measure this outer surface activity, so it cannot represent the transmembrane activity seen with whole cells. It could, however, represent an external feed to a part of the transmembrane electron transport chain analogous to the external NADH site on mitochondrial cristae in plants [193]. Since substrate–loaded vesicles of proper

orientation have not been obtained with animal plasma membranes; studies on proton transport accompanying the transmembrane electron transport have been restricted to intact cells. With intact cells the shift in the redox state of NADH/NAD^+ when an external oxidant is reduced is consistent with the idea of a transmembrane electron transport [194]. It does not necessarily mean that NADH is the primary substrate since the NADH may reduce the primary substrate. For example, NADH may reduce semi dehydroascorbate to ascorbate, and ascorbate may be the primary electron donor to the transmembrane electron transport [195].

3.1.2. Properties of animal cell transmembrane electron transport

With intact cells or perfused tissue, the rate of transplasma membrane electron transport measured by ferricyanide reduction can be quite rapid [196, 197]. For example, rat liver cells have ferricyanide reduction rates as high as 260 nmol per minute per g fresh weight of cells. If 4% of the liver cell protein is in the plasma membrane, then the rate of electron transport through the membrane would approach 100 nmol per minute per mg membrane protein. Rates of NADH–ferricyanide reductase up to 1000 nmol per minute per mg protein have been reported for erythrocyte membranes, and 780 nmol per min per mg for rat liver plasma membranes, but part of this activity will come from internal or external enzymes.

Of course, ferricyanide is not the natural electron acceptor, so these rates only give maximum electron transport capacities of the transplasma membrane enzyme.

Isolated rat liver plasma membranes have a unique NADH oxidase, which is not sensitive to cyanide and is stimulated by azide, transferrin, and hormones [198-201]. In isolated membranes the activity is up to 20 nmol per min per mg protein, unless diferric transferrin or hormones stimulate it. The measurement of the plasma membrane oxidase in cells is difficult because of the multifarious nature of cellular oxygen uptake. Since it is cyanide-insensitive enzyme, which may transfer electrons to impermeable external electron acceptors, transmembrane oxidase can be identified by measuring the effect of ferricyanide on cyanide–resistant oxygen uptake. The inhibition is postulated on the basis of ferricyanide–accepting electrons before the site where oxygen accepts electrons. In an experiment with well–oxygenated liver cells, it was found that 20% of the respiration was

insensitive to 1mM KCN and one–half of that oxygen uptake was inhibited by 0.2mM ferricyanide. In the absence of cyanide, 0.1 mM ferricyanide inhibits oxygen uptake by rat liver cells 10%. The overall indication is that with well–aerated liver cells the transplasma membrane oxidase activity can be 10% of the total respiration [202]. Studies on the effect of diferric transferrin and growth factors on cyanide–resistant, ferricyanide–inhibited respiration of cells have not been done. There are many reports in the literature of complete inhibition of oxygen uptake by cyanide, which actually should be unexpected, since internal endosomal cyanide–resistant oxidase (P_{450}) are known in addition to the plasma membrane enzyme. If the plasma membrane enzyme is involved in specialized functions or growth control, then it may be undetectable, unless diferric transferrin and growth factors are present. Transmembrane NADH–ferricyanide reductase activity is not necessarily connected to the oxidase activity, since open erythrocyte ghosts have no NADH oxidase activity, despite the high level of NADH–ferricyanide reductase [199, 200]. Mammalian erythrocytes also have no transferrin receptors and differric transferrin does not activate NADH oxidase in these membranes [203]. However, if transferrin receptors are inserted into the erythrocyte membranes by *Falciparum* infection, then the cells show a transmembrane diferric transferrin reductase activity [204]. Whether this is coordinated to induction of transmembrane oxidase and proton release remains to be seen.

The stimulation of the transmembrane oxidase by diferric transferrin brings up the question of how the transferrin acts. Does it act as a terminal oxidase by catalyzing the reoxidation of ferrous iron with oxygen as soon as the iron is reduced by the transmembrane electron transport [205], or does binding of the diferric transferrin to the transferrin receptor activate the oxidase in the membrane by a conformational change in the redox system? In support of the terminal oxidase hypothesis, we have demonstrated that the transmembrane electron transport system can act as a ferric transferrin reductase [206, 207]. Reduction of iron in diferric transferrin by cells can be demonstrated by direct spectrophotometric measurement of decrease in the absorbance of diferric transferrin at 465 nm under anaerobic conditions (unpublished), or by formation of ferrous bathophenanthroline disulfonate (BPS) in the media, when cells are incubated with diferric transferrin. BPS is an impermeable ferrous chelator. Reduction of the transferrin

iron at the membrane can also be measured with formation of ferrous dipyridyl trapped in the membrane [208]. The requirement for the transferrin receptor in these reactions with HeLa cells is indicated by inhibition with B3/25 and GB16 monoclonal antibodies to the transferrin receptor [207].

NADH diferric transferrin reductase activity can also be demonstrated using isolated liver membranes [186]. Three types of assay can be used to measure the activity.

(1) Direct measurement of a decrease of the diferric transferrin at 465nm absorbance in the presence of NADH and membranes under anaerobic conditions. The absence of oxygen is essential in this assay because the ferrous iron formed is immediately reoxidized by oxygen to re-form diferric transferrin.

(2) Oxidation of NADH by membranes is greatly increased when diferric transferrin is added [186, 201]. This reaction can also be interpreted as a stimulation of an NADH-oxygen: oxidoreductase by diferric transferrin binding to the membrane. If the assay is for an NADH transferrin reductase, then it should work under anaerobic conditions, which has not been tested. The requirement for the transferrin receptor in this reaction is likely, since the reaction does not occur in erythrocyte membrane [204].

(3) Ferrous BPS is formed when diferric transferin is added to liver plasma membranes with NADH.

This type of assay has been criticized by Thorstensen and Aisen (1990) on the basis that the BPS effectivity raises the redox potential of the ferric transferrin to the point that it can be reduced by the transplasma membrane electron transport. Since diferric transferrin in simple solution at pH 7.0 has a redox potential at –500 mV and NADH has a potential at – 320 mV, it is quite clear that they are correct that NADH cannot reduce diferric transferrin in simple solution. However, the presence of plasma membrane, transferrin receptor, and a complex transmembrane electron transport system introduces factors, which do not allow a simple theoretical analysis of the possibility for reduction of external diferric transferrin by cytosolic NADH. Both the surface of cells and isolated membranes has a negative ξ potential, which can modify surface pH. Transferrin iron is released at pH below 7.0 and reduced by ascorbate (+56 mV). The redox potential of diferric transferrin bound to the transferrin receptor is unknown, and it may be much

higher than free transferrin if binding changes the conformation of the transferrin. If a transferrin receptor site is not important, then reduction of ferric desferrioximine would also be expected (–430mV) in the presence of BPS. This reduction is not seen with HeLa cells [202]. Finally, the reduction of NAD^+ by succinate in mitochondria would be impossible, except for the fact that the cristae membrane can carry out reversed electron transport energized by the proton gradient created by the electron–transport system. The plasma membrane may have an energy–linked reverse electron transport.

Actually, on thermodynamic grounds diferric transferrin reduction at the plasma membrane is even less likely than Thorstensen and Aisen (1990) propose because the redox potential of the electron carrier on the outer surface of the plasma membrane has been titrated at – 160 mV [209], which means that reduction of diferric transferrin at that site is less energetically favoured than with NADH directly. The study of reduction of diferric transferrin by cells or membranes in the presence of BPS obviously will not answer the question whether diferric transferrin can be a natural acceptor for the plasma membrane electron transport. The fact that ferrous BPS formation occurs even in the Thorstensen and Aisen experiments is evidence for a transmembrane electron transport system, at least to high redox potential acceptors. Diferric transferrin in the presence of BPS can act as a high redox potential acceptor for the transmembrane electron transport. It should be noted that Thorstensen and Aisen [201] do confirm diferric transferrin stimulation of the plasma membrane NADH oxidase. In their studies they do not consider the direct measurement of diferric transferrin reduction by decline in absorbance at 465nm under anaerobic conditions [186]. The ESR evidence, which they present as direct assay for reduction, is by no means conclusive, since it is done in the presence of 1 mM BPS, which at this concentration acts as an inhibitor of the transmembrane [200]. A more decisive answer could have been obtained if the experiment had been done under anaerobic conditions in the absence of BPS so that the loss of the transferrin iron signal could have been observed directly.

If not a redox carrier, then the diferric transferrin can act by binding to the transferrin receptor to activate the oxidase. The binding site at which diferric transferrin stimulates the NADH oxidase in the isolated plasma membrane appears to have much lower affinity

for diferric transferrin than does the high–affinity binding site involved in iron uptake by endocytosis. Iron uptake is saturated at 1 μM diferric transferrin, whereas the stimulation of NADH oxidase by diferric transferrin is saturated at 40μM [210]. This low–affinity site is also involved in diferric transferrin reduction by cells and is probably the site involved in the "nonsaturable" iron uptake by liver [211]. In other words, NADH oxidase stimulation and diferric transferrin reduction require 40μM diferric transferrin to each saturation, which suggests that each of these activities occurs at the same site on the membrane. The inhibition of diferric transferrin reduction by intact HeLa cells with B3/25 and GB16 monoclonal antibodies but not by GB18 or 42/6 further indicates binding and reduction at a site different from the high-affinity binding site. GB18 and 42/6 bind an epitope at the high–affinity site on the transferrin receptor but B3/25 and GB16 bind elsewhere on the receptor. Cooperative effects of B3/25 and 42/6 on cell proliferation have been described [212].

In conclusion, the relationship between the plasma membrane NADH oxidase and diferric transferrin appears to involve a direct stimulation of the NADH oxidase when transferrin binds to a low–affinity site on the transferrin receptor, as well as slow reduction of iron in the diferric transferrin. The slow reduction at the low-affinity site may add to the total oxidase activity by recycling the ferric–ferrous iron by oxidation on the transferrin after the ferric iron is reduced by transmembrane electron transport.

3.1.3. Components of the trans PMET

Plasma membranes have been reported to contain flavin, cytochromes of the b type, nonheme iron, coenzyme Q, α-tocopherol, thiol groups, and possibly copper [200, 213-215]. Coenzyme Q is the only component for which there is good evidence for participation in transmembrane electron transport.

3.1.4. Evidence for coenzyme Q function

Reduction of ferricyanide and diferric transferrin is inhibited by analogs of coenzyme Q, and the inhibition is reversed by addition of coenzyme Q [216, 217]. Piericidin A is the most effective inhibitor among the coenzyme Q analogs. 2,3- dimethoxy-5-chloro-6-naphthyl-mercaptobenzoquinone and 2-methoxy-3-ethoxy-5-methyl-6-hexa-decyl merca-

ptobenzoquinone are also good inhibitors of diferric transferrin reduction by cells [216]. The NADH-ferricyanide reductase and NADH oxidase activity of rat liver plasma membranes are inhibited by the same concentrations of the above inhibitors and addition of coenzyme Q_{10} partially restores the activity.

Extraction of coenzyme Q from lyophilized plasma membranes with heptane partially inhibits NADH–ferricyanide reductase activity. Activity is restored by addition of coenzyme Q in heptane membranes, followed by evaporation of the heptane by the Norling *et al.* (1974) [218] procedure. Loss of activity is proportional to the amount of coenzyme Q removed [219].

A precedent for coenzyme Q function is transmembrane electron transport is seen in mitochondria [220, 221]. A similar function as electron and proton carrier in the lipid phase may be possible in plasma membranes. It should be emphasized that the coenzyme Q appears to function before the site of external ferricyanide reduction by plasma membrane, whereas in mitochondria it functions after the site of ferricyanide reduction by NADH dehydrogenase. For example, piericidin A inhibits ferricyanide reduction in the plasma membrane, whereas it does not inhibit ferricyanide reduction by mitochondrial cristae [222]. Antimycin A and rotenone do not inhibit electron transport in plasma membranes [223, 224]. Since they act at coenzyme Q binding sites in mitochondria, the coenzyme Q binding sites in the plasma membrane must differ from those in mitochondria [225].

3.1.5. Inhibitors of trans PMET

Inhibitors at specific sites in electron transport systems are useful in defining the sequence of the system or to see if the system contributes to a cellular function. The transmembrane electron transport from cells or the NADH dehydrogenase activity of plasma membranes has been found to respond to some unique inhibitors. For ferricyanide or diferric transferrin reduction by cells atebrin and chloroquine are effective at high concentrations [226, 227], whereas adriamycin, *cis*-dichlorodiamine platinum II, actinomycin D, and bleomycin inhibit at low concentrations [228]. These same compounds are good inhibitors of NADH–ferricyanide reductase or NADH diferric transferrin with isolated plasma membranes. Atebrin and chloroquine are effective

against malaria and the other compounds are used as antitumor agents, so the inhibitions may point to a vital role of the redox system in cancer and infections by protozoa.

The important antitumor drugs, which are strong inhibitors of transplasmalemma electron transport, include adriamycin and related anthracyclines-bleomycin, *cis*-diaminodichloroplatinum II (cis-platin), actinomycin D, anthramycin, and retinoic acid [217, 228]. Electron transport by transformed cells or tumor cells is more sensitive to these compounds than with normal cells and inhibition occurs at concentrations, which inhibit cell growth [229]. Except for retinoic acid [230], proton release associated with the redox activity is also inhibited at the same concentrations starting at 10^{-7} M [228]. Adriamycin coupled to transferrin with glutaraldehyde is more effective than adriamycin alone in inhibition of transmembrane electron transport and redox–induced proton release. Good inhibition is seen with HeLa cells at 10^{-8} M adriamycin equivalent. Since the effect is seen in 3 minutes, the effectiveness of the conjugate suggests that the adriamycin acts at the plasma membrane and the redox enzyme is close to the transferrin receptor [231-233]. The time is too short for the conjugate to release adriamycin to the nucleus.

3.1.6. Proton release associated with trans PMET: Animal cells

Transplasma membrane electron transport is associated with proton release from cells, as measured by a change in the external pH [234, 235]. Reduction of both ferricyanide and diferric transferrin is accompanied by proton release. The ratio of proton release to electron transfer is much lower for ferricyanide than for diferric transferrin [202]. Since ferrocyanide and apotransferrin do not stimulate proton release, an electron acceptor is necessary. Inhibitors of transplasma membrane electron transport, such as adriamycin [203], bleomycin [236], cis-platin, and piericidin A, as well as monoclonal antibodies to the transferrin receptor [237], inhibit the proton release at the same concentrations which inhibit electron transport; the redox system appears responsible for activation or driving the proton movement.

There are several known mechanisms by which proton transfer across the membrane could be coupled to the transplasma membrane electron transport. It could be based on:

(a) anisotropic arrangement of protonated and nonprotonated electron carriers as proposed by Mitchell (1983) [175] for mitochondria.

(b) the electron transport protein could act as a redox-controlled proton channel as proposed by Wikstrom [238], Wikstrom and Krab [239], and Wikstrom *et al.* [240], for cytochrome oxidase.

(c) the Q cycle with oxidation and reduction of coenzyme Q on opposite sides of the membrane might apply to plasma membrane, since coenzyme Q is present in the membrane [215].

As an alternative, the redox-generated proton release could be based on activation of a channel or pump, such as the Na^+/H^+ antiport or a proton-pumping ATPase.

The analysis of how redox-induced proton release occurs in the plasma membrane is far from complete. In early studies with ferricyanide as an electron acceptor, the stoichiometry of portons released to ferricyanide reduced was around 2 or 3, which would be consistent with proton transfer through redox carriers during their oxidation-reduction cycle [235]. Later studies find 5 to 15 protons released per ferricyanide reduced, which would be more appropriate for activation of a channel [202]. Evidence that the Na^+/H^+ antiport could be the channel activated by ferricyanide was developed by Garcia-canero *et al.* [241], when they showed that ferricyanide reduction stimulated Na^+ uptake by liver cells. They also showed Na^+ dependence and amiloride inhibition of the ferricyanide reduction. With HeLa cells the ferricyanide–induced electron transport was inhibited by amiloride and increased in Na^+-containing media [242]. Fuhrmann *et al.* [243] have also reported Na^+ influx into crythrocytes in presence of 5 mM ferricyanide.

The lack of inhibition of proton release by 4, 4′– diisothiocyanatostilbene–2,2′–disulfonic acid (DIDS) and 4-acetamido-4′ isothiocyanatostilbene-2,2′-disulfonic acid (SITS) treatment of cells indicates that the HCO^-_3/Cl^- anion exchanger is not the basis for ferricyanide-induced proton trnasfer [237].

Diferric transferrin reduction is accompanied by a much greater proton release than with ferricyanide [174]. These are transformed cells, which would tend to have high levels of transferrin receptor, which may optimize the transferrin–related redox function. The

stoichiometry of proton release to ferrous BPS formation as a measure of diferric trnsferrin reduction is often over 100. An H^+/e^- ratio this large would be consistent with activation of a H^+ channel rather than a carrier-dependent H^+ transfer.

These observations are subject to two major caveats:

(1) The transferrin–stimulated NADH oxidase has not been measured as part of the diferric transferrin–stimulated electron transport, so the ferrous BPS formation may represent only a part of the electron transfer, which is inducing H^+ release.

(2) Some preparations of diferric transferrin have adventitious loosely bound iron, which greatly stimulates the rate of ferrous BPS formation by cells.

An indication of the effect of extra iron is seen where ferrous BPS formation is 140nmol min^{-1} gww^{-1} for HeLa cells with 10μM diferric transferrin. The addition of 10μM apotransferrin to convert all iron to the tightly bound form decreases the reduction rate to 80nmol min^{-1} gww^{-1}. Further decrease may be achieved by incubating the ferric transferrin with apotransferrin before starting the reaction [210,244].

An extensive series of studies on redox–induced proton release by rat pineal cells in the transformed and untransformed phenotype based on temperature-sensitive SV40 [245] is consistent with dependence of a major part of the proton release on the Na^+/H^+ antiport activation with a small part possibly dependent on some other pathway [237].

3.1.7. Inhibition of proton release

Good evidence for the requirement for electron transport to activate the antiport is seen in the specific inhibition of oxidant–induced proton release by agents, which inhibit the transplasma membrane electron transport. These agents include adriamycin, cis–platin, bleomycin, and actinomycin D [217, 228], as well as inhibitory coenzyme Q analogs, piericidin A, and 2-methoxy-3-ethoxy-5-methyl-6-hexadecylmercapto-1, 4-benzoquinone. The effects of the coenzyme Q analogs are reversed by added coenzyme Q [210].

Retinoic acid inhibits transmembrane electron transport without inhibition of proton release [246]. Retinoic acid also stimulates proton release in the absence of oxidants or other agonists to activate the antiport, so the retinoic acid, which is permanent acid, may

activate the antiport by direct acidification of the allosteric site [247]. The continued proton release with retinoic acid, even with inhibition of transmembrane electron transport, is in contrast to the inhibition of both functions by adriamycin and other antitumor drugs. This difference may relate to the ability to retinoic acid to induce differentiation of transformed cells [248].

The lack of retinoic acid inhibition of electron transport in SV40 trnasformed cells is further evidence that the portion of large T antigen inserted into the plasma membrane modified the electron – transport system [230].

3.1.8. Electron transport activate the antiport: The mechanism

The mechanism for activation of the Na^+/H^+ antiport by the transmembrane electron transport is not known. There are logical consequences of electron transport or some experimental observations, which suggest mechanisms for transfer of a stimulus from the redox system to the antiport based on the current ideas concerning the site of activation on the antiport itself. These mechanisms could be

(1) activation of a protein kinase to phosphorylate the antiport.

(2) localized proton increase as a result of oxidation of a protonated electron transport carrier (e.g., coenzyme Q) with subsequent direction of the proton to the allosteric activation site on the antiport through a closed channel.

(3) changes in pH set point of the antiport by conformational change in a closely associated redox protein during oxidation–reduction [249].

(4) reduction of disulfide bonds which control antiport activity [243, 250].

The evidence that the antiport is regulated by phosphorylation on a serine [251] opens up a new approach to control of the antiport through the plasma membrane redox system. Tyrosine kinase can activate serine/threonine kinase (PKC or MAP). Isolated tyrosine kinase has activated by low levels of H_2O_2 [252]. Low *et al.* [196] have shown that band 3 in erythrocytes (note that the erythrocyte antiport at 110kDa should be included in the band 3 proteins) is phosphorylated when H_2O_2 is added to the cells and that external ferricyanide can also cause phosphorylation of band 3. H_2O_2 has long been known to increase phosphorylation of other membrane proteins, e.g., the insulin receptor to mimic

the action of insulin [253]. Quinones, such as coenzyme Q, can generate H_2O_2 in membranes by autooxidation of semiquinones formed during the electron transport [220, 254]. Since there is now evidence that coenzyme Q functions in the plasma membrane electron transport [216, 219], and H_2O_2 generation occurs during NADH oxidation with isolated liver plasma membrane [255], one must consider if generation of a low level of H_2O_2 is the basis for antiport activation by the plasma membrane redox system [196].

The redox state of a quinone in a membrane has been shown to control protein kinase activity. The redox state of platoquinone in chloroplasts controls phosphorylation of the light-harvesting complex protein [256]

Addition to permeant acids to cells also activates the antiport [249]. The protons are considered to act at an allosteric activator site on the cytosolic domain of the antiport, which may be associated with the phosphorylated site. Oxidation of NADH on the cytosolic side produces protons, which would be close to the allosteric activator site. Oxidation of cellular NADH by external ferricyanide and diferric transferrin has been shown [194]. If the protons from the redox activity are released into a closed channel, which communicates with the activator site, then activation by redox action could occur without decreasing the bulk cytosolic pH. A channel of this type, controlled by calcium, has been described in chloroplasts [257].

The possible relation between the transmembrane electron transport system, the transferrin receptor, and the Na^+/H^+ antiport is diagrammed in [Fig.3.1]. Redox activation of a proton channel has also been described during the respiratory burst of erythrocytes, where rapid H_2O_2 formation occurs [167]. A role of protein kinase C in this process has also been indicated [258].

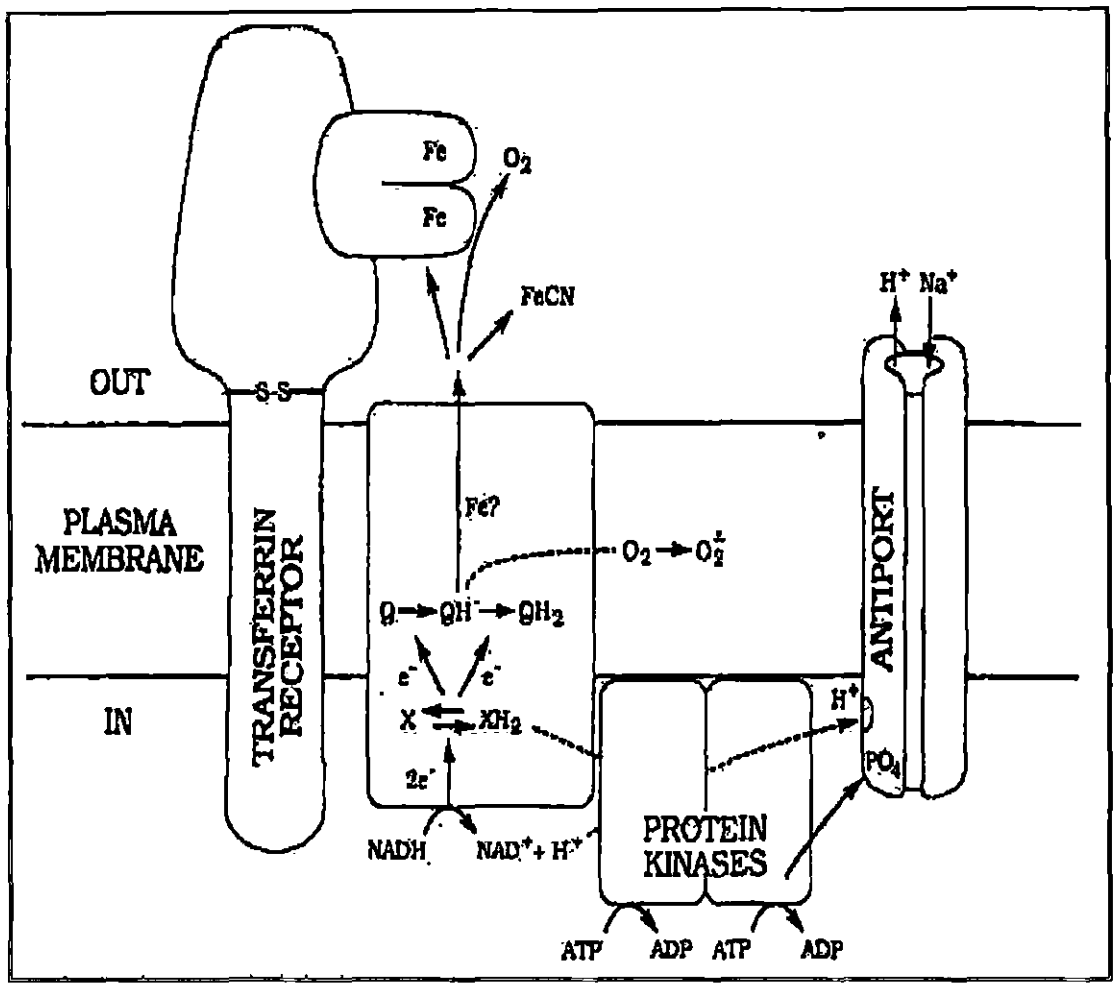


Fig.3.1. *Diagram showing the proposed relation between the transplasma membrane electron transport system, the transferrin receptor, and the Na^+/H^+ antiport for mammalian cells. Electron transport across the membrane is stimulated by ferric transferrin associated with the transferrin receptor. As a consequence of the electron transport activity, the antiport is activated either by proton release from protonated electron carriers or by generation of peroxide from superoxide to active protein kinases to phosphorylate the antiport. Oxidation of a coenzyme Q semiquinone is the most likely source of superoxide. External NADH may also be oxidized by the redox enzyme (not shown). X may be flavoprotein.*

Chapter- 4

Chemotherapy Studies

4. CHEMOTHERAPY STUDIES

4.1. Studies of various agents

4.1.1. Antibacterial compounds

A large number of antibacterial compounds have been tested against leishmaniasis in both in vivo and in vitro at high concentrations. But not even a simple compound showed good antileishmanial of activity. Even the combination of trimethoprim and sulphadiazine did not show activity in mice infected with *L. major* [259]. The rifampicin, isoniazid potentiation was observed against mice infected with *L. mexicana amazonensis,* but high dose levels were required [260]. Russian Physician has used Monomycin against zoonotic cutaneous leishmaniasis caused by *L. major* [261].

4.1.2. Antimalarial agents

A large number of antimalarial compounds have been tested for antileishmanial activities both in vivo and in vitro. But not even a single compound showed prominent antileishmanial effects. Quinine showed moderate activity against *L. mexicana, L. major* and *L. donovani* amastigote cell line in vitro [262]. Chloroquine is reported to have low activity against *L. donovani* promastigotes in vitro [263]. Hanson et al., have reported moderate activity of two experimental antimalarials, a phosphonium (WR 179, 422) and quinazoline (WR 152, 412) derivative against hamsters infected with *L. donovani* [264]. The effect of 2–styrylquinazolines was also observed only at maximum tolerated doses against leishmaniasis [265]. 4-Nitroquinoline-1-oxide showed high activity against *L. donovani* promastigotes in vitro [266]. Cycloguanil showed less activity in vivo and in vitro against *L. mexicana mexicana, L. braziliensis paramenesis, L. donovani* [259, 267]. Pyrimethamine showed high activity in vitro against *L. mexicana* [262]. Mepacrine showed consistent but moderate activity in vivo and in vitro. It appeared to be inactive when given by local injection into cutaneous lesions in mice due to *L. major* [262, 264].

4.1.3. Antifungals and Anthelmentics

A large number of antifungal agents were tested against leishmaniasis. The highest activity was reported with ketocanazole against cutaneous leishmaniasis [268, 269]. Its use to treat patients with CL was suggested by its activity against the fungal cytochrome P450. It blocks demethylation of the enzyme substrate at C-14, an essential step in

formation of ergosterol [270]. Terbinafine is an allylamine broad-spectrum antifungal compound active against many fungal pathogens particularly dermatophytes. It is capable of inhibiting squalene-epoxidase [271], a key enzyme in the biosynthesis of ergosterol, an essential component of cell membrane.

In a murine model of *Leishmania infantum* visceral leishmaniasis, terbinafine was less effective than antimonial agents in reducing hepatic parasite load [272]. Khalil et al., observed the failure of a combination of two antifungal drugs, terbinafine and itracanazole in Sudanese PKDL [273]. The antiproliferative effects and ultra structural alterations induced in vitro by terbinafine and ketocanazole on *Leishmania amazonensis* are reported. Combination of terbinafine and ketocanazole produced additive effects on promastigote axenic growth and synergistic effects on intracellular amastigote proliferation [274].

Simoesmattos et al., studied the effect of terbinafine treatment in hamsters infected with *Leishmaina chagasi*. Terbinafine alone at the dose of 10 mg/kg had no effect on spleen parasite burden or relative spleen weight of *L. chagai* –hamsters. Glucantime alone at the dose of 80 mg/kg and combination of glucantime (80 mg/kg) plus terbinafine (100 mg/kg) significantly reduced the weight of the spleen in comparison with the infected untreated groups [275]. No high activity was found with anthelmentics such as TAC Pamoate, Trichlorphan, Niridazole, Oxamniquine etc.

4.1.4 Allopurinol and its derivatives

Allopurinol was shown to be active against promastigotes of *L. donovani* and *L. mexicana* [276]. Allopurinol ribonucleoside, metabolite of allopurinol, was found to be more effective against promastigotes and intracellular amastigotes than allopurinol [277, 278]. Thiopurinol and thiopurinol ribonucleoside were found to have similar activity to that of allopurinol against promastigotes and amastigotes [279].

4.1.5. Aurones

Aurones are the secondary metabolite natural compounds belong to the flavanoids family (Rubiaceae; Cyperaceae), and structurally are the isomers of flavones, widely distributed in flowers and fruits. They play a significant role in the pigmentation of the parts of the plant in which they occur. Aurones are plant flavanoids that provide yellow colour to the

flowers of some popular ornamental plants, such as Snapdragon (Antirrhinum majus) and cosmos. The yellow coloration is mainly provided by the 6–glucosides of aureusidin and bracteatin. Literature survey indicates that flavones, chalcones and isoflavones have been studied largely for their therapeutic potential. However, aurones still are less studied and it is only recently that these compounds have been to be investigated. Three unusual, highly oxygenated novel phenylpropanoids and two novel isoflavones, 8-prenylmucronulatol and smiranicin, were isolated from *Smirnowia iranica* together with isoflavon, glyasperin H [280].

The isoflavones significantly inhibited the growth of extracellular stages of *leishmania* in vitro their activity against the intracellular stages being considerably lower [280]. A series of aurones with drug-potential for *Leishmania* infections was identified in vitro using both a direct cytotoxicity test against extracellular promastigotes of *L. donovani*, *L. infantum*, *L. enriettii*, and *L. major*, and a test against intracellular amastigote *L. donovani* residing within murine macrophages. The compounds proved to be active at concentrations in a microgram range between 0.4 and 5.0 μg/ml. When tested against murine bone marrow–derived macrophages as a mammalian host cell control, all compounds showed only moderate cytotoxicity EC 50 (2.32-25.0 μg/ml) [281]. 6-hydroxy-2-[phenylmethylene]-3(2H)-benzofuranone had an EC_{50} of 0.45 μg/ml in the extra-, and an EC_{50} of 1.4 μg/ml in the intracellular assay against extracellular promastigotes of *L. donovani L. infantum, L. enriettii* and *L. major*, and intracellular amastigotes of *L. donovani* residing within murine macrophages [282].

A series of aurones were analyzed for the ability to inhibit respiratory functions of mitochondria of *Leishmania* parasites. In a cell-free assay mitochondrial fumarate reductase from *L. donovani* was inhibited in a concentration-dependent manner. The most active compounds were 4^{l}, 6-dihydroxyaurone and 6-methoxyaurone, which inhibited parasite enzyme activity at 25 nM by over 90% [283].

4.1.6. 8–Aminoquinolines

Studies of 6-methoxy-8-alkylpiperazinoalkyl- aminoquinolines showed good antileishmanial activity [284]. Among the 8-aminoquinolines, primaquine finally emerged as the compound of choice. From a study of the activity of over 260 compounds against hamsters experimentally infected with *L. donovani*, two compounds, viz..

Wellcome 125C and Wellcome 171C were selected for clinical trails against kala-azar in Kenya. But it was observed that the therapeutic activity of these two compounds was less than that of sodium stibogluconate [284]. The Walter Reed Army Institute of Research Group has reinvestigated the structure-activity relationship of 8-aminoquinolines. The outcome of their studies has revealed a new series of agents with activities upto several hundred times greater than pentavalent antimonial and meglumine antimoniate [285, 286]. The antileishmanial activity was also observed with 6–aminoquinolines and 7–aminoquinolines; but the activity was lower than 8–aminoquinolines [264].

4.1.7. Sitamaquine

The oral drug that might have an impact on VL is the 8-aminoquinoline derivative sitamaquine, currently in development with Glaxo SmithKline [287]. The antileishmanial activity of this compound was first identified in the 1970s at the Walter Reed Army Institute of Research. Limited Phase I/II clinical trials have been completed with varying levels of success, for instance, 67 per cent of patients were cured of *L. chagasi* in Brazil when treated with 2 mg/kg daily for 28 days [288], and 92 per cent were cured of VL when treated with 1.7 mg/kg daily for 28 days in Kenya [289] and a 89 per cent cure rate with 1.75 mg/kg daily for 28 days in India [290]. Sitamaquine is rapidly metabolized, forming desethyl and 4-CH_2OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild, it causes mild methemoglobinaemia, and further studies are underway on this drug.

Moxipraquine showed antileishmanial activity against cutaneous leishmaniasis. The observation of foetal toxicity precluded further development [291]. Among that new series, the compound WR6026 was shown to be 474 times as active as meglumine antimoniate when given by the standard intra–muscular route. Compound WR 6026 was more active when given orally, being reported to be 708 times as active as meglumine antimoniate [285].

4.1.8. α–DFMO

Several new approaches to the chemotherapy of leishmaniasis are being explored. Kaur et al., [292] have shown that DFMO inhibits the growth of *L. donovani* in culture. Bacchi [293, 294] has reviewed the role of polyamines in trypanosomatids, including several species of *Leishmania*. He studied the effect of α-difluromethylornithine (DFMO) on

infections of *Trypanosoma brucei* in mice. This compound inhibits ornithine decarboxylase, blocking putrescine and spermidine synthesis. These compounds work in cell growth as co–factors and as membrane stabilizers. Another novel approach is based on the studies of Fairlamb and his co-workers [295-297]. They isolated a unique molecule, trypanothione, which in part controls redox potentials in trypanosomatids and protect them against oxidant stress. The two key enzymes that work together with this molecule are peroxidase and a reductase.

4.1.9. Chalcones

Chalcones exhibited potent antileishmanial and antitrypanosomial activity in vitro and in vivo [298]. Licochalcone A inhibited the activity of fumarate reductase (FRD) in the permeabilized *Leishmania major* promastigote and in the parasite mitochondria, and it also inhibited solubilized FRD and a purified FRD from *L. donovani* [299]. Two other chalcones, 2, 4-dimethoxy-4′-allyloxychalcone (24m 4ac) and 2, 4-dimethoxy-4′-butoxy chalcone (24 mbc) also exhibited inhibitory activity on the solubilized FRD in *L. major* promastigotes [299].

4.1.10. Diamidine compounds

A large number of diamidines and related cyclic compounds were tested for antileishmanial effects against *L. donovani* in vivo. Diminazene aceturate (berenil), which was developed for treating bovine trypanosomiasis, was found to have antileishmanial activity [300].

4.1.11. Emetine and its related compounds

Emetine and its related compounds showed good activity, but were highly toxic in vivo and to the cell lines in vitro Berberine, a plant alkaloid, was found to have moderate effect against promastigotes of *L. donovani* in vitro [263]. Al-Khateeb and Molan (1981) concluded that dehydroemetine showed anti-*L.donovani* properties by weight reduction of infected transfer liver and spleen [301].

4.1.12. Imiquimod

Imiquimod (Aldara, 3M Pharmaceuticals) is an antiviral compound [1-(2-methylropyl)-1H- imidazo (4,5-c) quinolin-4-amine] used extensively for the topical treatment of genital warts caused by the human papillomavirus. It is an immunomodulator, stimulating

a local immune response at the site of application, which in turn resolves the infection. Imiquimod induces the production of cytokines and nitric oxide in macrophages and has been shown to have an effect in experimental infections of cutaneous leishmaniasis [302]. It is suggested that the topical treatment activates localized macrophages to kill the parasite, while the antimonial eliminates systemic amastigotes, which are responsible for persistence of infection [303, 304].

4.1.13. Tranquillizers [Antipsychotics]

The phenothiazine tranquillizers and tricyclic antidepressants are toxic to *Leishmania* [305, 306]. Compounds of both groups kill *L. donovani* and *L. major* amastigotes within macrophages as well as extracellular promastigotes in vitro [307]. Neal and Allen [308] have shown that amitriptylin, an analog of imapramine, and chlorprothixene, a derivative of promazine, are high toxic to *L. donovani.* Previously it was suggested that antidepressants are toxic because they inhibit membrane functions, which are essential for the survival of *Leishmania* within its hosts. Evidence for this hypothesis arose from experiments, which demonstrated that imapramine and clomipramine inhibit transport of L–proline in promastigotes of *L. donovani* [305]. Proline actively accumulated in *L. donovani* promastigotes and the transport is driven by the proton electrochemical gradient across the plasma membrane [309]. Tricyclic drugs reduce proton motive force in *L. donovani* promastigotes [310].

4.1.14. Trifluralin

Antileishmanial activity studied by Rabinovitch (1989) reported that addition of L–amino acid esters to the culture media of macrophages infected with amastigotes killed the parasites [311]. Man–Ying Chan and Fong (1990) observed that leishmanial growth is inhibited by trifluralin, a dinitro aniline herbicide. At a concentration of five parts per million, it inhibited the growth of amastigotes in cultured macrophages by 50%. At lower concentrations, it prevented amastigote–promastigote transformation [312].

4.1.15. Trypanocides

Nifutrimox, benznidazole, ethidium, suramins and melarsaprol were tested for antileishmanial activity. Nifutrimox has been reported to have effective against human

cutaneous leishmaniasis in Brazil, but further development is precluded because of toxic side effects [313].

4.1.16. Plant glycoproteins

The ribosome–inactivating proteins (RIPs), such as plant glycoproteins cleave the glycosidic bond of adenine in 28SrRNA. They have been shown to inhibit the ribosomal function of *L. d. infantum* [314].

4.1.17. Sinefungin

Sinefungin [5–deoxy–5'–(1,4–diamino–4–carboxy butyl) adenosine] is a naturally occurring antifungal antibiotic nucleoside in which an ornithine residue is linked to the 5' end of adenosine by a carbon–carbon bond. Sinefungin was shown to be significantly suppressive against *L. donovani* and *L. braziliensis paramensis* infections in hamsters when compared with meglumine antimoniate [Glucantime] [315]. It inhibits the incorporation of thymidine into DNA [316]. Sinefungin was also shown to be effective in the treatment of American leishmaniasis [317].

4.1.18. Azoles and other steroid biosynthesis inhibitors

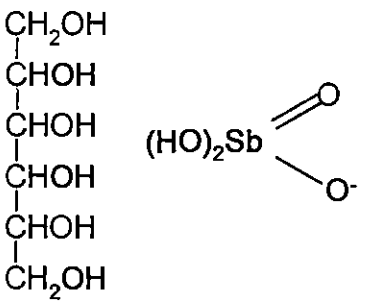
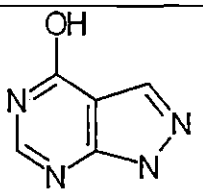
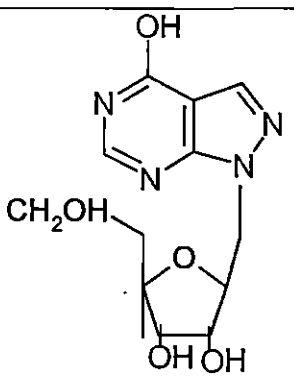
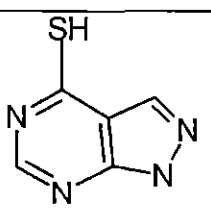
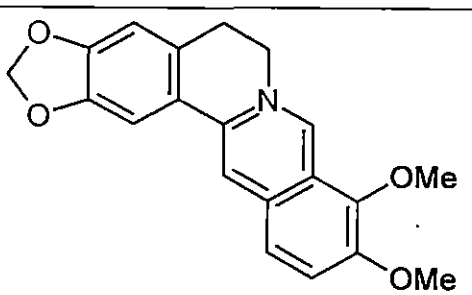
The azoles, like ketoconazole and triazoles, itraconazole and fluconazole produce an anti-leishmanial effect by blocking ergosterol synthesis [318]. Varying results have been reported from small-uncontrolled poorly designed clinical trials in both VL and cutaneous leishmaniasis (CL). In a study in Saudi Arabia, fluconazole showed a cure rate of 79 % in patients of CL caused by *L. major* [319]. Till date this drug has not been tried in India.

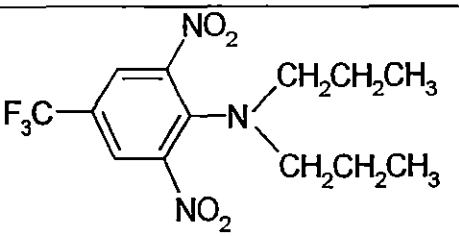
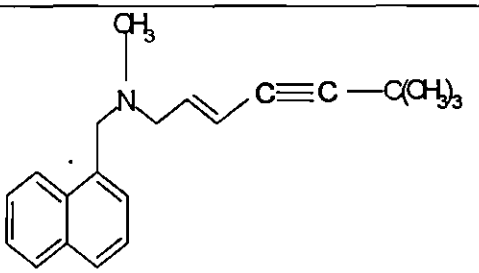
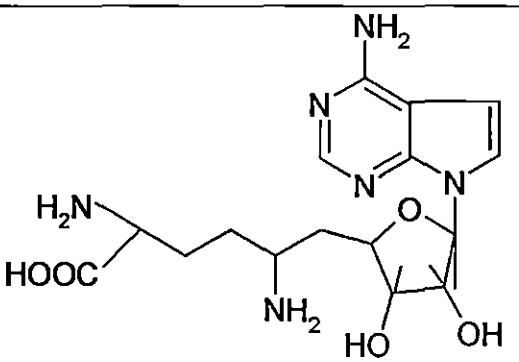
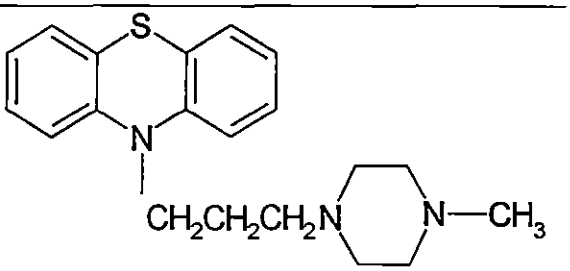
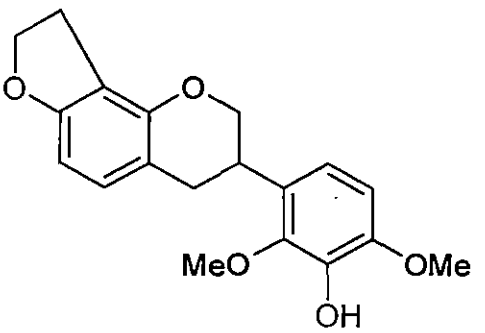
4.1.19. Cytokines

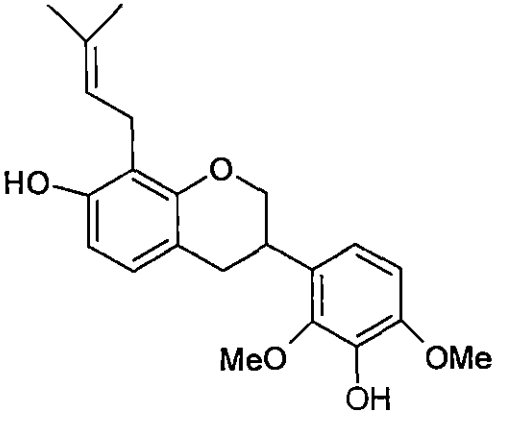
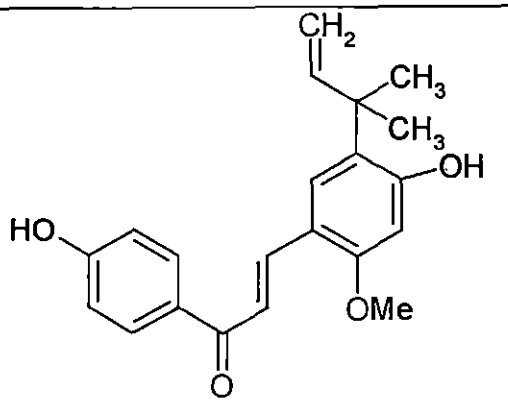
Leishmania infection progresses to kala-azar in individuals who fail to initiate Th1 response, which is mediated by IL-2 and IFN-r [320, 321]. Interferon-r is one of the principal activators of macrophages. Interferon-r as adjutants to Sbv has been used successfully in VL with high cure rate in comparison to Sbv alone [322]. Later, it was observed that interferon-r (daily dose 100 μg/m2) though improved the response rate to antimony, but overall cure rate was less than 50 per cent [323]. However, steep decline in the response rate to antimony rendered the addition of IFN-r ineffective.

Structure of some drugs studied for antileishmanial activity shown in (Table 3.1.)

Table 4.1. Name and structure of antileishmanial drugs

Name(s)	Chemical type	Structure(s)
Meglumine antimoniate (Glucantime)	Pentavalent antimonal	
Allopurinol	Xanthine oxidase inhibitor	
Allopurinol ribonucleoside	Purine analog	
Thiopurinol	Purine analog	
Berberine	Plant alkaloid	

Trifluraline	Herbicide	
Terbinafine	Antifungal	
Sinefungin	Antifungal nucleoside antibiotic	
Trifluoperazine	Phenothizine	
Smiranicin	Isoflavans	

8 - Prenylmucronulatol	Isoflavans	
Licochalcone A	Chalcones	

4.2. Drugs used in the treatment of leishmaniasis

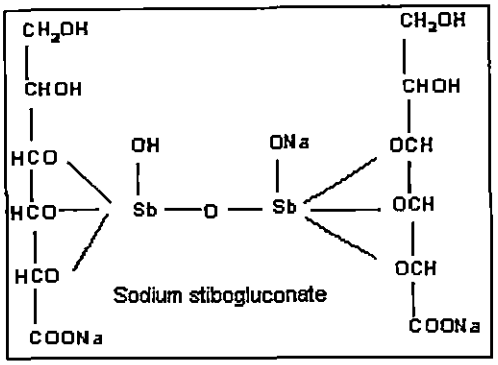
Leishmaniasis is a significant cause of morbidity and mortality in several countries. The basic treatment consists in the administration of sodium stibogluconate (Pentostam) and meglumine (Glucantime). Treatment failure, especially in kala-azar, mucosal leishmaniasis and diffuse cutaneous leishmaniasis, is becoming a common problem in many areas where diseases are endemic. There is now strong indication that treatment failure may be partly due to the drug resistance of the parasite [324-327]. In cases of treatment failure, second line agents such as pentamidine and amphotericin B are used [328, 329]. In some cases, even this agent failed to eradicate the parasite, [330-336]. In addition, the low efficacy of pentavalent antimony in the treatment of patient's co–infected with AIDS and *Leishmania* spp. is often noticed.

Table 4.2. Represents Current drugs used for the treatment of leishmaniasis [337]

	Visceral Leishmaniasis
First line drugs	Sodium stibogluconate (Pentostam, SSG)
	Meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)
	Liposomal amphotericin B (AmBisome)
	Pentamidine
Clinical trials	Miltefosine (oral, Phase IV; registered in India)
	Paromomycin (Phase III)
	Sitamaquine (oral, Phase II)
	Other amphotericin B formulations

	Cutaneous Leishmaniasis
First line drugs	Sodium stibogluconate (Pentostam)
	Meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)
	Pentamidine
	Paromomycin (topical formulations with methylbenzethonium chloride or urea)
Clinical trials	Miltefosine (oral, Phase III, registered in Colombia)
	Paromomycin (topical formulation with gentamicin and surfactants, Phase II)
	Imiquimod (topical immunomodulator, Phase II)
	Also anti-fungal azoles – ketoconazole, fluconazole, itraconazole

4.2.1. Antimony compounds

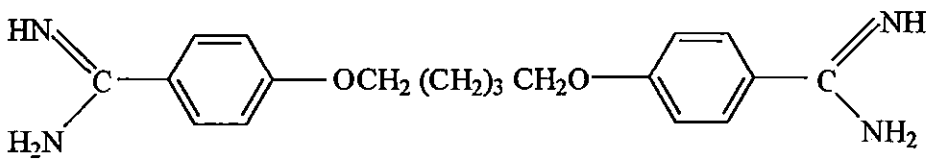


In 1937, Kikuth and Schmidt [338] have reported the antileishmanial activity of solustibosan (Sodium Stibogluconate), pentavalent antimony compound. In 1946, Durand et al [339] were the first to list pentavalent antimony, N–methylgucamine antimoniate (glucantime) in man against leishmaniasis. Today these two pentavalent antimonides (PVAs) are the most widely used leishmanicides. Although it is usually reported that both have similar efficacy and toxicity in relation to their pentavalent antimony content, sodium stibogluconate (SSG) contains 10% antimony (100 mg sb/ml, whereas meglutamine antimoniate (MA) contains about 8.5% antimony (85 mg sb/ml) [340]. The dose of pentavalents recommended by WHO is 20 mg Sb/kg body weight per day to a maximum of 600mg daily for 10–14 days. The course can be repeated in resistant cases after a resting period of 14 days [341]. Pentavalent antimonials appear to have number of modes of action against leishmaniasis. Berman et al., [342] were able to show that sodium stibogluconate inhibits the purine nucleotide triphosphate and macromolecular synthesis. Sodium stibogluconate can inhibit glucose uptake by promastigotes of *L. tropica* [343]. In addition, both aerobic and anaerobic glucose oxidation are inhibited, resulting in ATP and GTP reduction in the amastigotes exposed to sodium stibogluconate [344]. Now it is clear that the pentavalent antimonial compounds have to be converted into trivalent antimony to show their antileishmanial activity [337].

Even though pentavalents are considered to be initial treatment of choice, still their use is controversial. Treatment failures have been frequently observed [345]. Recently, strains of *Leishmania* resistant of pentavalent antimonials have been emerged, and this has reached 'alarming' proportions in some countries [346, 347]. Antimonials are

contraindicated in pregnancy and in patients with significant renal, hepatic or cardiac diseases. The first signs to toxicity are myalgia, joint stiffness, malaise, anorexia and bradycardia with ECG changes of prolongation of the QT interval and T–wave inversion. Hepatotoxicity, haemolytic anaemia, nephrotoxicity, pancreatitis and anaphylaxis are rare occurrences [348].

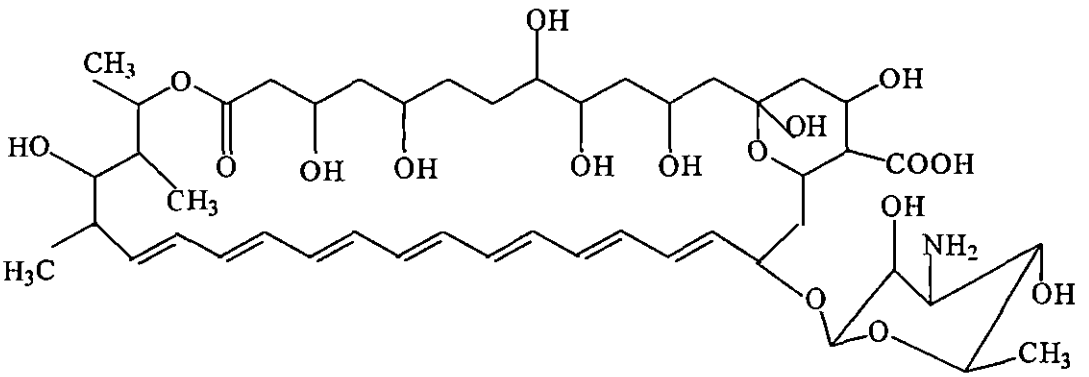
4.2.2. Pentamidine



Pentamidine is used in the treatment of African trypanosomiasis, babesiosis, leishmaniasis and pneumocystosis. In the early 1940s pentamidine emerged as the safest of the diamidines [349]. It is effective against all forms of leishmaniasis. Pentamidine become the backbone of anti kala-azar therapy. It has been used extensively for many years in the treatment of visceral leishmaniasis in India and is still being used [350]. Pentamidine was the first registered drug to be used in patient's refractory to Sbv, and high cure rates were reported [351]. Thus it is apparent that Sbv continues to be effective in the state of UP, but in North Bihar, where most (approximately 90%) of the disease occurs, it is ineffective in most patients. The magnitude of resistance varies in different areas of Bihar, peaking in the 11 districts most patients from southern districts of Bihar respond well to Sbv therapy, as do patients from West Bengal or UP. But its efficacy has declined over the years, and now it cures only approximately 70% of patients [352]. It acts against Leishmania spp. by damaging the kinetoplast DNA – mitochondria complex [353]. It is effective against all forms of leishmaniasis. Pentamidine in a dose of 3 – 4 mg/kg once or twice weekly until resolution occurs is recommended in resistant cases of leishmaniasis [341]. Although 50% of injected dose of pentamidine is excreted mainly in the urine in five days, traces can be detected in the urine up to 217 days, and in the kidney up to 240 days after a single injection. Cumulative effects, which often limit dose or frequency of administration, include weakness, nausea, vomiting and abdominal

pain, which may indicate pancreatic damage [354]. The unusually high rate of hyperglycemia (50%) associated with its use has been attributed to be high rate of pancreatic flybosis [355]. Others have also attributed the observed hypertension, tachycardia and electrocardiographic changes in T waves to its cardiotoxicity [356].

4.2.3. Amphotericin B



It is a polyene microlide antibiotic, act on sterols and phospholipids in cell membranes of *Leishmania* and fungi [357]. It has shown itself to be an effective antileishmanial drug. Amphotericin B (Amp B) amphotericin B deoxycholate (Fungizone) is the drug of choice for second-line of treatment for visceral leishmaniasis, if the patient either fails to respond to treatment with antimonial drugs or relapses thereafter [329]. Amphotericin B is the most effective antileishmanial drug; it is originally developed as an antifungal agent. The activity of the drug induces highcure rates. Use of formulation of amphotericin B, a pollen antibiotic, for treatment of leishmaniasis is biochemically rational because the target of amphotericin B is ergosterol like sterols, which are the major membrane sterols of *Leishmania* species [357]. Due to high affinity of amphotericin B for sterols, aqueous pores are formed in the membrane leading to increased membrane permeability and killing of *Leishmania* [358]. Amphotericin B is now being more widely used for VL and constitutes the major advance in antileishmanial chemotherapy during the last 10 yr. At dose of 0.75-1.0 mg per kg for 15 infusions on alternate days, it cures more than 97 per cent of patients [359, 360]. Occasional relapse (1%) might occur with amphotericin B, which can be treated successfully with the same drug. It has been recommended as first line drug in India by the National Expert Committee for Sbv in refractory regions of VL. Primary resistance to this drug is

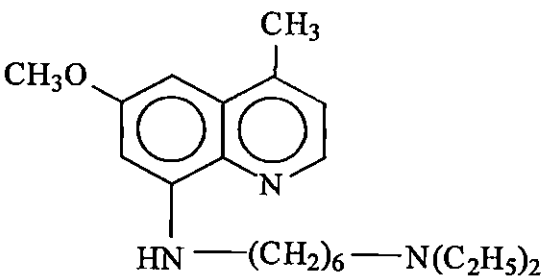
unknown. It is administered over a period 4-6 hours by slow intravenous infusion in 500 ml glucose 5%; starting at 0.1mg/kg doses and gradually increasing to 1mg/kg. It is only stable at neutral pH and is unstable when exposed to light and air.

The use of amphotericin B for leishmaniasis in human has been limited. In addition to nephrotoxicity other side effects include anaemia, thrombophlebitis and hypokalemia.

4.2.4. Lipid formulation of Amphotericin B

The need to develop less toxic, more effective formulation of amphotericin B has led to three new clinical formulation of amphotericin B in which deoxycholate has been replaced by other lipids. These formulations are liposomal ampho B (L-AmB: Ambiosome), amphotericin B colloidal dispersion (ABCD: Amphocil) and amphotericin B lipid complex (ABL: Abelcit). Novel amphotericin B formulations have been used successfully to treat canine visceral leishmaniasis [361] and cutaneous leishmaniasis in immunocompromised patients [362] and paediatric CL [363]. These substitutes are well taken by reticuloendothelial system and poorly taken by kidney, the major target of organ toxicity [364]. Adverse effects of the conventional amphotericin B can be circumvented without compromising with the efficacy of the drug. It is possible to deliver high doses of drugs over short periods. The dose requirement varies from region to region. In Indian subcontinent a small dose (3.75 mg/kg) of ambiosome for five consecutive days induces high cure rates [365].

4.2.5. 6–Methoxy–8–(Diethylaminohexylamino) lepidine

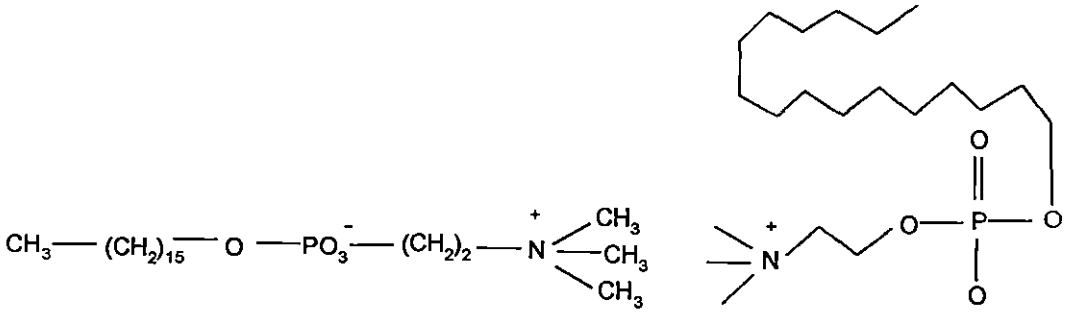


WR6026 was first synthesized and studied by scientists from the Water Reed Army Institute of Research under the code name WR6026 [366, 367]. The compound is in the public domain.

The bioavailabilities and pharmacokinetics of intravenous and oral dose of this compound were studied in beagle dogs [368]. In addition, tests conducted by Water Reed Army Institute of Research with healthy male subjects with oral doses upto 60mg revealed no significant drug related symptoms and no physical or laboratory abnormalities [369]. WR 6026 was effective for the treatment of visceral leishmaniasis in 16 patients at a dose of 0.75 to 1.00 mg/kg/day. The therapy was associated with minimal toxicity; adverse effects included gastrointestinal distress, headache and methemoglobinaemia.

4.2.6. Miltefosine

There are few new antileishmanial drugs in the pipeline. Recently a progress has been achieved in the treatment of leishmaniasis by introducing a drug called miltefosine.



Miltefosine

(N–hexadecylphosphoryl choline)

Miltefosine, an alkylphospholipid was originally developed as an oral antineoplastic agent, is the most advanced drug in the treatment of leishmaniasis. It is the first effective oral drug for the treatment of visceral Leishmaniasis and received the market registration in India in March 2002.

Miltefosine was originally developed as an anticancer agent first by ASTA Medica and since 2001 in collaboration with the Max Planck Institute in Gottingen (Prof. H. J. Eibl) and the Universitatsklinik in Gottingen (Prof. G. Nagel, Prof. C. Unger). Its antileishmanial activity was initially discovered in the mid-1980s. In 1988, Simon Croft [370] and his group reported anti–leishmanial activity of miltefosine and related compounds after parenteral use in mice. Considering the good oral bioavailability of

miltefosine, as evident from the studies in tumour patients, Kulencord, Unger and others demonstrated for the first time an excellent oral activity in their leishmaniasis models [371]. In 1995, ASTA Medica/Zentaris signed an agreement with WHO for the clinical development of miltefosine for visceral leishmaniasis. The Task Force for this development was introduced by TDR, with Prof. Anthony Bryceson as chairman, Dr. Jonathan Berman as co chair, and clinical investigators from India [372, 373].

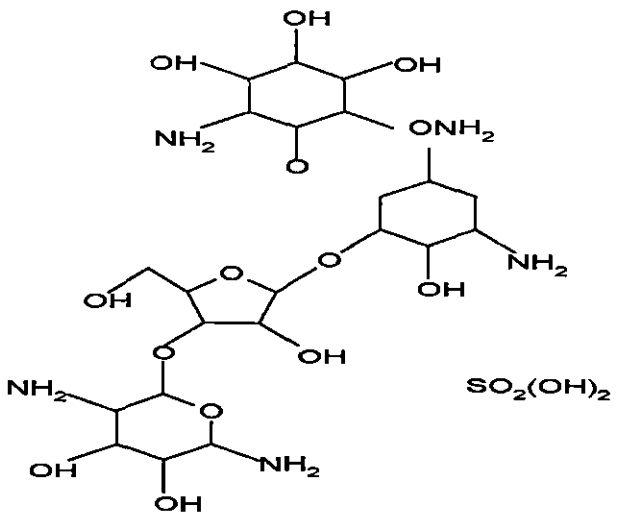
On the basis of a small scale proof of concept study in India, which was organized by ASTA Medica/Zentaris, Prof. Shyam Sundar and Prof. Henry Murray of Cornell University, a clinical development programme was initiated under the supervision of the TDR Task Force. A dose ranging and pharmacokinetic phase I/II study and a large phase III trial comparing miltefosine with amphotericin B were organised, initiated and completed in adult patients. In addition, dose ranging and pharmacokinetic study, and a confirmatory phase III study, were conducted in children. Recently, the Indian Council of Medical Research (ICMR) has involved in developing the drug and a phase IV study, to be conducted under the auspices of ICMR, has been organised jointly by ICMR, WHO, Zentaris and German Remedies Ltd. In all clinical studies, a cure rate >94 per cent has been found consistently with this drug. This drug has mild gastrointestinal adverse events like vomiting and diarrhoea in 40 and 20 per cent patients, respectively. Miltefosine has been used in India for treatment of VL at a dose of 50-100 mg (~2.5 mg/kg) for four weeks [374, 375]. It has also been found safe and effective in paediatric patients [376, 377]. This drug with mild side effects can become an important tool in containing the epidemics of VL. However, there are certain major limitations. Miltefosine has a median long terminal half-life of 154 h, which could encourage development of clinical resistance, and the best way to use this drug would be to use as a combination multi drug therapy. It is teratogenic and abortifacient, which means the drug, cannot be used in pregnancy, and females with child bearing potential must observe contraception for the duration of treatment and an additional two months. Further, rapid therapeutic response coupled with unsupervised treatment can severely affect compliance, and bring a premature end to this very important arsenal against *Leishmania*.

The Indian partner company of Zentaris received the approval for miltefosine for the treatment of visceral leishmaniasis in India. Very encouraging therapeutic responses have been seen even in patients with multiple pretreatment. In addition, a placebo

controlled phase III study in cutaneous leishmaniasis is currently ongoing in South America, with the aim to confirm the efficacy seen in an earlier dose finding study. Finally, a multinational programme is now being sponsored by the European Community to study the mechanism of action of miltefosine in leishmaniasis.

The mechanism of action of miltefosine is not well established, but it probably interacts with the cell membrane of *Leishmania* [378]. The antiproliferative effect may be mediated by an increase in cellular ceramide, which results in apoptosis. Treatment of cells with 25 μM miltefosine results in a 53% increase in ceramide concentration relative to control. The drug acts as a membrane signaling pathway inhibitor. Miltefosine inhibits CTP: phospho cholineytidyl transferase. It also possesses antimetastatic properties [379].

4.2.7. Paromomycin sulfate



Paromomycin (PM), an aminoglycoside antibiotic, was originally identified as an antileishmanial in the 1960s and has been used in clinical trials for both VL and CL. As with miltefosine, resistance to paromomycin could be induced in *L.donovani* promastigotes experimentally in vitro. The resistance was specific to PM and stable and its mechanism seems to be due to decreased drug uptake [380]. Ribosomes have been implicated as target [381] and inhibition of RNA synthesis followed by protein synthesis shown, along with induction of respiratory dysfunction [382]. Its efficacy has been demonstrated in India and a dose of 16 mg per kg intramuscularly for 21 days has cured 93 per cent of patients [383, 384].

In India under the aegis of the Institute for One World Health Founder and CEO Victoria G. Hale, and Gland Pharma Limited has registered the Paromomycin, Intramuscular (IM) Injection for the treatment of Visceral Leishmaniasis (VL), in 2006.

A combination of PM and sodium stibogluconate has been the subject of various clinical trials in Sudan and India [385] but further studies to optimize the combination and define drug-drug interactions are required. PM might also be a drug suitable for the topical treatment of CL. The report by El-On and colleagues in 1984 [386] that a topical formulation containing 15 per cent PM and 12 per cent methyl benzethonium chloride (a skin-penetrating agent) was effective against experimental CL led to the clinical trials. One such trial demonstrated that 77 per cent were cured after 20 days treatment compared with 27 per cent cured in the placebo group [387]. Other topical formulations with lower skin irritancy have recently been on clinical trial, including one containing 15 per cent PM with 10 per cent urea and another containing 15 per cent PM with 0.5 per cent gentamicin in a 10 surfactant vehicle (WR279 396) that cured 64 per cent of CL patients after 20 days treatment in Colombia [388]. In an endemic area of Iran the 15 per cent PM/10 percent urea showed no efficacy on cutaneous leishmaniasis and it was argued that the response to PM varied with the species and the type of lesion being treated [389]. These studies have also highlighted the need for a rational pharmaceutical design of formulations optimal for cutaneous leishmaniasis and the need for species-specific diagnosis [390].

4.3. Clinical manifestations

Currently, the most used methods for diagnosis of visceral leishmaniasis are direct agglutination test (DAT) and enzyme linked immunosorbent assay (ELISA). DAT was introduced about two decades ago rapidly followed by its improved version for field use [391, 392].

ELISA is now being used as potential serodiagnostic tool for visceral leishmaniasis. Though this technique is highly sensitive, its specificity depends upon the antigen used.

4.4. Drug resistance in human leishmaniasis

Treatment failures have long been observed in the chemotherapy of leishmaniasis [267, 348]. Reasons postulated for failure include derangements in the typical host-parasite

interaction, poor host immune response, improper dosing and lack of understanding of pharmacokinetics, poor drug penetration into sites of infection, insensitivity of promastigotes, species and strain differences in drug sensitivity [259]. Unfortunately, little is known about the mechanism of underlying drug resistance as seen inhuman visceral leishmaniasis. After administration, pentavalent antimonials are converted into trivalent compounds for its antileishmanial effect. The reduction of pentavalent to trivalent compound takes place either in macrophages [393] or in the parasite. In the later case, loss of reductase activity of parasite may lead to resistance. This is supported by the observation that Sbv resistant *L. donovani* amastigotes loose their reductase activity [344]. Molecular studies have identified an ATP binding cassette (ABC) transporter system, p-glycoprotein A (PGPA) involved in the metal resistance [394, 395]. PGPA is a member of multidrug resistance protein family, whose substrate includes organic anions and drugs conjugated to glutathione, glucoroate or sulphate. *Leishmania* contains glutathione as well as trypanothione (TSH) formed by conjugation of glutathione with spermidine. Transport experiments using radioactive conjugates clearly showed that PGPA recognized and actively transported the metal conjugates [394]. Thus, PGPA might be conferring resistance either through efflux from *Leishmania* [344] or by sequestering metal thiol conjugates into a vacuole [396, 397]. In a laboratory generated multidrug resistant (MDR) *L. tropica* line overexpressing a P-glycoprotein-like transporter displayed significant cross-resistance to miltefosine [398]. Defective uptake of miltefosine by resistant *L. donovani* [399] lines appeared to be through point mutations on a plasma membrane aminophospholipid translocase [400].

Chapter- 5

Materials and Methods

Table 5.3.1.1. Composition of modified Ray's blood agar medium

Modified Ray's blood agar medium	
Composition	**Quantity**
NaCl	2.5g
Glucose	7.0g
Peptone	4.0g
Agar	7.0g
Panmede	2.5g
Rabbit blood	10.0ml

All the above-mentioned ingredients, except glucose and rabbit blood, were taken in a 500 ml conical flask. The volume was made upto 350 ml with double distilled water. Then the materials were melted on water bath and pH 7.5 was adjusted by using 20% w/v sodium hydroxide solution. The 7 gm glucose was taken in a separate culture test tube and 10 ml of double distilled water added. Then the medium, glucose solutions and culture tubes were sterilized in an autoclave at 15 lbs pressure for 15 – 20 minutes. Then the medium was allowed to cool to 50 – 60°C and sterilized culture tubes, glucose solution and required amount of rabbit blood were kept in UV chamber. The blood, glucose solution and 10 – 15 mg of ampicillin–cloxacillin were added to the tubes aseptically and mixed thoroughly. About 10 ml of media was poured into each sterilized tubes, allowed to cool in UV–chamber and then kept at 24°C in B.O.D incubator for 3 days and finally stored at 4°C in a refrigerator. Subcultures were made on these slants with *L. donovani* strain. The logarithmic growth phase was reached within 72 hours after subculture.

The peptone serves an amino acid source and glucose as carbohydrate and energy source. They need at least 10 amino acids for their growth [96, 104]. Haemin of rabbit blood acts as growth promoter [97]. Protozoa fails to synthesize it [98, 99]. Other compenents of blood, other than haemin, are also responsible for growth of haemoflagellates [100]. Sodium chloride acts as the osmotic pressure stabilizer. Agar is used as solidifying agent.

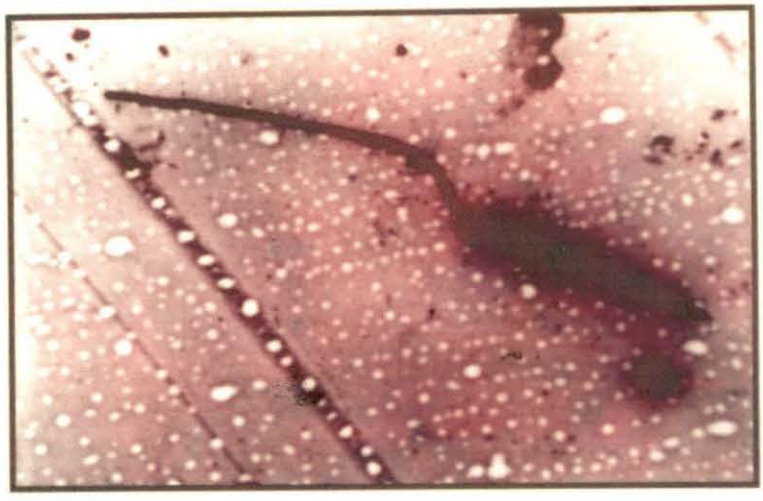


Fig. 5.3.A. 1% Uranylacetate stained micrograph of a single *Leishmania donovani* promastigote cell (magnification X 15000)

Fig. 5.3.B. 1% Uranylacetate stained micrograph of a field containing *Leishmania donovani* promastigote cells (magnification X 15000)

5.3.2. Preparation of Buffers

Table 5.3.2.1. Composition of major buffers

Composition ↓	Different Major Buffers				
	Washing buffer	**Phosphate buffer saline (PBS)**	**EDTA–PBS buffer**	**Acetate buffer**	**Digitonin permeabilization buffer**
NaCl	125mM	135mM	135mM	120 mM	–
KCl	5mM	2.75mM	2.75mM	–	50mM
Na_2HPO_4	–	0.08mM	0.08mM	–	–
KH_2PO_4	–	0.15mm	0.15mM	–	–
$MgSO_4$	–	–	–	5 mM	–
EDTA Na_2	1mM	–	1mM	–	–
Sucrose	–	–	–	–	300 mM
Glucose	5mM				
Tris- HCl	–	–	–	–	50 mM
Hepes	20mM				
Potassium acetate	–	–	–	20 mM	–
EGTA	–	–	–	–	2 mM
pH ⇨	**7.5**	**7.2**	**7.2**	**6.4**	**7.0**

Table 5.3.2.2. Composition of minor buffers

Composition ⇩	Different Minor Buffers				
	Standard buffer	**Choline buffer**	**Sodium free buffer**	**Potassium free buffer**	**Chloride free buffer**
NaCl	135 mM	–	–	135 mM	–
KCl	5 mM	–	5 mM	–	–
Gluconate - Na	–	–	–	–	135 mM
Potassum acetate	–	–	–	–	5 mM
$MgSO_4$	10 mM	1 mM	1 mM	1 mM	1 mM
Hepis	10 mM	10 mM	10 mM	10 mM	10 mM
Choline chloride	–	140mM	140mM	5 mM	–
pH ⇨	**6.4**	**6.4**	**6.4**	**6.4**	**6.4**

5.3.3. Preparation of cell suspension

The subcultured colonies on modified Ray's blood agar medium after 72 hours were scrapped off carefully with a sterilized loop and suspended in isolation and washing buffer of pH 7.5. After centrifugation of the homogeneous suspension at 3000x g for 10 minutes, the supernatant was discarded and the pellets were again suspended in isolation and washing buffer (pH 7.5). Homogeneous suspension was made by cyclomixer and absorbance was measured at 660nm with the help of spectrophotometer. The amount of protein of *Leishmania donovani* was calculated from a standard graph. The suspension was again centrifuged at 3000 x g for 10 minutes and pellets were again suspended by vortexing either in acetate buffer, pH 6.4 or phosphate buffer saline (PBS), pH 7.2 according to the requirement.

5.3.4. Protein estimation

The amount of protein was determined by the biuret method in the presence of 0.2% deoxycholate [402]. Bovine serum albumin (BSA) was used as standard. 1 mg whole–cell protein corresponds to 1.4 x 10^8 cells.

5.3.5. Cell surface iodination of intact cells

Leishmania cell surface proteins were labeled with ^{125}I by a modification of the lactoperoxidase-glucose oxidase method of Hubbard and Cohn [403]. Briefly, cells were harvested, washed twice in PBS buffer, pH 7.2 and resuspended in 5 ml ice-cold PBS buffer, pH 7.2 that contained 50 mM glucose and 3 μMNaI. 5 mCi of carrier free Na (^{125}I) was added to this mixture. Lactoperoxidase (700 odianisidine units) were added to initiate the labeling reaction. Cells were agitated every 2 min. After 15 min, cells were washed with PBS, spun through 20 ml of bovine serum, and washed twice with PBS, all by centrifugation at 2000×g for 5 min. Samples of cells from controls (incubated without enzymes) and from the full incubation mixture were saved for determination of cell viability and ^{125}I incorporation. Cell viability, as monitored by trypan blue exclusion, remained N90%. The amount of ^{125}I into protein of controls was b1% of the enzyme mediated incorporation.

5.3.6. Plasma membrane preparation

Parasites were harvested at a concentration of 108 cells/ml (5000×g, 10min, 4°C). The cell pellet was suspended to 2×107 cells/ml in PBS buffer, pH 7.2 that contained 10 mM $MgCl_2$ and rapidly mixed with equal volume of 1mg/ml concanavalin A in the same buffer. Cell aggregation was apparent within 1min. After 5min, cells were gently spined at 1000×g for 1min to remove excess concanavalin A. The supernate was discarded, and the cell pellet was re-suspended in 12ml of 10mMTris–HCl buffer pH 7.5, that contained 10μg leupeptin/ml and 1mM $MgCl_2$. After swelling for 10min in that hypotonic buffer, cells were homogenized by 18–20 strokes in tight fitting Dounce-type homogenizer. Cell lyses and formation of membrane sheets were verified by phase-contrast microscopy. The homogenate was layered over a two- step gradient consisting of 8ml of 0.5 M mannitol over 4ml 0.58 M sucrose, both in Tris buffer, and spin at 1000×g for 20min. For analysis, material remaining at the top of the 0.5 M mannitol (supernate I) was saved. Large crude plasma membrane fragments were separated as a tight pellet at the bottom of the gradient (pellet I). This pellet was resuspended in Tris buffer that contained 1M α-methylmannoside and left on ice for 40 min with occasional mixing. This plasma membrane, free from bulk concanavalin A, was diluted into three volumes of Tris buffer and homogenized by 80 strokes with a glass Dounce-type homogenizer. This second homogenate was layered on a single-step gradient that consisted of 20% sucrose in Tris buffer and spun for 30 min at 500×g. Scrolls and large plasma membrane sheets above the 20%(w/v) sucrose layer (supernate III) was collected by centrifugation at 40,000×g for 1 h. The pellet (pellet IV) containing the enriched plasma membrane was resuspended in Tris buffer. All samples were either assayed immediately or frozen at −20 °C for further use.

5.3.7. Marker enzymes

The following enzymes were used as markers of the subcellular fractions: tartarate-resistant acid phosphatase [404] for plasma membrane, cytochrome oxidase [405] for the inner mitochondrial membrane and NADPH-cytochrome c reductase [406] for microsomes.

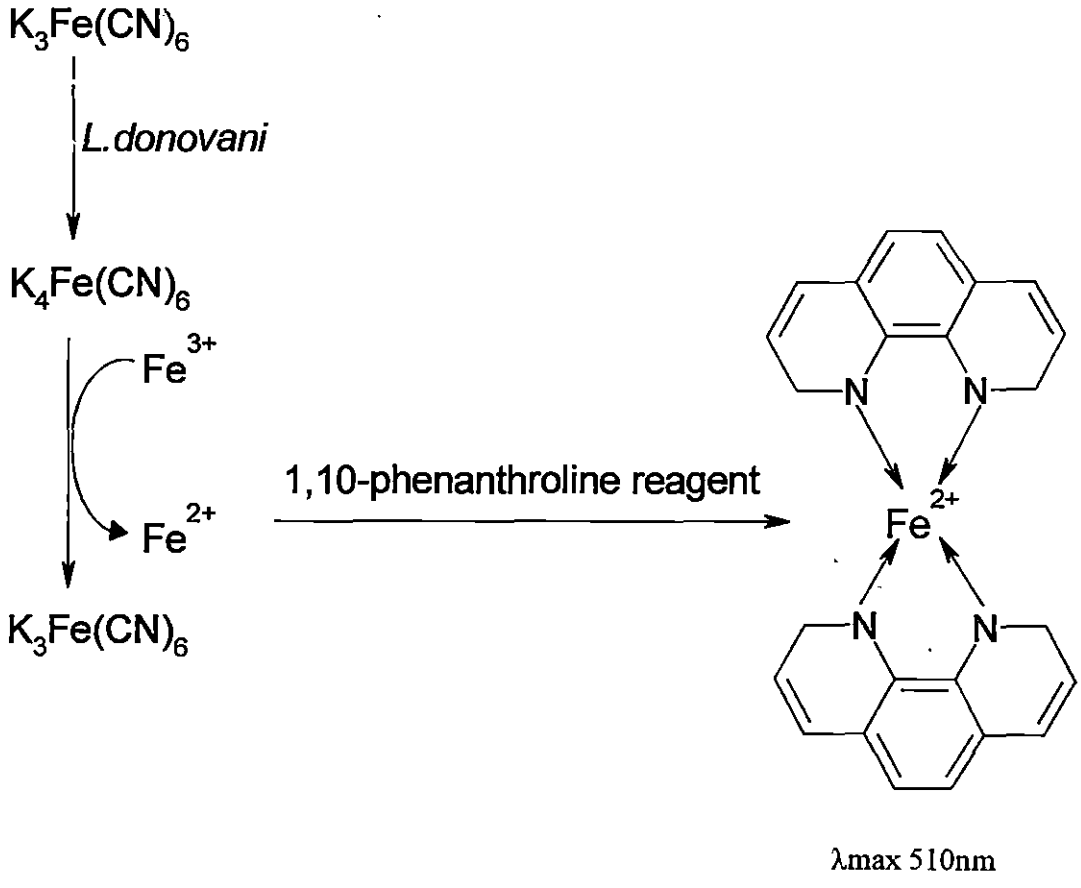
5.3.8. Measurement of ferricyanide reduction by *L. donovani* cells (LDC)

Ferrocyanide quantitation was performed using 1, 10–phenanthroline complex (Scheme 5.1) as described by Avron and Shavit [407]. The incubation mixture contained acetate buffer (potassium acetate 20 mM, sodium chloride 120 mM, magnesium sulphate 5 mM), pH 6.4, 3 mg LDC, 5 µmoles D–glucose and 3 µmoles $K_3Fe(CN)_6$ in a final volume of 1 ml. The incubation was carried out at 25°C. The reaction was terminated by the addition of 0.1 ml 30% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 10,000 xg for 15 mins. Ferrocyanide in supernatant was measured by 1.5 ml of 1,10–phenanthroline reagent containing 1.5 mmoles sodium acetate, 0.1 mmoles citric acid, 0.75 µmoles ferric chloride and 12.6 µmoles 1,10–phenanthroline at 510 nm. The blanks were carried out with all reagents except LDC.

5.3.9. ALA, DTNS and NQSA reduction assay

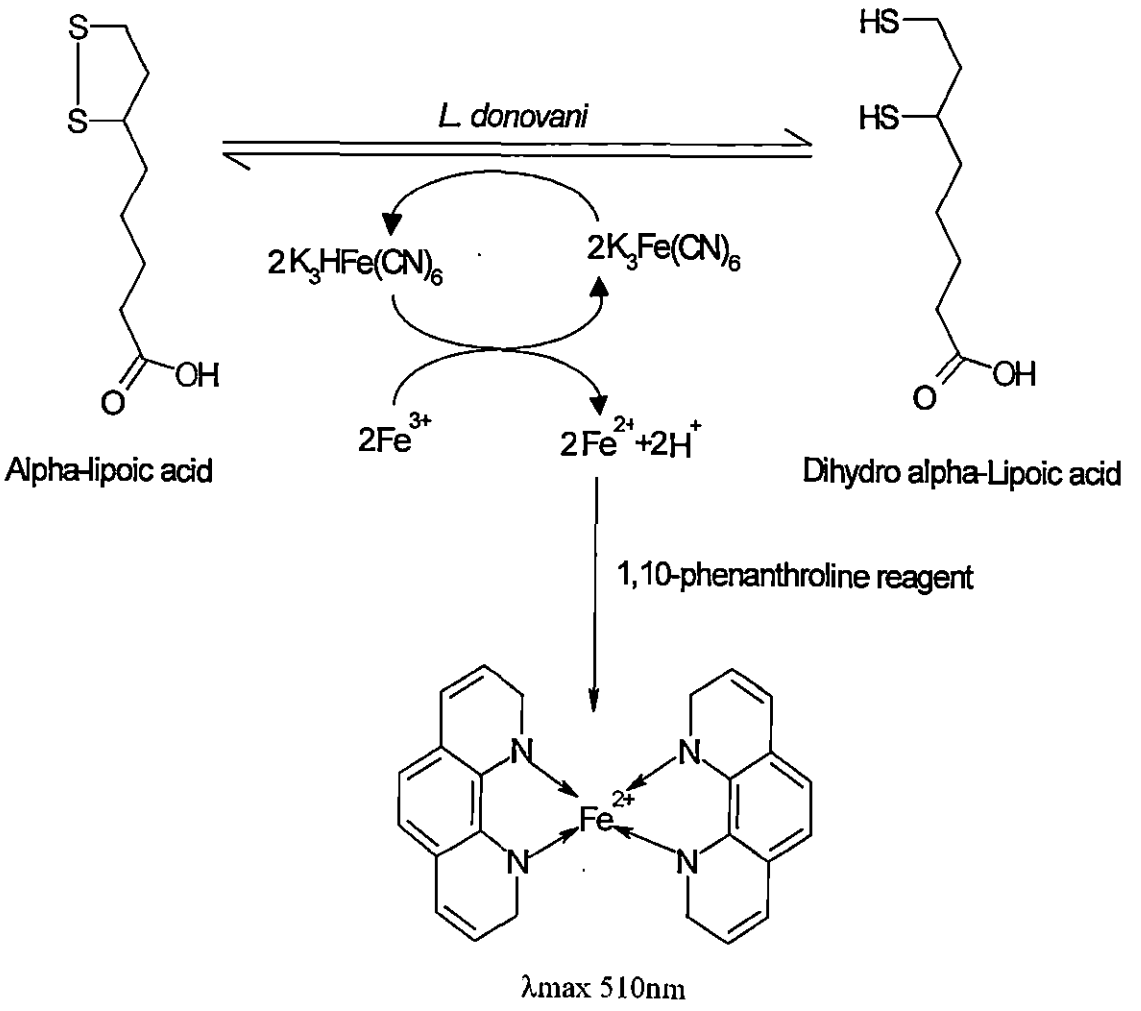
ALA, DTNS and NQSA reduction by *L. donovani* promastigote cells was assayed as the formation of ferrocyanide [$K_3HFe(CN)_6$]as a result of the reduction of ferricyanide by dihydrolipoic acid, 5-mercapto-2–nitroaniline-N-sulfonic acid and hydroquinone of NQSA (Scheme 5.2, 5.3 and 5.4).

Ferrocyanide was estimated according to the method of Avron and Shavit [407]. The reaction mixture consisted of 3 mg LDC, acetate buffer (potassium acetate 20 mM, sodium chloride 120 mM, magnesium sulphate 5 mM), pH 6.4, 5 mM D-glucose and 1 mM ALA or 0.05 mM DTNS or 0.4 mM NQSA in a final volume of 3 ml. The reaction mixtures were incubated for 10 min at 25°C. After incubation, the reaction mixtures were kept in ice and then centrifuged at 4°C at 10000xg for 10 min. 1.4 ml of the supernatant was removed and added to 0.1 ml of 75mM $K_3Fe(CN)_6$, followed by 1.5 ml 1,10–phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except LDC. Two molecules of ferricyanide have been considered to react with one molecule of dihydrolipoic acid or one molecule of hydroquinone of NQSA or two molecule of 5–mercapto–2–nitroaniline–N–sulfonic acid.

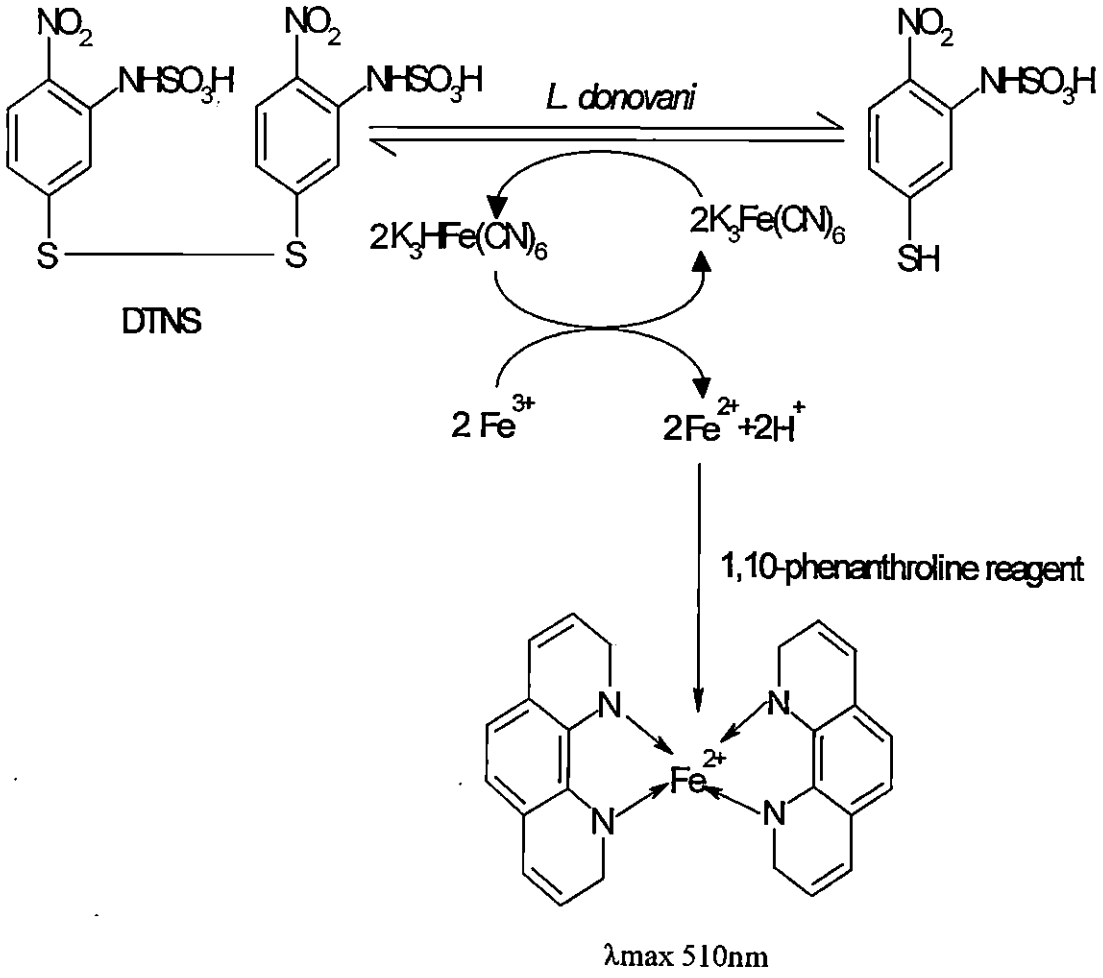


λmax 510nm

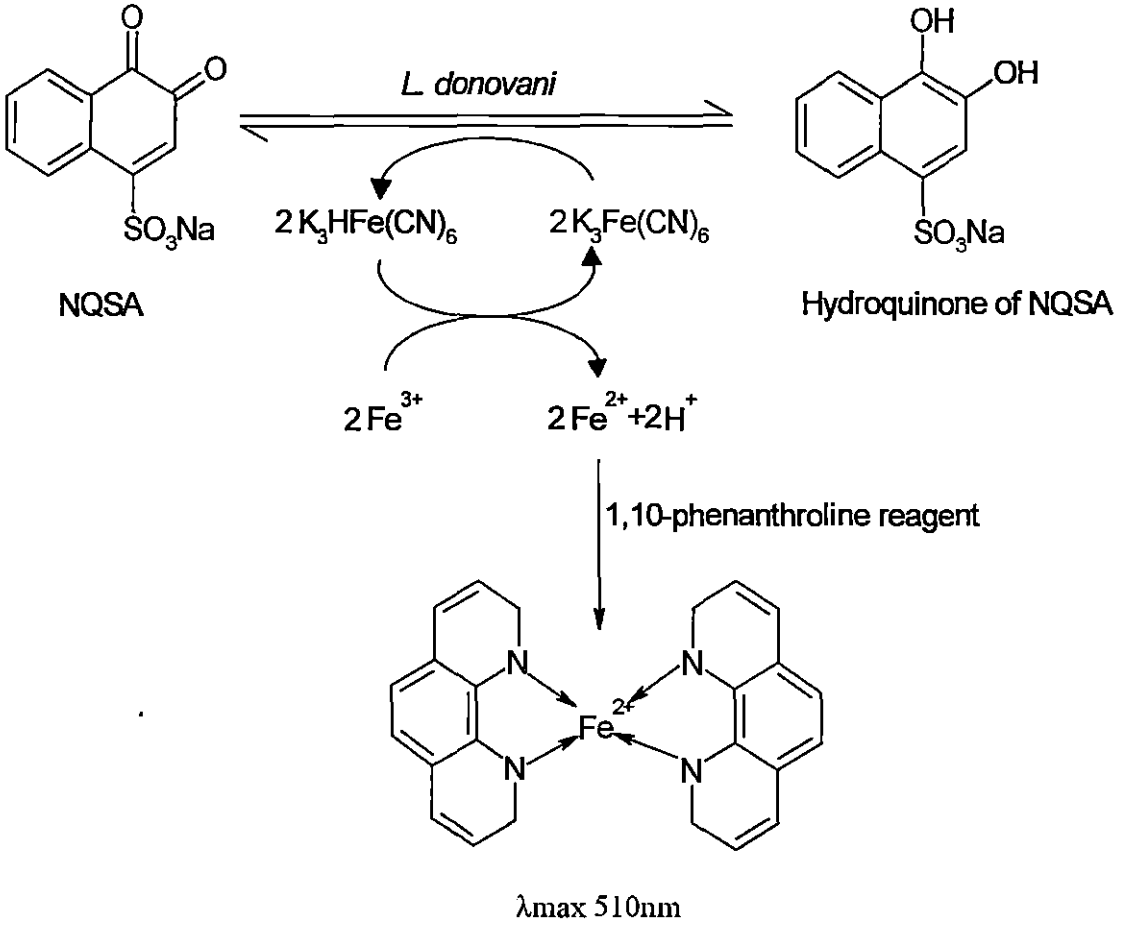
Scheme 5.1. Ferricyanide reduction by *L.donovani* promastigote cell.



Scheme 5.2. ALA reduction by *L.donovani* promastigote cell.



Scheme 5.3. DTNS reduction by *L.donovani* promastigote cell.



Scheme 5.4. NQSA reduction by *L.donovani* promastigote cell.

5.3.10. Exposure of *L. donovani* promastigotes to Capsaicin, TTFA, Trifluoperazine and Amiloride

The liquid media used for growth experiments was a semisynthetic medium developed by Kar et al [408]. Cultures of promastigotes in 100 ml conical flask where incubated for 96 h with added compound supplied as a solution in DMF (5 µl/ml medium) with proper vehicle control (DMF). After incubation, the numbers of elongated motile promastigotes were counted.

5.3.11. Measurement of Oxygen Uptake

Oxygen uptake of the *Leishmania donovani* promastigote cells was determined with late log-growth-phase cells (72h) from blood agar slants. After washing in sterile PBS (pH 7.2) and protein estimation, cells were suspended in PBS (pH 6.4) containing 10mM glucose to give a cell density of 4mg/ml. Oxygen consumption was measured at 25°C with a DW1 Hansatech oxygraph plus (Hansatech Instruments Ltd., Norfolk,UK [409]. Respiration of 2mg cells in a 1-ml final volume was measured, and effect of the addition of effectors was evaluated. Water insoluble compounds were given as a solution in dimethyl formamide (DMF; 5µl/ml PBS). Appropriate vehicle controls (DMF) were used for each experiment. Respiration rates are expressed as nanomoles of oxygen consumed per milliliter per minute.

5.3.12. Measurement of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction by LDC under anaerobic condition

Anaerobiosis was carried out in glass centrifuge tube in presence of oxygen scavenging enzymic system [410]. The tube contained 5 mg LDC, 120 mM NaCl, 20 mM potassium acetate, 20 mM Hepes, pH 6.4, 5 mM $MgCl_2$, 5 mM D–glucose, 10 units of glucose oxidase, 200 units of catalase in a volume of 2.6 ml. Open surface of liquid was covered by 1 ml heavy liquid paraffin. After 10 minute incubation, ALA, DTNS, NQSA or $K_3Fe(CN)_6$ was injected through the paraffin layer in a final concentration of 1 mM, 0.05 mM, 0.4 mM or 3 mM respectively. The assay system was incubated for 10 min at 25°C. The reaction was terminated by immersing the tubes in ice and centrifuged at 3000xg for 10 min at 4°C. 1.3 ml of supernatant was removed by syringe and added to 0.1 ml of 70

mM $K_3Fe(CN)_6$, followed by 1.6 ml 1,10–phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except LDC.

5.3.13. UV irradiation of *L.donovani* promastigote cells

L. donovani promastigote cells were UV irradiated by modifying the procedures as described by Brodie et al. [411]. This treatment was performed as follows: LDC at a protein concentration of 10 mg/ml of PBS, 140 mM, pH 6.4, was placed in Petri dishes at 4 °C. A 30-cm long Eye G8T5 lamp (maximum emission 360 nm) was placed at a distance of 3 cm from the petridishes and they received 36 W/m2. Control (UV-unexposed) cells were treated similarly without UV irradiation.

5.3.14. Preparation of digitonin permeabilized *L.donovani* cells

After protein estimation, the suspension was centrifuged at 3000xg for 10 minutes to obtain the pellets. Digitonin (50µg/mg protein) and buffer for digitonin permeabilization (sucrose 300mM, Tris-Hcl 50mM, KCl 50mM, EGTA 2mM), pH 7 were added to the resulting pellets and vortexed for 10 minutes followed by incubation in ice for 5 minutes. Then the suspension was again centrifuged at 3000xg for 10 minutes and pellets were suspended by vortexing with same buffer according to the requirement.

5.3.15. Measurement of ATPase activity in digitonin permeabilized LDC

ATPase is an enzyme, which converts ATP to ADP and P_i. Both exoplasmic and endoplasmic ATPase activity was measured by phosphate release method [412]. After protein estimation and washing with PBS-EDTA buffer, pH 7.5, cells were suspended in assay buffer. The incubation mixture contained buffer for digitonin permeabilization, pH 7, $ATP.Na_2$, magnesium acetate and digitonin permeabelized LDC in a final volume of 0.86 ml. The reaction mixture was incubated for 10 min at RT and the reaction was stopped by the addition of 0.43ml of 12% SDS followed by centrifugation at 31,304xg (20,000 rpm for 5min). To the supernatant solution 0.86ml of 6% ascorbic acid in 1N HCl and 1% ammonium molybdate solution was added followed by 0.86ml of 2% sodium citrate, 2% sodium meta-arsenite and 2% acetic acid mixture. The absorbance was recorded at 850 nm. The blanks were carried out with all reagents except LDC.

5.3.16. Measurement of PPase activity in digitonin permeabilized *L.donovani* cell

PPase activity in terms of phosphate release was assayed as described in ATPase measurement [412]. After protein estimation and washing with PBS-EDTA buffer, pH 7.5, cells were suspended in assay buffer. The incubation mixture contained buffer for digitonin permeabilization (sucrose 300mM, Tris-Hcl 50mM, KCl 50mM, EGTA 2mM), pH 7, pyrophosphate 0.25mM, magnesium acetate 0.5mM and 0.1mg of digitonin permeabelized LDC (50µg digitonin/mg protein), in a final volume of 0.86 ml. The reaction mixture was incubated for 10 min at RT°C and the reaction was stopped by the addition of 0.43ml of 12% SDS followed by centrifugation at 31,304xg (20,000 rpm for 5min). To the supernatant solution 0.86 ml of 6% ascorbic acid in 1N HCl and 1% ammonium molybdate solution was added followed by 0.86 ml of 2% sodium citrate, 2% sodium meta-arsenite and 2% acetic acid mixture. The absorbance was recorded at 850 nm. The blanks were carried out with all reagents except LDC.

5.4. Synthesis of 5, 5′-dithiobis (2-nitroaniline-N-sulfonic acid) [DTNS]

The various steps in our synthesis are shown on the Scheme 5.5.

- **Synthesis of 5-chloro-2-nitroaniline-N-sulfonic acid (II)**

17g (0.098mol) of 5–chloro–2–nitroaniline was dissolved in a mixture of 80ml of anhydrous acetone and 12 ml dry pyridine in a conical flask cooled to 4°C. 7 ml (0.05mol) of chlorosulfonic acid was added slowly with stirring. Water was added to the reaction product and the resultant solid was separated. The product 5–chloro–2–nitroaniline–N–sulfonic acid was recrystalized from ethanol (23% yields).

- **5,5′-dithiobis (2-nitroaniline-N-sulfonic acid) (III)**

5.4g (0.22 mol) of 5–chloro–2–nitroaniline–N–sulfonic acid (II) was suspended in 150 ml of water and concentrated NaOH was added to bring the pH 7.2. To this solution 2.4 g (0.03 mol) of Na_2S (anhydrous) in 30 ml water was added. This solution was stirred and heated to 50–60°C for 2 hours. Reaction mixture was oxidised with iodine in potassium iodide solution (4g I_2 in 100 ml of 5% KI). Iodine solution was added until the orange colour of the thiol anion disappeared and was replaced by pale reddish yellow colour of the disulfides. When the solution was acidified with acetic acid, oil, which crystallizes on further stirring, was formed. The product was recrystallized from glacial acetic acid

(0.79g, 15% yield) : mp 145–147°C; TLC (2–butanone/n–butanol/chloroform 30 : 40 : 30) R_f = 0.87; IR (KBr pellet) 3380, 3339, 1621, 1562, 1371, 1244, 1162, 802, 434 cm^{-1}; ^1H NMR (dimethylsulfoxide – d_6) δ 8.01 (2H, d, aromatic proton), 7.59 (2H, s, NH), 7.13 (2H, d, aromatic proton), 6.70 (2H, d, aromatic proton). Anal. ($C_{12}H_{10}N_4O_{10}S_4$) C, H, N.

5.5. Synthesis of 2-methyl-3-(1'-oxooctadecyl)-1,4-napthoquinone [MOON]

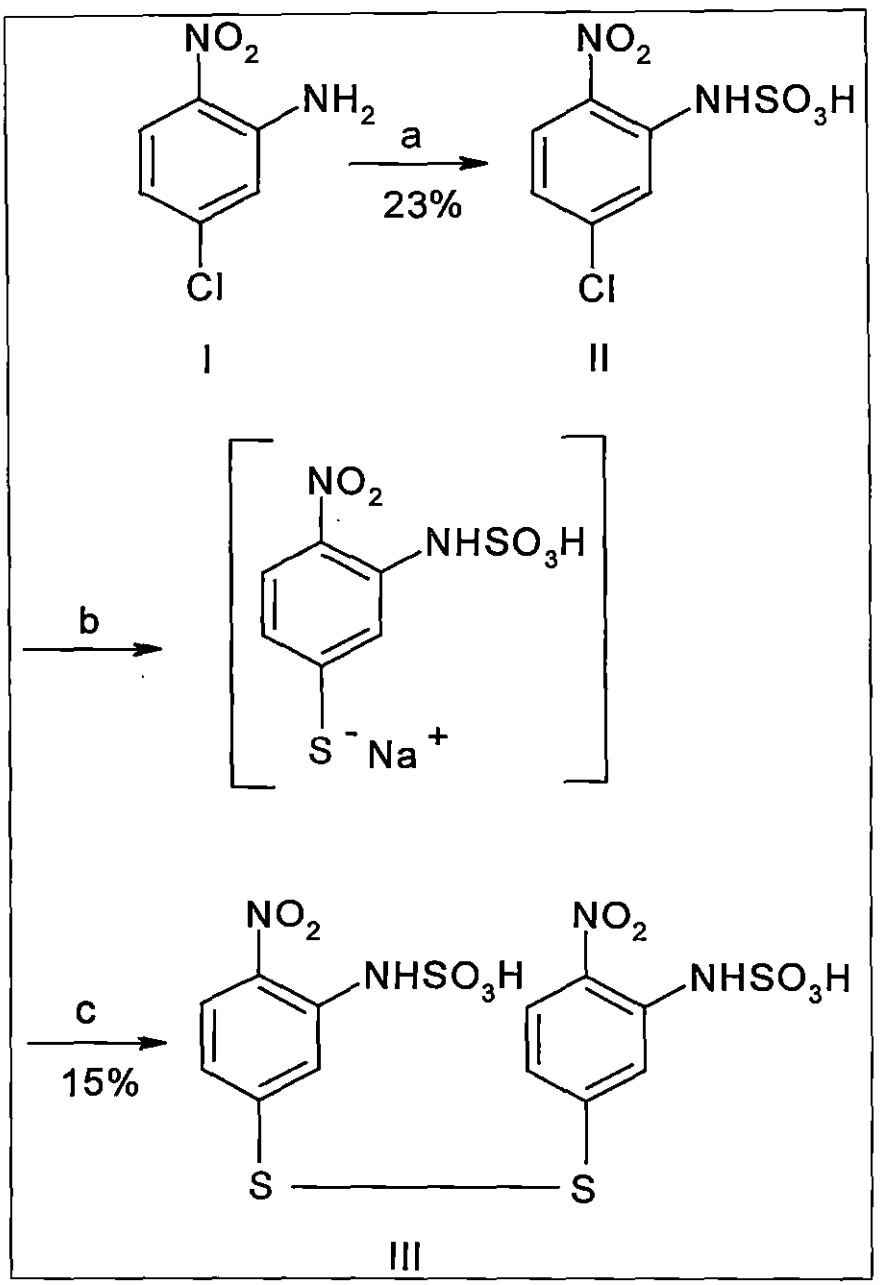
Synthesis of MOON is mentioned in scheme 5.6.

7.74 g (0.045 mol) of dihydromenaquinone and 2.5 g (0. 0045 mol) of stearic anhydride was dissolved in 100 ml of dry diethyl ether. To this solution, 1.2 g (0. 0045 mol) of anhydrous AlCl3 was added and stirred for 30 min at room temperature under the atmosphere of nitrogen. This solution was stirred and heated to 60°–70 °C for further 1 h. Solution was separated from $AlCl_3$ by filtration and ether was removed in vacuum. Solid mass was extracted with 40°–60°C petroleum ether. Petroleumether of the extract was removed in vacuo. Light yellowsolidmass was recrystallized from methanol (1.5 g, 75% yield): mp 42°–45 °C; TLC (2-butanone/n-butanol/chloroform 30:40:30) Rf=0.92; 1H NMR (CDCl3) δ7.56 (2H, m, aromatic proton), 7.47 (2H, d, aromatic proton), 2.2 (3H, s, ring methyl), 2.33–2.36 (2H, t, methylene adjacent to keto), 1.25(26H, m, chain alkane methylene), 0.86–0.89 (3H, t, terminal methyl of alkane chain). (M+23)+,461.

5.6. Synthesis of 4-aminophenyl arsineoxide-N-sulphonic acid (APAOS)

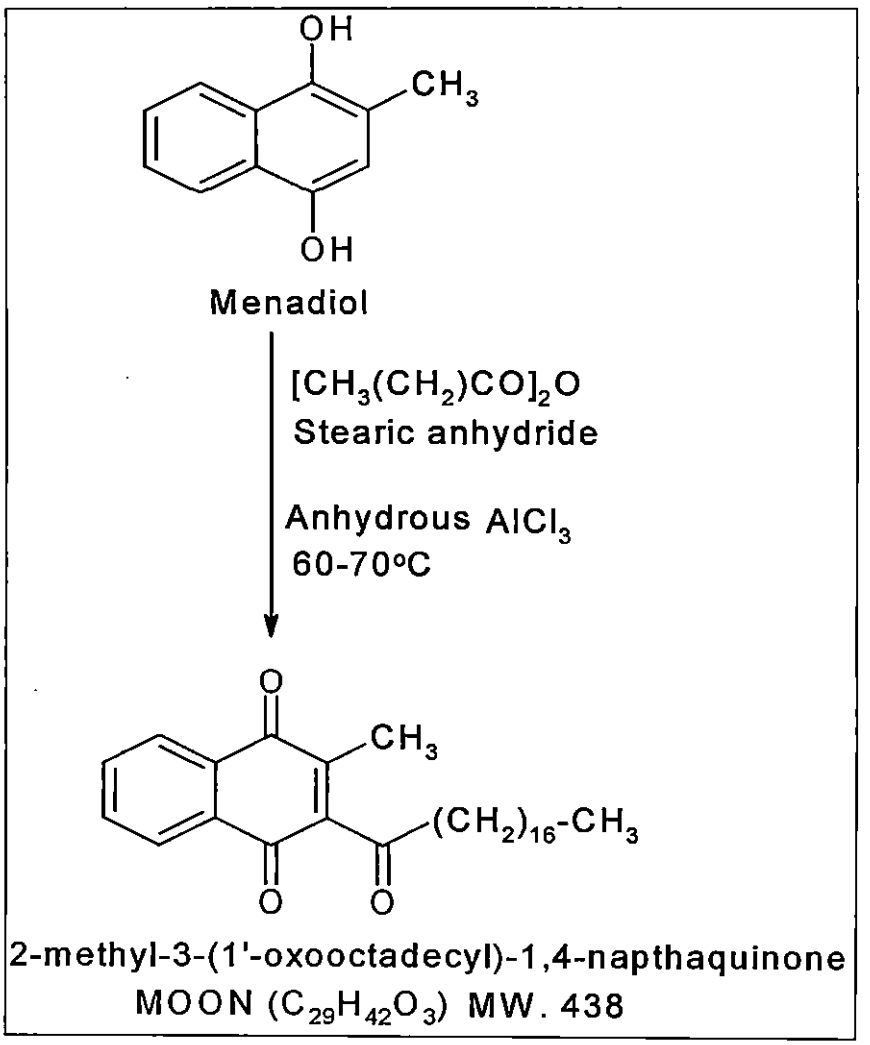
4-aminophenyl arsineoxide-N-sulphonic acid (APAOS) was synthesized in our laboratory by one of our senior scholar, which has mentioned in scheme 5.7.

Scheme 5.5.

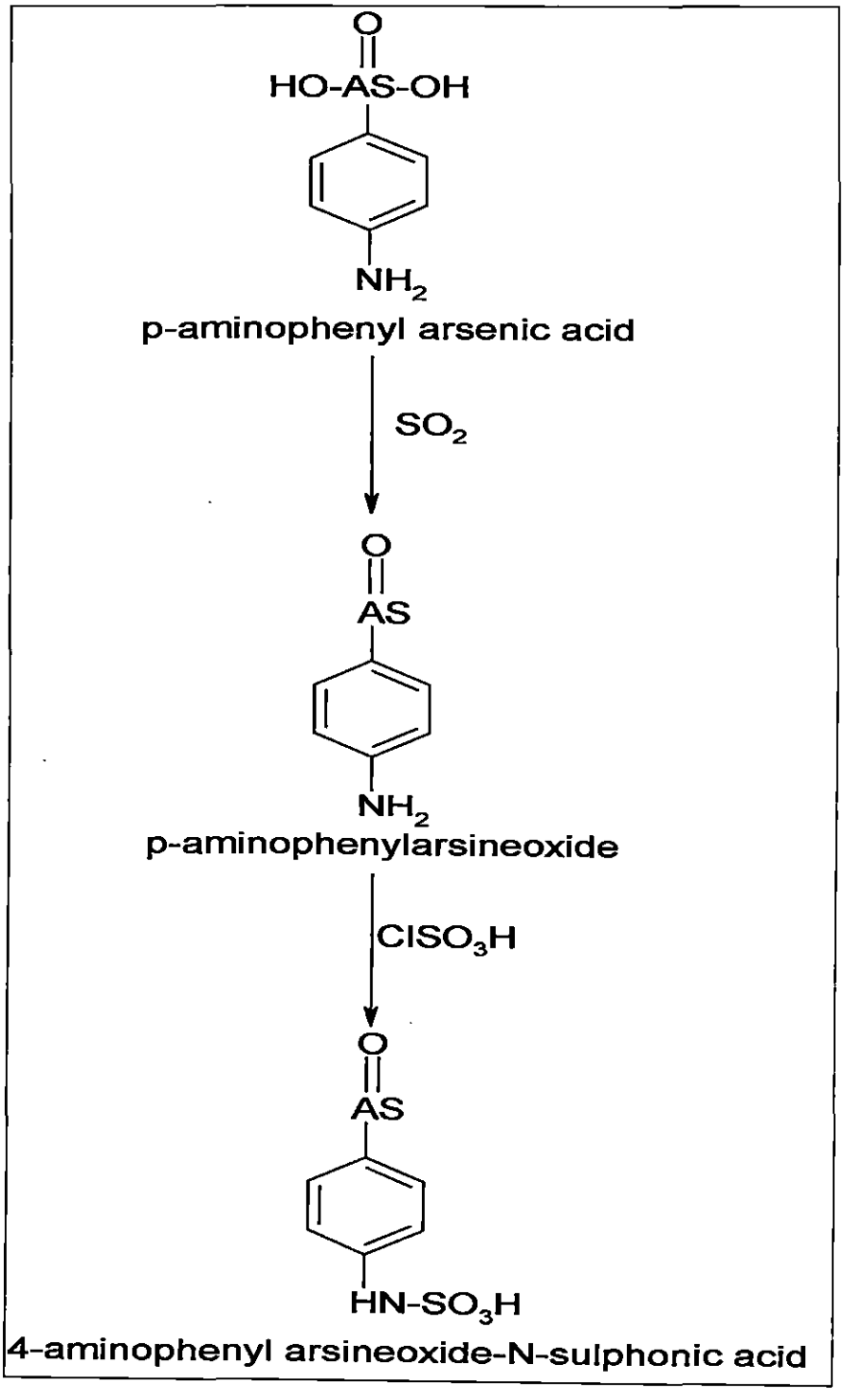


[a] Reagents and conditions: (a) acetone, pyridine, $ClSO_3H$, 4°C, 30 min (23% for II); (b) Na_2S, NaOH, 55°C, 2h; (c) iodine in potassium iodide solution, RT (15% for III).

Scheme 5.6.


OH
CH3
OH
Menadiol
[CH3(CH2)CO]2O
Stearic anhydride
Anhydrous AlCl3
60-70°C
O
CH3
(CH2)16-CH3
O
O
2-methyl-3-(1'-oxooctadecyl)-1,4-napthaquinone
MOON (C29H42O3) MW. 438

Scheme 5.7.


HO-AS-OH
O
NH2
p-aminophenyl arsenic acid
SO2
AS
NH2
p-aminophenylarsineoxide
ClSO3H
AS
HN-SO3H
4-aminophenyl arsineoxide-N-sulphonic acid

Chapter- 6

Results

6. RESULTS

6.1. Concentration of electron acceptors

ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction by *L. donovani* cell shows typical saturation kinetics with increasing ALA, DTNS, NQSA and $K_3Fe(CN)_6$ concentrations. A double reciprocal plot of the data yielded a K_m value of 0.66 mM, 0.37 mM, 4 mM and 1 mM for ALA, DTNS, NQSA and $K_3Fe(CN)_6$, respectively (Table 6.1)

6.2. pH optimum of electron acceptors for assays

ALA and DTNS reduction by *Leishmania* cell gives optimum rates at pH 6.4 whereas NQSA and $K_3Fe(CN)_6$ reduction by *Leishmania* cell gives optimum rates at pH 6.2 (Table 6.1).

6.3. Concentration of ATP and pyrophosphate for ATPase and PPase assays

ATP and pyrophosphate were assayed with Mg^{++} and maximum rates were achieved at the concentration of 0.5mM for both ATP (Fig. 6.18) and pyrophosphate (Fig. 6.19), whereas for Mg^{++} the concentration was found to be 1.00mM.

6.4. pH optimum for ATPase and PPase assays

Both ATPase and PPase assay gave optimum rates at pH 7 (Fig. 6.17.A. and B).

6.5. Reduction of electron acceptors

Leishmania cells can reduce disulfide electron acceptors, such as ALA and DTNS. LDC can also reduce naphthoquinone electron acceptor NQSA and iron co-ordinate complex $K_3Fe(CN)_6$. Table 6.2 lists the concentrations of those compounds, which gave a maximum reduction rate with *Leishmania* cells. The tested compounds that are active have standard redox potential at pH 7.0 ranging from +360 mV to –290mV.

Lack of ferricytochrome C, ferric EDTA, Fe(III) ammonium citrate and indigodisulphonate reduction by LDC cells signifies the specificity of electron acceptors in transPMET system. Lack of ferricytochrome C reduction in this system also indicates absence of superoxide anion radical generation.

6.6. Effect of extracellular ions on ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction rate

To examine the effect of external ions on the reduction rate of electron acceptors, parasites were centrifuged and re-suspended in specific ion free buffer. K^+ free buffer significantly inhibited ALA and DTNS reduction whereas Na^+ free buffer failed to show any effect. In contrast Na^+ free buffer significantly inhibited ferricyanide reduction. Cl^- free buffer stimulated reduction of all the four electron acceptors (Table 6.3).

6.7. Comparison of the aerobic and anaerobic rates of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction by LDC

It is evident from Table 6.4 that under anaerobic condition all four electron acceptors, e.g. ALA, DTNS, NQSA and $K_3Fe(CN)_6$ showed substantial stimulation of their rate of reduction. The magnitude of anaerobic stimulation showed similarity between ALA, DTNS and NQSA, $K_3Fe(CN)_6$.

6.8. Specific inhibitors and stimulators

Effect of various electron transport inhibitors and stimulators on transPMET system is illustrated in table 6.5. Extracellular reduction of ALA, DTNS, NQSA, and $K_3Fe(CN)_6$ was inhibited 83%, 24%, 49% and 89%, respectively by antimycin A at 4μM concentration. In the presence of 0.01mM HQNO, 35%, 31%, 60% and 70% inhibition of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction, respectively was attained. Rotenone at 0.02mM concentration showed 74%, 64%, 79% and 83% inhibition of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction, respectively.

Capsaicin, an inhibitors of energy coupling NADH dehydrogenase (complex I) at 0.025 mM concentration brought about an almost complete inhibition of NQSA and $K_3Fe(CN)_6$ reduction, while at 0.1 mM capsaicin concentration ALA, and DTNS reduction was inhibited 75% and 73%, respectively. IC_{50} value of capsaicin on $K_3Fe(CN)_6$ and NQSA reduction was found to be 3μM and 10μM, respectively whereas IC_{50} value of capsaicin on ALA and DTNS reduction was found to be 37 μM for both (Fig. 6.1.A). Capsaicin can inhibit the growth of LDC maximally 85% at 100μM concentration and the IC_{50} value of growth inhibition by capsaicin was found to be 37μM (Fig. 6.3.A). In the presence of 1.6mM TTFA, an inhibitor of mitochondrial complex II iron-sulphur center, 85%, 50%

and 75% inhibition of ALA, DTNS, and ferricyanide reduction was attained, while 65% inhibition of NQSA reduction was attained at 1.16mM concentration (Table 6.5). TTFA showed almost complete inhibition of LDC growth at 0.6mM concentration. TTFA showed its IC_{50} at 25μM concentration (Fig. 6.3.B).

Cyanide at 2mM concentration brought about complete inhibition of NQSA and ferricyanide reduction, while 76% and 59% inhibition of ALA and DTNS reduction, respectively was achieved at the same concentration. In the presence of 0.4mM trifluoperazine, a P-type ATPase inhibitior [413-415], 92%, 88% and 30% inhibition of ALA, DTNS, and NQSA reduction, respectively was attained, while at the same concentration trifluoperazine showed 50% stimulation of ferricyanide reduction (Table 6.5). Complete obliteration of growth was observed in the presence of trifluoperazine. The IC_{50} value of growth inhibition by trifluoperazine was found to be 4μM (Fig. 6.4.A).

Effect of various sulfhydril group inhibitors on extracellular reduction of ALA, DTNS, NQSA and ferricyanide are shown in Table 6.6. Nonpermeable sulfhydril inhibitor PCMBS at 0.1mM concentration showed complete inhibition on ALA, DTNS reduction and 40%, 76% inhibition on NQSA, ferricyanide reduction, respectively. Vicinal sulphhydryl group inhibitor cadmium chloride [416] at 0.1mM concentration showed complete inhibition on DTNS, NQSA reduction whereas at the same concentration 40% and 55% inhibition was achieved for ALA and ferricyanide reduction respectively. IC_{50} values of cadmium chloride on ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was found to be 130μM, 20μM, 14μM and 90μM, respectively (Fig. 6.1.B). On the other hand vicinal sulfhydryl group inhibitor sodium arsenite at 2mM concentration showed 16% inhibition on ferricyanide reduction whereas at the same concentration sodium arsenite showed complete inhibition on ALA, DTNS, and NQSA reduction.

Effect of various ionophore, ion channel inhibitor and protonophorous uncoupler on extracellular reduction of ALA, DTNS, NQSA and ferricyanide are shown in Table 6.7. K^+- ionophore valinomycin at 2μM concentration showed 70%, 58%, 68% and 79% inhibition of ALA, DTNS, NQSA and ferricyanide reduction, respectively. K^+- channel blocker 4-aminopyridine at 0.1mM concentration showed 89% and 86% inhibition of ALA and ferricyanide reduction, respectively; in contrast at the same concentration 4-aminopyridine showed 17% and 41% stimulation of DTNS and NQSA reduction,

respectively. Na^+- ionophore monensin showed 63% inhibition of ALA, and ferricyanide reduction at 2μM and 5μM, respectively, whereas at 10μM concentration monensin showed 38% and 60% inhibition of DTNS and NQSA reduction, respectively. IC_{50} values of monensin sodium on ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was found to be 0.6μM, 10μM, 3μM and 1.8μM, respectively (Fig. 6.2.A). K^+- sparing Na^+-channel blocker amiloride at 0.1mM concentration showed 90%, 22%, 32% and 31% inhibition of ALA, DTNS, NQSA and ferricyanide reduction, respectively. IC_{50} values of amiloride on ALA and DTNS reduction were found to be 25μM and 160μM, respectively. 50% inhibition of NQSA and ferricyanide reduction was not achieved by amiloride at the saturated concentration (Fig. 6.2.B). The IC_{50} value of growth inhibition by amiloride was found to be 0.1mM (Fig. 6.4.B). With LDC addition of 1μM FCCP, a protonophore uncoupler resulted in the 43%, 34% and 52% inhibition of ALA, DTNS and ferricyanide reduction, respectively. Strikingly, however, at the same concentration of FCCP, NQSA reduction was not inhibited.

It is evident from Table 6.8 that all electron acceptors except $K_3Fe(CN)_6$ at the identical concentration as in ALA, DTNS, NQSA and ferricyanide reduction assay showed substantial O_2 uptake inhibition. Complete O_2 uptake inhibition was achieved by antimycin A, HQNO, PCMBS, FCCP and trifluoperazine. Most noteworthy is the relative insensitivity of cyanide in O_2 uptake. K^+- ionophore, valinomycin showed 86% O_2 uptake inhibition, but Na^+- ionophore monensin showed only 40% inhibition. O_2 uptake was 50% and 58% inhibited by complex I inhibitor rotenone and capsaicin, respectively [417]. Iron-sulphur protein (complex II) inhibitor TTFA at 0.2mM concentration showed 62% inhibition of O_2 uptake. Thiol reagent PCMBS showed complete inhibition of O_2 uptake, but dithiol reagent $CdCl_2$ and Na_3AsO_3 showed 47% and 76% inhibition, respectively.

The effect of vicinal sulfhydryl group inhibitor 4-aminophenyl arseneoxide-N-sulphonic acid (APAOS) on extracellular reduction of ALA, DTNS, NQSA, $K_3Fe(CN)_6$ is shown in Fig.6.5. It appears from same figure that APAOS showed almost 75-80% inhibition of DTNS, NQSA, and $K_3Fe(CN)_6$ at 4mM and complete inhibition at 5mM concentration and the IC_{50} is achieved at 0.5mM for ALA reduction. This observation also tells that plasma membrane mediated electron transport system requires the involvement of vicinal suiphhydryl groups.

Effect of ATPase inhibitor sodium azide, dicyclohexylcarbodiimide (DCCD), triethyltin bromide (TTB), resveratrol and quercetin dihydrate [418-421] showed variable inhibition of ALA, DTNS, NQSA, and $K_3Fe(CN)_6$ reductase activity with the exception for triethyltin bromide mediated $K_3Fe(CN)_6$ reduction, which showed stimulation rather than inhibition. It is apparent from Fig.6.6 that sodium azide was most potent inhibitor on $K_3Fe(CN)_6$ reduction followed by ALA, NQSA and DTNS reductase activity. However DCCD in Fig.6.7 at 50μM concentration showed its highest inhibitory action on $K_3Fe(CN)_6$ reduction followed by NQSA, ALA, and DTNS reductase activity. In contrast $K_3Fe(CN)_6$ reductase activity was not inhibited, rather stimulated by triethyltin bromide (Fig.6.8). Triethyltin bromide showed its highest sensitivity on ALA reduction followed by DTNS, NQSA reductase activity. Fig.6.9 indicates that the resveratrol shows more ALA reductase activity than others. Sensitivity of quercetin dihydrate on ALA, DTNS, NQSA, and $K_3Fe(CN)_6$ reductase activity in Fig.6.10 was very close to each other. However $K_3Fe(CN)_6$ reductase activity appears to be least sensitive to quercetin.

Sodium orthovanadate and verapamil are P-type ATPase inhibitor [422]. From Fig.6.11 it appears that ALA reductase activity has highest sensitivity to sodium orthovanadate at 1mM concentration followed by DTNS, NQSA and finally $K_3Fe(CN)_6$ but at higher concentrations NQSA reductase activity is more than compare to DTNS and $K_3Fe(CN)_6$. From Fig.6.12 it appears that ALA reductase activity has lowest sensitivity to verapamil whereas $K_3Fe(CN)_6$ shows highest activity.

Sodium fluoride and imidodiphosphate are pyrophosphatase enzyme inhibitors. ALA and NQSA reductase activity was progressively inhibited by sodium fluoride, whereas DTNS and $K_3Fe(CN)_6$ reductase activity was inhibited at low concentration followed by stimulation at higher concentrations (Fig.6.13). At low concentration imidodiphosphate showed stimulation of ALA, DTNS, NQSA, and $K_3Fe(CN)_6$ reductase activity followed by inhibition of same at high concentration (Fig. 6.14).

According to Dwayer DM, plasma membrane of *L. donovani* promastigote were isolated by concanavalin-A [423]. Based on that observation plasma membrane of *L. donovani* promastigote was selectively isolated by concanavalin-A with high purity. Lactoperoxidase-glucose oxidase mediated iodination, when performed at low temperature, has been shown to selectively label the externally disposed peptides [424].

When intact promastigotes of *L. donovani* were labeled in this manner, lysed and the homogenate spun at 40,000×g for 1h, >90% of the incorporated label was recovered in the membrane pellet, suggesting that only membrane associated proteins were iodinated. When ^{125}I-labelled promastigotes were treated with concanavalin-A, homogenized, and spun gently through the mannitol/sucrose gradient, large membrane scrolls as evidenced by phase-contrast microscopy, were concentrated in the pellet fraction, whereas smaller vesicles were retained above mannitol phase of the gradient. As shown in Table 6.9, the bulk of the radiolabel was recovered in pellet I. This observation suggests that pellet I consisted of crude plasma membrane. The crude plasma membrane fraction was treated with α-methyl mannoside to release bound concanavalin-A and vesiculated in a second homogenization step. The plasma membranes retained above the sucrose (supernate III) was concentrated by centrifugation at 40,000×g for 1h (pellet IV). Less than 1% of the total protein was associated with this fraction, for a final enrichment of 29-fold. Overall recovery of the ^{125}I marker was 81%.

The purity of the plasma membrane fraction was assessed on the basis of the distribution of three characteristic enzymes:

(1) plasma membrane-bound tartarate resistant acid phosphatase which was previously described and demonstrated to be a useful marker for such membranes [425]

(2) cytochromes c oxidase activity for mitochondria

(3) NADPH-cytochrome c reductase for microsome.

The isolated plasma membrane fraction (pellet IV) contained trace amount of NADPH-cytochrome c reductase and cytochrome oxidase. In contrast, acid phosphatase activity was mostly found in plasma membrane fraction.

Depending on the redox potential of the electron acceptors, UV inactivation of napthoquinone co-enzyme resulted in either inhibition or stimulation of reduction rate (Fig.6.15). UV exposed *L. donovani* promastigote cells also showed parallel inhibition of oxygen uptake with respect to ferricyanide reduction stimulation (Fig.6.16.B). It therefore seems likely that oxygen uptake inhibition in UV exposed cells may lead to stimulation of ferricyanide reduction. On the other hand ALA reduction was inhibited by UV exposed cells (Fig.6.16.A) and appears to be independent of oxygen linked pathway.

Greater restoration of ALA reduction rate, ferricyanide reduction rate and oxygen uptake rate (Table 6.10) in UV exposed cell was also observed for the conjugate of 2-methyl-3-(1'-oxooctadecyl)-1,4-napthoquinone (MOON) and octadecylamine. Restoration of ALA and ferricyanide reductase activity by MOON was potently inhibited by quinone analog inhibitor HQNO.

The plasma membrane bound p-type ATPase and PPase activities were assayed in digitonine permeabelized cell of *Leishmania donovani* promastigote. The effect of extracellular ions was examined on ATPase and PPase activities and mentioned in Table 6.11. From the table it was confirmed that in presence of Na^+ and K^+ the rate was increased in both the activities whereas in the absence of Na^+ and K^+, 71% inhibition occurred on ATPase activity but the PPase activity was increased only about 6%. When the rates were compared, maximum rate was achieved in presence of K^+ on both ATPase and PPase activities.

From Table 6.12 it was apparent that NADH (0.5mM), NADPH (0.5mM) and DL-lactate (5mM) showed 14%, 32% and 16% inhibitions on ATPase activity whereas at the same concentrations PPase activity was stimulated. Surprisingly fumarate at the concentration of 5mM stimulated the ATPase enzyme activity but 13% inhibition was occurred incase of PPase enzyme.

Some sulfhydryl group inhibitors like mercuric chloride ($HgCl_2$), p-chloro mercuric benzoic acid (PCMB) and 4-aminophenyl arsenineoxide-N-sulphonic acid (APAOS) were assayed for their ATPase and PPase activities. All these inhibitors were first treated with *Leishmania donovani* cell (LDC) then washed followed by permeabilization with digitonine. From Table 6.13 it was evident that $HgCl_2$ at the concentration of 0.5mM showed 83% inhibition on ATPase enzyme and 74% inhibition on PPase enzyme. Similarly PCMB (1mM) and APAOS (2mM) were achieved 50% and 32% inhibition for ATPase whereas 37% and 39% inhibition for PPase enzyme respectively.

Effect of sodium chloride (NaCl) and sodium fluoride (NaF) were compared on ATPase and PPase assay system. From Table 6.14, it can be concluded that for both ATPase and PPase enzymes NaCl is a weak inhibitor whereas NaF showed 47% inhibition on ATPase activity and 82% inhibition on PPase activity.

Sodium azide and sodium orthovanadate are P-type ATPase inhibitors. Sodium azide and sodium orthovanadate (Fig. 6.20 and 6.21) were showing progressive inhibition on ATPase activity but PPase enzyme activity was inhibited at low concentration followed by stimulation. Similarly other inhibitors like trifluroperazine, N, N-dicyclohexyl carbodimide (DCCD) and quercetin dihydrate were also assayed for their ATPase and PPase activities. From Fig. 6.24 it was apparent that quercetin dihydrate in μM label showed more inhibition on ATPase activity whereas trifluroperazine (Fig. 6.22) and DCCD (Fig. 6.23) were more sensitive towards PPase activity.

From Fig. 6.25 it can be concluded that imidodiphosphate (IMD) was a potent inhibitor for PPase enzyme, whereas ATPase activity was stimulated in the presence of imidodiphosphate. Verapamil, a Ca^{++} cannel blocker was achieved almost complete inhibition for PPase enzyme at the concentration of 40μM but variable inhibition was seen in case of ATPase enzyme activity (Fig. 6.26). The effect of triethyltin bromide (TTB) was showing more inhibition for both ATPase and PPase activities at low concentrations (2μM) followed by stimulation in higher concentrations. The sensitivity of TTB towards ATPase rather more than PPase enzymes (Fig. 6.27).

It was evident from Fig. 6.28 that ATPase and PPase activities were progressively inhibited by sodium fluoride when the concentration was increased. In the presence of resveratol (Fig. 6.29) more inhibition was achieved at low concentration but ATPase enzyme activity was more potently inhibited than PPase enzyme. In higher concentration steady inhibition was seen for both the enzymes.

TABLE.6.1. Comparison of K_m, V_{max} and pH optimum values of nonpermeable electron acceptors reduced by *L. donovani* cells[a]

Electron acceptors	K_m (mM)	V_{max} (nmol/min/mg/protein)	pH optimum
ALA	0.66	8.33	6.4
DTNS	0.37	5.00	6.4
NQSA	4.00	3.70	6.2
$K_3Fe(CN)_6$	1.00	1.82	6.2

Methods for the reduction of various electron acceptors are given in Materials and methods.

TABLE.6.2. Comparison of the rates of nonpermeable electron acceptors reduced by *L. donovani* cells[a]

Electron acceptors	Concentration (mM)	Rate of reduction (nmol/min/mg protein)	Redox potential E^{o}pH (7.0) (mV)
Ferricyanide	5	1.07 ± 0.160	+ 360
Ferricytochrome C	0.10	0.030 ± 0.008	+ 225
NQSA	2	1.01 ± 0.080	+ 187
Ferric EDTA	0.10	0.020 ± 0.006	+ 90
Fe(III) amonium citrate	4.00[b]	0.020 ± 0.006	–
Indigo disulphonate	0.05	0.001	–125
DTNS	0.5	2.39 ± 0.190	–
ALA	1	2.25 ± 1.170	–290

[a] activities were measured on one cell culture using four aliquots of cells computed ± standard deviation. 3-day-old cell cultures were used in all experiments. Methods for the reduction of various electron acceptors are given in Materials and methods.

TABLE.6.3. ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction rates in different buffers

Composition[a]	Rate of electron acceptor reduction (nmol/min/mg protein)[b]							
	ALA reduction rate	RR[c]	DTNS reduction rate	RR[c]	NQSA reduction rate	RR[c]	$K_3Fe(CN)_6$ reduction rate	RR[c]
Standard buffer	1.31 ± 0.24	100	1.35 ± 0.22	100	0.82 ± 0.16	100	1.61 ± 0.31	100
Na^+ free buffer	1.35 ± 0.22	104	1.43 ± 0.18	106	1.36 ± 0.26	166	1.13 ± 0.28	70
K^+ free buffer	0.13 ± 0.19	10	0.75 ± 0.17	56	1.02 ± 0.20	125	2.65 ± 0.18	165
Na^+ and K^+ free buffer	1.14 ± 0.21	87	1.37 ± 0.17	101	1.07 ± 0.23	130	0.82 ± 0.26	51
Cl^- free buffer	1.81 ± 0.30	138	2.18 ± 0.21	162	1.38 ± 0.03	168	2.65 ± 0.06	165

[a] Standard buffer was composed of 135mM NaCl, 5mM KCl, 1mM $MgSO_4$ and 10mM Hepes–Tris, pH 6.4; Na^+, K^+ free buffer was composed of 140mM choline chloride, 1mM $MgSO_4$, 10mM Hepes-Tris, pH 6.4; Na^+ free buffer was composed of 135mM choline chloride, 5mM KCl, 1mM $MgSO_4$, 10mM Hepes–Tris, pH 6.4; K^+ free buffer was composed of 140mM NaCl, 1mM $MgSO_4$, 10mM Hepes–Tris, pH 6.4; Cl^- free buffer 13 mM sodium gluconate, 5mM potassium gluconate, 1mM $MgSO_4$, 10mM Hepes–Tris, pH 6.4.

[b] ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was assayed in different buffers according to procedure as given in Materials and methods. The values represent average of four experiments.

[c] RR: relative rate.

TABLE.6.4. Comparison of the aerobic and anaerobic rate of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction by LDC[a]

Electron acceptor	Aerobic rate[b] nmol/min/mg protein	Anaerobic rate[b] nmol/min/mg protein	Anaerobic rate/ Aerobic rate
ALA	1.56 ± 0.36	2.40 ± 0.41	1.54
DTNS	1.40 ± 0.26	2.01 ± 0.36	1.43
NQSA	0.71 ± 0.26	1.40 ± 0.30	1.97
$K_3Fe(CN)_6$	1.04 ± 0.37	2.06 ± 0.48	1.94

[a]Aerobic and anaerobic reduction of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ by LDC was carried out as described in Materials and methods.

[b]Values represent average of four experiments.

TABLE.6.5. Effect of electron transport inhibitors on extracellular reduction of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ by LDC

Addition	Rate of electron acceptor reduction in nmol/min/mg protein[a]											
	Conc.[c] (mM)	Rate of ALA reduction	RR[d]	Conc.[c] (mM)	Rate of DTNS reduction	RR[d]	Conc.[c] (mM)	Rate of NQSA reduction	RR[d]	Conc.[c] (mM)	Rate of $K_3Fe(CN)_6$ reduction	RR[d]
None[b]	–	1.21± 0.22	100	–	1.25 ± 0.19	100	–	0.75 ± 0.12	100	–	1.47 ± 0.26	100
Antimycin A	$4x10^{-3}$	0.21 ± 0.03	17	$4x10^{-3}$	0.94 ± 0.13	76	$4x10^{-3}$	0.38 ± 0.07	51	$4x10^{-3}$	0.16 ± 0.02	11
HQNO	0.01	0.78 ± 0.11	65	0.01	0.86 ± 0.11	69	0.01	0.30 ± 0.05	40	0.01	0.44 ± 0.07	30
Rotenone	0.02	0.32 ± 0.04	26	0.02	0.45± 0.05	36	0.02	0.16 ± 0.03	21	0.02	0.25 ± 0.03	17
Capsaicin	0.10	0.03 ± 0.04	25	0.10	0.34 ± 0.05	27	0.025	0.02 ± 0.01	3	0.025	0.01 ± 0.00	1
TTFA	1.60	0.18 ± 0.03	15	1.60	0.62 ± 0.69	50	1.16	0.26 ± 0.04	35	1.6	0.38 ± 0.06	25
Cyanide	2.00	0.29 ± 0.13	24	2.00	0.51 ± 0.12	41	2.00	0	0	2.00	0	0
Trifluoperazine	0.40	0.10 ± 0.02	8	0.40	0.15 ± 0.02	12	0.40	0.53 ± 0.08	70	0.40	2.20 ± 0.39	150

[a] ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was assayed according to procedure as given in Materials and Methods. Electron transport inhibitors were added to LDC, 10 minutes before the addition of electron acceptors. Incubation time with ALA, DTNS, NQSA and $K_3Fe(CN)_6$ was 10 minutes. Control experiments, which received an equal volume of solvent given along with the electron transport inhibitors, had no effect on electron acceptor reduction in LDC. The values represent the average of four experiments.

[b] Control incubation contained LDC, D–glucose and electron acceptor as described in Materials and methods.

[c] Value represents the concentration required for maximum inhibition or stimulation.

[d] RR: relative rate.

TABLE.6.6. Effect of sulfhydryl group inhibitors on extracellular reduciton of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ by LDC

Addition	Rate of electron acceptor reduction in nmol/min/mg protein[a]											
	Conc.[c] (mM)	Rate of ALA reduction	RR[d]	Conc.[c] (mM)	Rate of DTNS reduction	RR[d]	Conc.[c] (mM)	Rate of NQSA reduction	RR[d]	Conc.[c] (mM)	Rate of $K_3Fe(CN)_6$ reduction	RR[d]
None[b]	-	1.21± 0.22	100	-	1.25 ± 0.19	100	-	0.75 ± 0.12	100	-	1.47 ± 0.26	100
PCMBS	0.10	0.01 ± 0.00	1	0.10	0.01 ± 0.00	1	0.10	0.45 ± 0.07	60	0.10	0.35 ± 0.05	24
Cadmium chloride	0.10	0.73 ± 0.14	60	0.10	0.01 ± 0.00	1	0.10	0.01 ± 0.00	1	0.10	0.66 ± 0.16	45
Sodium arsenite	2.00	0.02 ± 0.00	2	2.00	0.01 ± 0.00	1	2.00	0.10 ± 0.00	1	2.00	1.23 ± 0.22	84

[a] ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was assayed according to procedure as given in Materials and Methods. Electron transport inhibitors were added to LDC, 10 minutes before the addition of electron acceptors. Incubation time with ALA, DTNS, NQSA and $K_3Fe(CN)_6$ was 10 minutes. Control experiments, which received an equal volume of solvent given along with the electron transport inhibitors, had no effect on electron acceptor reduction in LDC. The values represent the average of four experiments.

[b] Control incubation contained LDC, D–glucose and electron acceptor as described in Materials and methods.

[c] Value represents the concentration required for maximum inhibition or stimulation.

[d] RR: relative rate.

TABLE.6.7. Effect of ionophore and ion channel inhibitors on extracellular reduciton of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ by LDC.

Addition	Rate of electron acceptor reduction in nmol/min/mg protein[a]											
	Conc[c] (mM)	Rate of ALA reduction	RR[d]	Conc[c] (mM)	Rate of DTNS reduction	RR[d]	Conc[c] (mM)	Rate of NQSA reduction	RR[d]	Conc[c] (mM)	Rate of $K_3Fe(CN)_6$ reduction	RR[d]
None[b]	-	1.21 ± 0.22	100	-	1.25 ± 0.19	100	-	0.75 ± 0.12	100	-	1.47 ± 0.26	100
Valinomycin	0.002	0.36 ± 0.06	30	0.002	0.52 ± 0.08	42	0.002	0.24 ± 0.04	32	0.002	0.31 ± 0.05	21
4-Aminopyridine	0.10	0.13 ± 0.02	11	0.10	1.46 ± 0.22	117	0.10	1.07 ± 0.17	141	0.10	0.20 ± 0.03	14
Monensin sodium	0.002	0.45 ± 0.08	37	0.01	0.77 ± 0.10	62	0.01	0.30 ± 0.05	40	0.005	0.54 ± 0.08	37
Amiloride	0.10	0.12 ± 0.01	10	0.10	0.97 ± 0.11	78	0.10	0.58 ± 0.07	68	0.10	1.01 ± 0.16	69
FCCP	0.001	0.69 ± 0.12	57	0.001	0.82 ± 0.12	66	0.001	0.75 ± 0.12	100	0.001	0.70 ± 0.12	48

[a] ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction was assayed according to procedure as given in Materials and methods. Electron transport inhibitors were added to LDC, 10 minutes before the addition of electron acceptors. Incubation time with ALA, DTNS, NQSA and $K_3Fe(CN)_6$ was 10 minutes. Control experiments, which received an equal volume of solvent given along with the electron transport inhibitors, had no effect on electron acceptor reduction in LDC. The values represent the average of four experiments.

[b] Control incubation contained LDC, D–glucose and electron acceptor as described in Materials and methods.

[c] Value represents the concentration required for maximum inhibition or stimulation.

[d] RR: relative rate.

TABLE.6.8. Effect of several effectors on O_2 uptake by LDC

Addition	Concentration (mM)	O_2 uptake rate[a] (nmol/min/mg protein)	Relative rate
None	-	18 ± 1.78	100
ALA	1.0	8.28 ± 0.90	46
DTNS	0.05	8.64 ± 0.86	48
NQSA	0.4	5.22 ± 0.52	29
$K_3Fe(CN)_6$	3.0	18 ± 1.70	100
Antimycin A	4×10^{-3}	0	0
HQNO	0.02	0	0
Rotenone	0.01	9 ± 0.91	50
Capsaicin	0.025	7.56 ± 0.76	42
TTFA	0.2	6.84 ± 0.67	38
Cyanide	2.0	6.30 ± 1.70	35
PCMBS	0.1	0.18 ± 0.020	1
Cadmium chloride	0.4	9.54 ± 0.94	53
Sodium arsenite	0.5	4.32 ± 0.45	24
FCCP	0.001	1.26 ± 0.23	7
Valinomycin	0.002	2.52 ± 0.26	14
Monensin sodium	0.002	10.81 ± 1.01	60
Trifluoperazine	0.4	0.54 ± 0.06	3

[a] O_2 uptake was assayed according to the procedure as given in Materials and methods. The incubation time with effectors was 10 min. Control experiment that received an equal volume of solvent given along with the effectors had no effect on O_2 uptake in LDC. Values represent average of four experiments.

TABLE.6.9. Distribution of enzyme markers in the subcellular fractions of *L. donovani* promastigote

Cell fraction [a]	Enzyme activity[b] (nmol/min/mg protein)			
	NADPH-cytochrome c reductase	Cytochrome c oxidase	Tartarate - resistant acid phosphatase	^{125}I label
Total homogenate	20 ± 5	470 ± 22	12 ± 3	1
Crude plasma membrane (pellet I)	9 ± 2	207 ± 11	108 ± 7	15.6
Soluble protein (supernate I)	250 ± 16	169 ± 8	2 ± 0.3	0.4
Plasma membrane (pellet IV)	7.4 ± 1	37 ± 5	279 ± 14	28.8

[a] Cell fractionation was carried out as indicated under Materials and methods.

[b] NADPH: cytochrome c reductase, cytochrome c oxidase and tartarate-resistant acid phosphatase activities were used as microsomal, mitochondrial and plasma membrane markers, respectively. Specific activities are in nmol/min/mg protein. Specific activity of ^{125}I labeled cell homogenate is arbitrarily set equal to 1.0 and is calculated as counts/min/mg protein. Each value represents average of four experiments.

TABLE.6.10. Effect of UV irradiation and MOON on extracellular ALA, ferricyanide reduction and oxygen uptake by *L. donovani* promastigote cell

Cell pretreatment [a]	Electron acceptor					
	ALA[b]		Ferricyanide[b]		Oxygen[c]	
	Rate[d]	RR[e]	Rate[d]	RR[e]	Rate[d]	RR[e]
UV unexposed LDC	1.68±0.17	100	0.49±0.06	100	12.12±1.78	100
UV unexposed LDC + HQNO	0.76±0.09	45	0.15±0.01	30	-	-
UV unexposed LDC + MOON	1.29±0.13	77	0.91±0.12	186	13.22±1.49	109
UV unexposed LDC + MOON + octadecylamine	1.48±0.19	88	2.35±0.28	470	13.33±1.50	110
UV exposed LDC + MOON	0.84±0.10	50	2.16±0.22	440	5.80±0.61	48
UV exposed LDC + MOON + octadecylamine	1.34±0.15	80	2.64±0.30	527	8.12±0.86	67
UV exposed LDC + MOON + octadecylamine + HQNO	0.77±0.10	46	0.24±0.03	48	-	-

[a] UV-unexposed and UV-exposed LDC was prepared according to procedure as given in Materials and methods. Octadecylamine and HQNO were added to LDC at a concentration of 30 μM,and 10 μM 10 min before the addition of electron acceptor. LDC was exposed to UV irradiation for 40 min (UV-exposed LDC) and control LDC was kept in dark under similar conditions (UV-unexposed LDC).

[b] ALA and ferricyanide reduction was assayed according to the procedure as given in Materials and methods. Incubation time with ALA and ferricyanide was 10 min.

[c] Oxygen uptake was assayed according to the procedure as given in Materials and methods. Rate values represent the oxygen uptake in presence of 5 mM glucose.

[d] Rate expressed in nmol/min/mg protein, values represents average of four experiments.

[e] RR: relative rate

Control experiments received equal volume of solvent (DMF).

TABLE.6.11. Rates of ATPase and PPase activity of digitonine permeabilized *L. donovani* promastigotes in different buffers

Composition[a]	ATPase activity (nmol/min/mg protein)		PPase activity (nmol/min/mg protein)	
	Rate[b]	RR[c]	Rate[b]	RR[c]
Standard buffer	42.13±5.63	100	20.45±3.78	100
K^+ free buffer	48.54±7.14	115	27.56±3.82	135
Na^+ free buffer	49.47±7.89	117	34.48±5.63	169
K^+ and Na^+free buffer	12.42±2.56	29	21.78±5.39	106

[a] Standard buffer was composed of 300mM sucrose, 50mM Tris, 50mM NaCl, 50mM KCl and 2mM EGTA(pH 7); K^+ free buffer was composed of 300mM sucrose, 50mM Tris, 50mM NaCl, and 2mM EGTA(pH 7); Na^+ free buffer was composed of 300mM sucrose, 50mM Tris, 50mM KCl and 2mM EGTA (pH 7); K^+ and Na^+free buffer was composed of 200mM sucrose, 50mM Tris, 60mM choline chloride, and 2mM EGTA(pH 7).

[b] The values represents average of four experiments.

[c] RR: relative rate.

TABLE.6.12. Effect of NADH, NADPH, DL-lactate and fumarate on ATPase and PPase activity of digitonine permeabilized *L. donovani* promastigote cells

Incubation[a]	ATPase activity (nmol/min/mg protein)		PPase activity (nmol/min/mg protein)	
	Rate[b]	RR[c]	Rate[b]	RR[c]
DPC+ ATP+ Mg^{++}	31.58±4.72	100		
DPC+ ATP+ Mg^{++}+ NADH	27.23±3.83	86		
DPC+ ATP+ Mg^{++}+ NADPH	21.54±3.12	68		
DPC+ ATP+ Mg^{++}+ DL-lactate	26.67±3.14	84		
DPC+ ATP+ Mg^{++}+ Fumarate	38.46±4.59	122		
DPC+ PP_i+ Mg^{++}			30.15±3.96	100
DPC+ PP_i+ Mg^{++}+ NADH			33.78±4.13	112
DPC+ PP_i+ Mg^{++}+ NADPH			37.57±4.85	125
DPC+ PP_i+ Mg^{++}+ DL-lactate			32.39±4.60	110
DPC+ PP_i+ Mg^{++}+ Fumarate			27.19±3.81	87

[a] DPC: digitonine (50µg/mg protein) permeabilized cell; ATP (0.25mM); pyrophosphate (0.25mM); Mg^{++} (0.5mM); NADH (0.5mM); NADPH (0.5mM); DL-lactate (5mM); fumarate (5mM)

[b] The values represents average of four experiments.

[c] RR: relative rate

TABLE.6.13. Effect of sulfhydryl group inhibitors on ATPase and PPase activity of digitonine permeabilized *L. donovani* promastigote cells

Incubation[a]	ATPase activity (nmol/min/mg protein)		PPase activity (nmol/min/mg protein)	
	Rate[b]	RR[c]	Rate[b]	RR[c]
DPC+ ATP+ Mg^{++}	30.23±3.92	100		
$HgCl_2$-DPC+ ATP+ Mg^{++}	05.17±0.78	17		
PCMB- DPC+ ATP+ Mg^{++}	15.28±2.01	50		
APAOS- DPC+ ATP+ Mg^{++}	20.58±3.14	68		
DPC+ PP_i + Mg^{++}			30.47±4.23	100
$HgCl_2$-DPC+ PP_i + Mg^{++}			07.82±1.31	26
PCMB- DPC+ PP_i + Mg^{++}			19.20±2.57	63
APAOS- DPC+ PP_i + Mg^{++}			18.45±3.15	61

[a] DPC: digitonine (50µg/mg protein) permeabilized cell; $HgCl_2$-DPC: LDC treated with 0.5mM of $HgCl_2$ and washed, then permeabilized with digitonine (50µg/mg protein); PCMB-DPC: LDC treated with 1mM p-chloro mercuric benzoic acid (PCMB) and washed then permeabilized with digitonine (50µg/mg protein); APAOS-DPC: LDC treated with 2mM 4-aminophenyl arseneoxide-N-sulphonic acid (APAOS) and washed then permeabilized with digitonine (50µg/mg protein).

[b] The values represents average of four experiments.

[c] RR: relative rate.

TABLE.6.14. Effect of NaCl and NaF on ATPase and PPase activity of digitonine permeabilized *L. donovani* promastigote cells

Incubation[a]	ATPase activity (nmol/min/mg protein)		PPase activity (nmol/min/mg protein)	
	Rate[b]	RR[c]	Rate[b]	RR[c]
DPC+ ATP+ Mg^{++}	33.24±5.34	100		
DPC+ ATP+ Mg^{++}+ NaCl(10mM)	30.56±4.87	92		
DPC+ ATP+ Mg^{++}+ NaF(10mM)	17.52±2.58	53		
DPC+ PP_i+ Mg^{++}			36.52±5.82	100
DPC+ PP_i+ Mg^{++}+ NaCl(10mM)			29.25±4.65	80
DPC+ PP_i+ Mg^{++}+ NaF(10mM)			06.51±0.96	18

[a] DPC: digitonine (50µg/mg protein) permeabilized cell.

[b] The values represents average of four experiments.

[c] RR: relative rate.

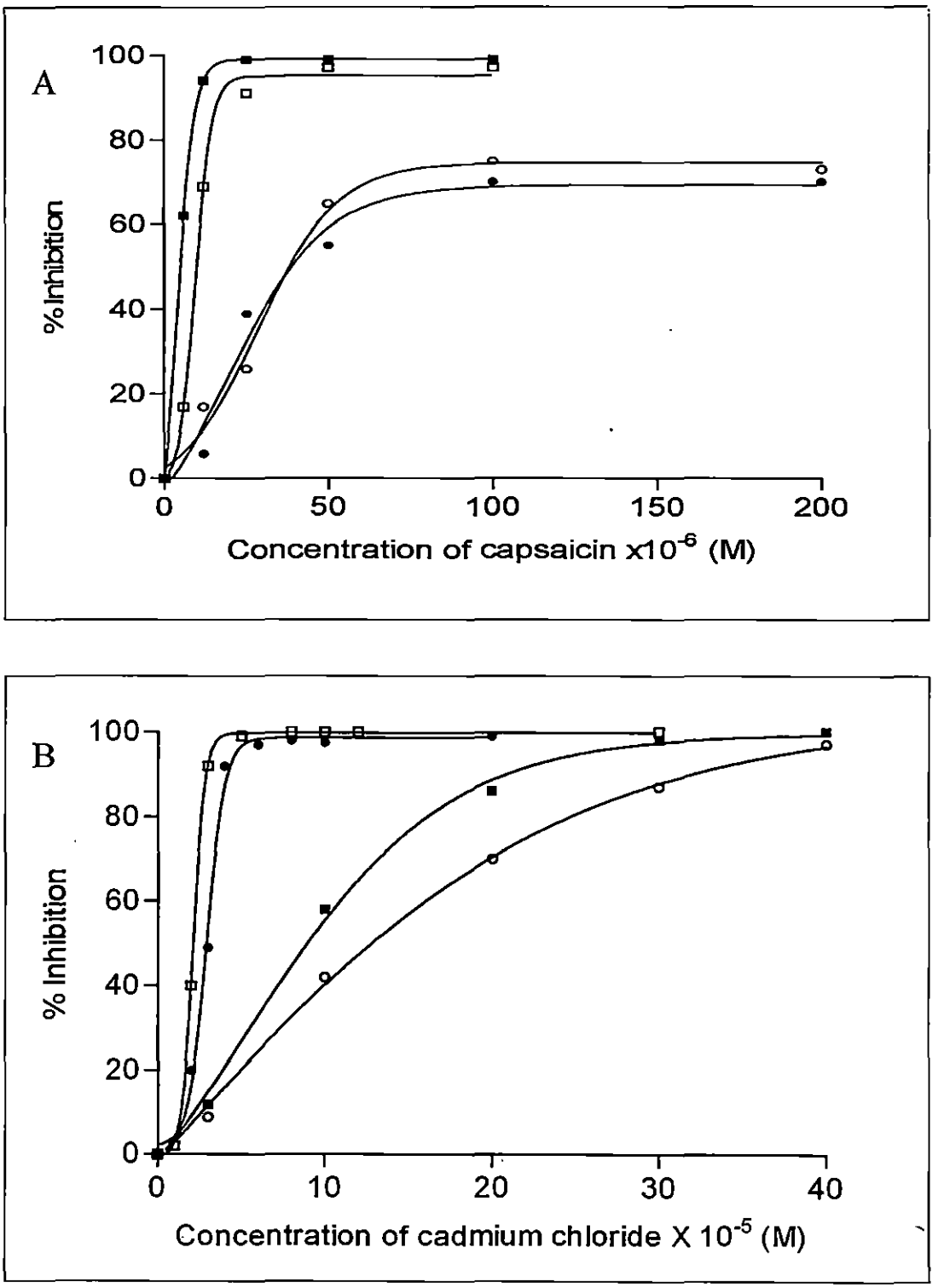


Fig. 6.1. Concentration–response curve describing the ALA (○), DTNS (●), NQSA (□) and $K_3Fe(CN)_6$ (■) reduction inhibition in *L. donovani* promastigotes by capsaicin (A) and cadmium chloride (B). Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

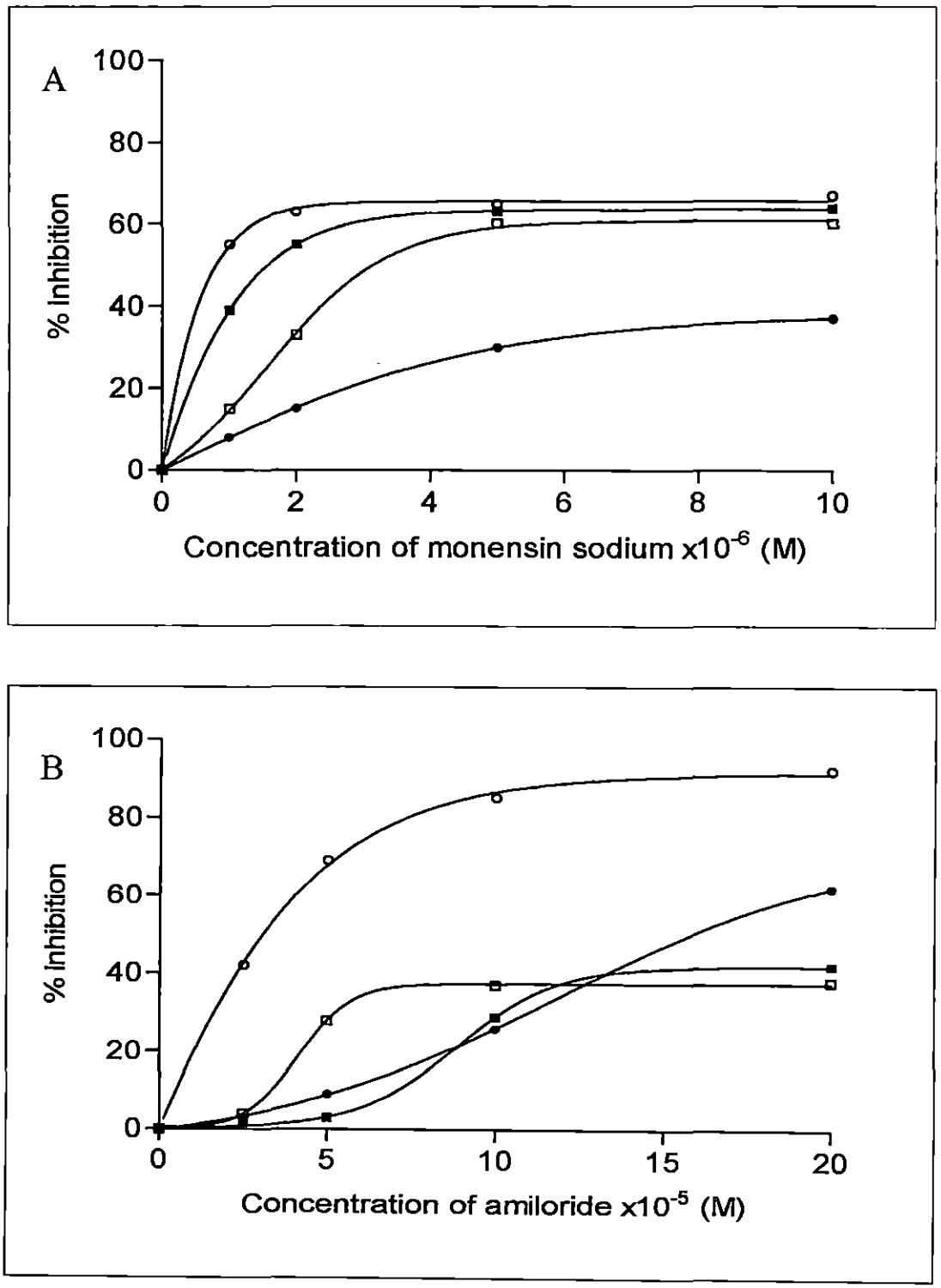


Fig. 6.2. Concentration–response curve describing the ALA (○), DTNS (●), NQSA (□) and $K_3Fe(CN)_6$ (■) reduction inhibition in *L. donovani* promastigotes by monensin sodium (A) and amiloride (B). Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

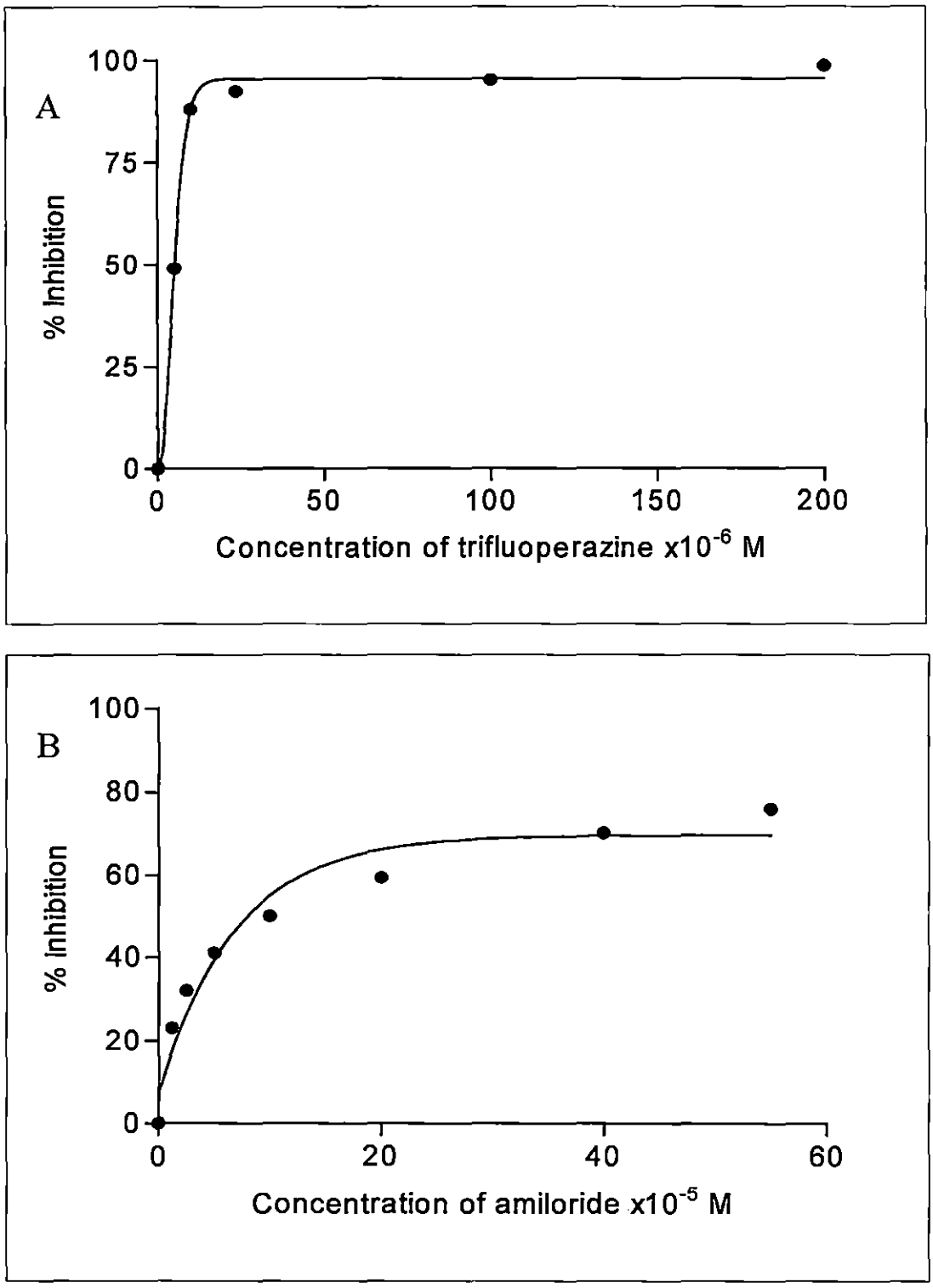


Fig. 6.4. Concentration–response curve describing the inhibition of *L. donovani* promastigote growth by trifluoperazine (A) and amiloride (B). The culture was exposed for 96hrs with the compounds. Each point represents the mean of four experiments.

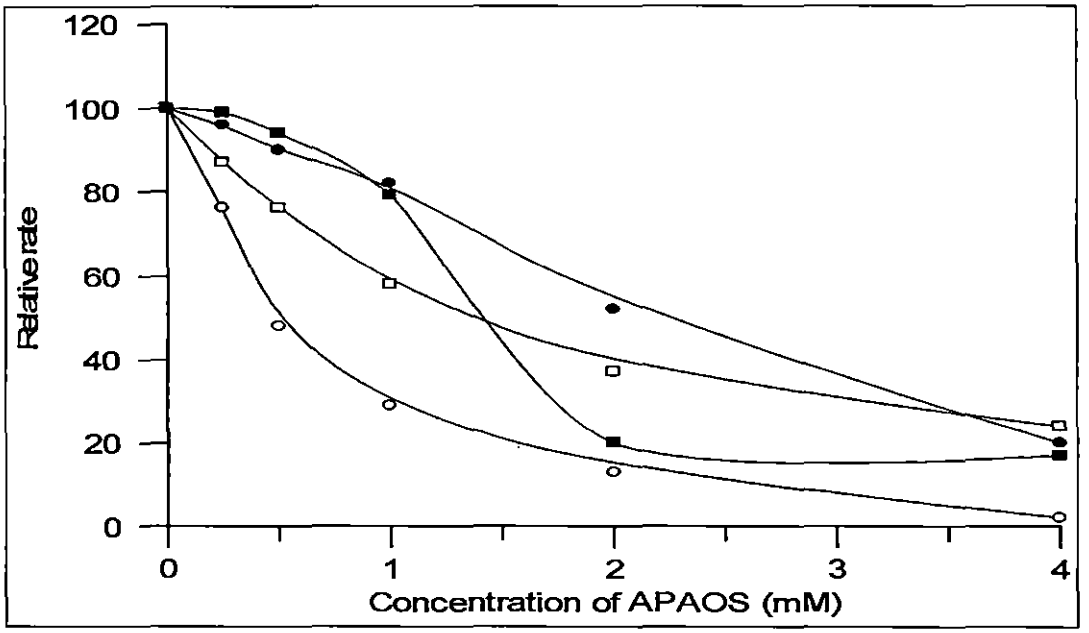


Fig. 6.5. Effect of ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by 4-aminophenylarseneoxide-N-sulphonic acid [APAOS]. Assay was made as described in Materials and Methods. Each point represents the mean of four experiments.

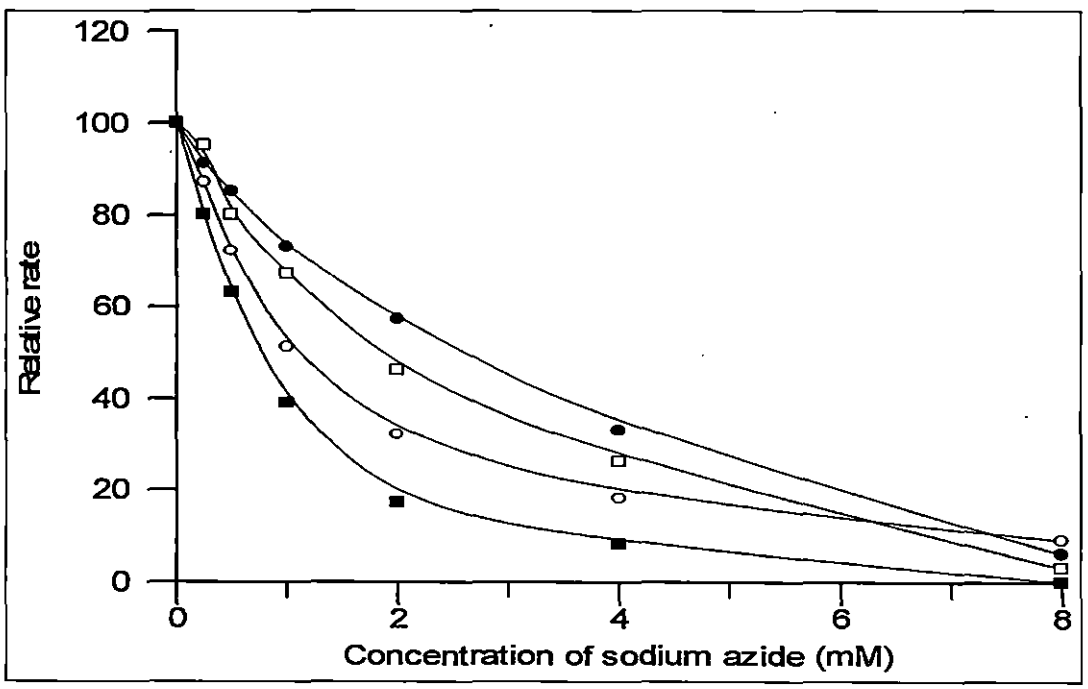


Fig. 6.6. Effect of ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by sodium azide. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

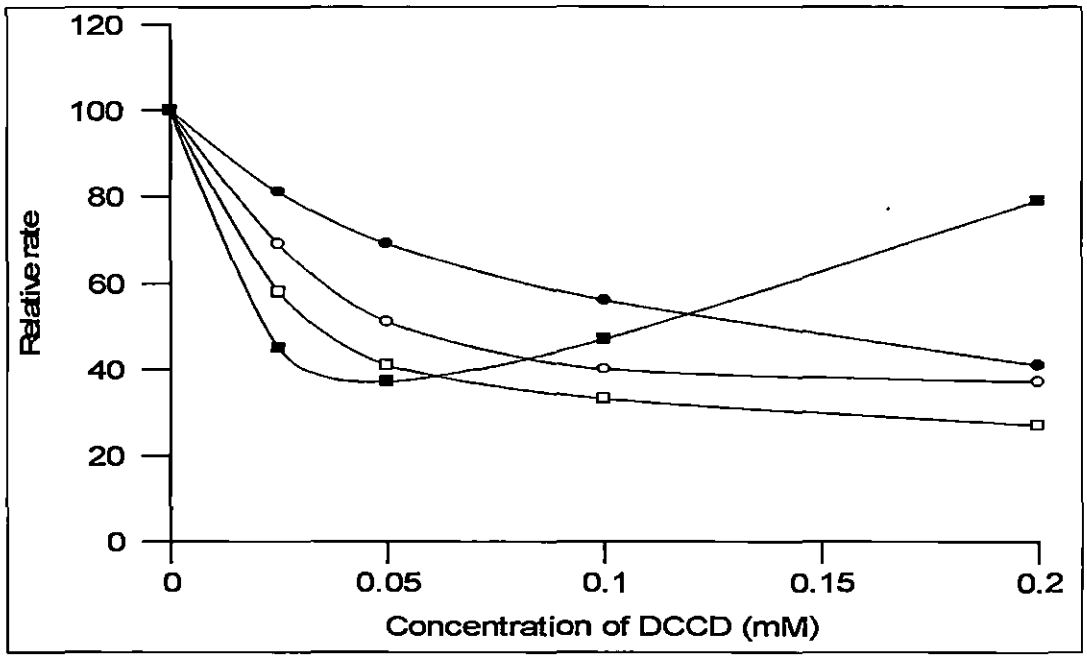


Fig. 6.7. Effect of ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by dicyclohexylcarbodiimide [DCCD]. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

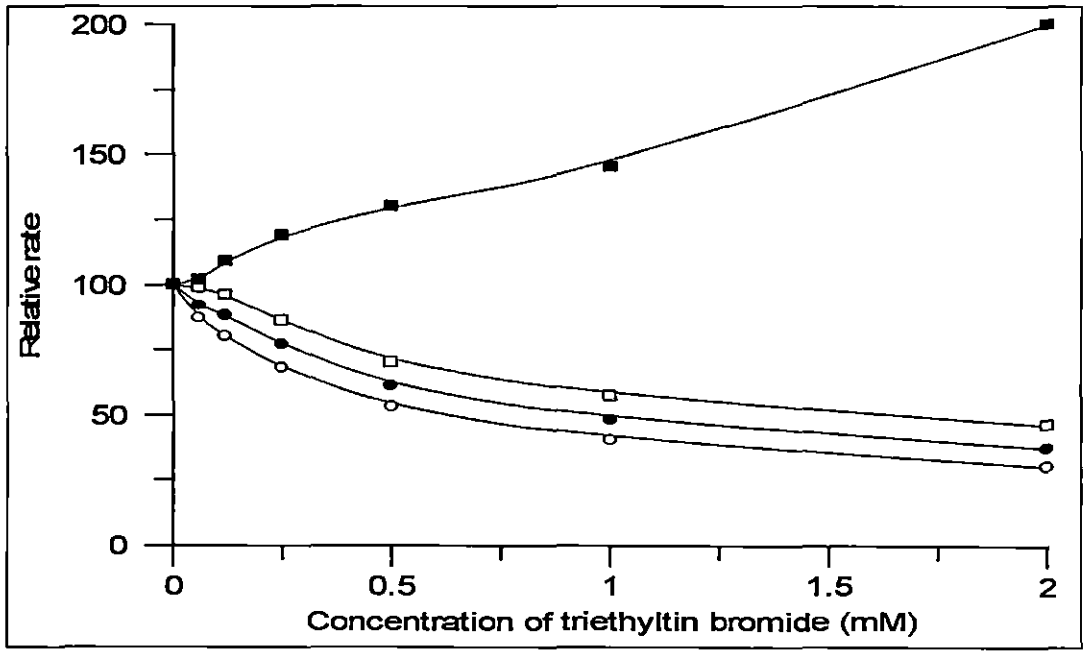


Fig. 6.8. Effect of ALA, ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by triethyltin bromide (TTB). Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

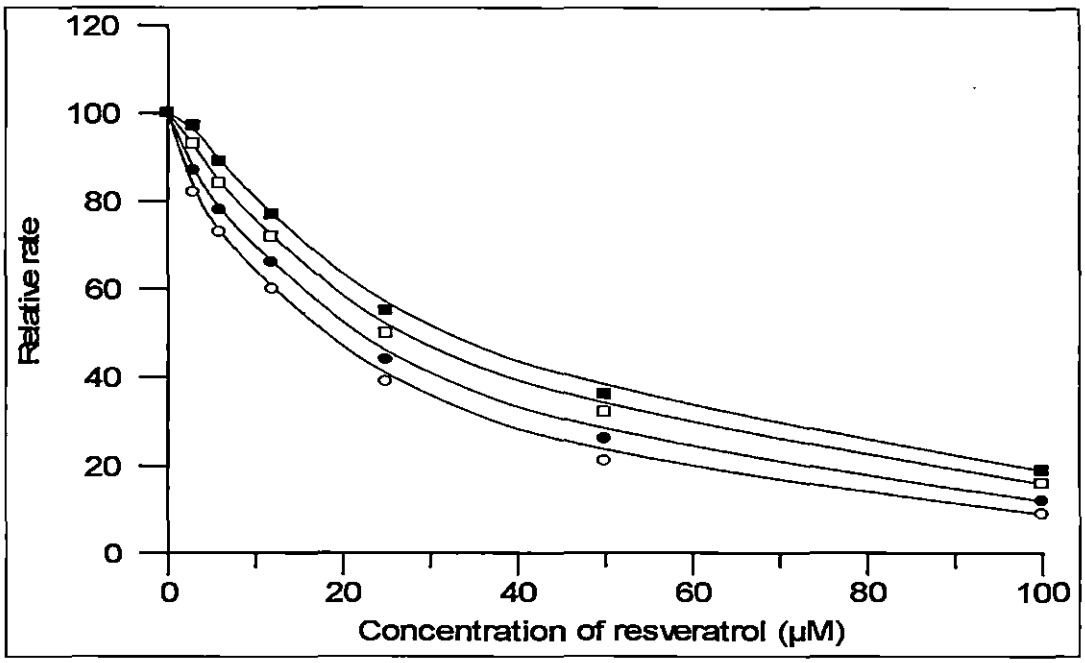


Fig. 6.9. Effect of ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by resveratol. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

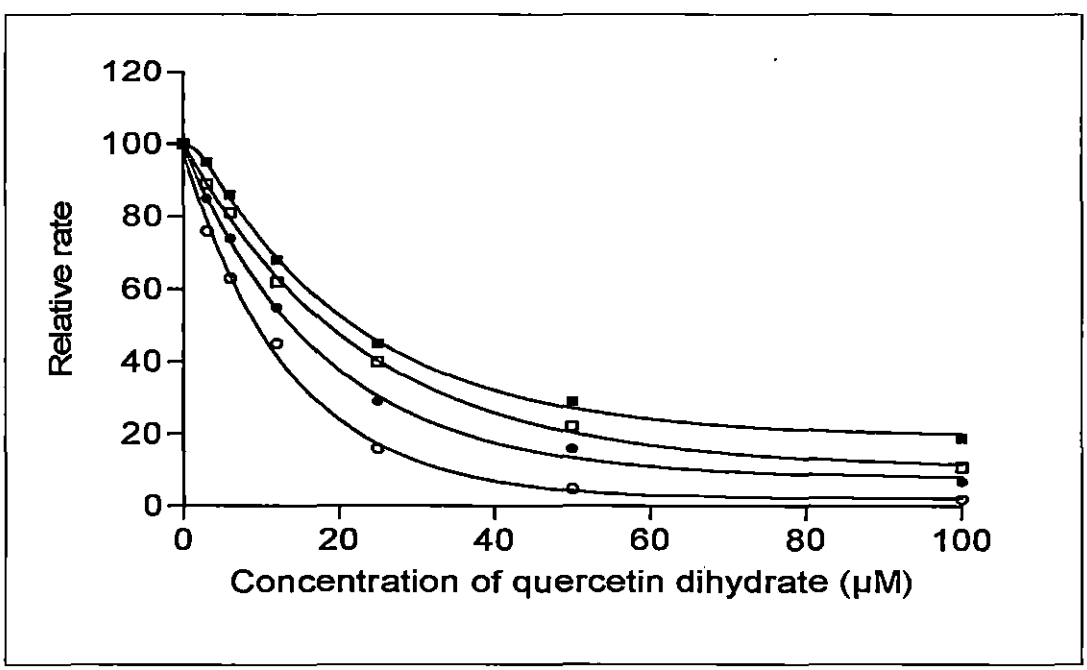


Fig. 6.10. Effect of ALA, ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by quercetin dihydrate. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

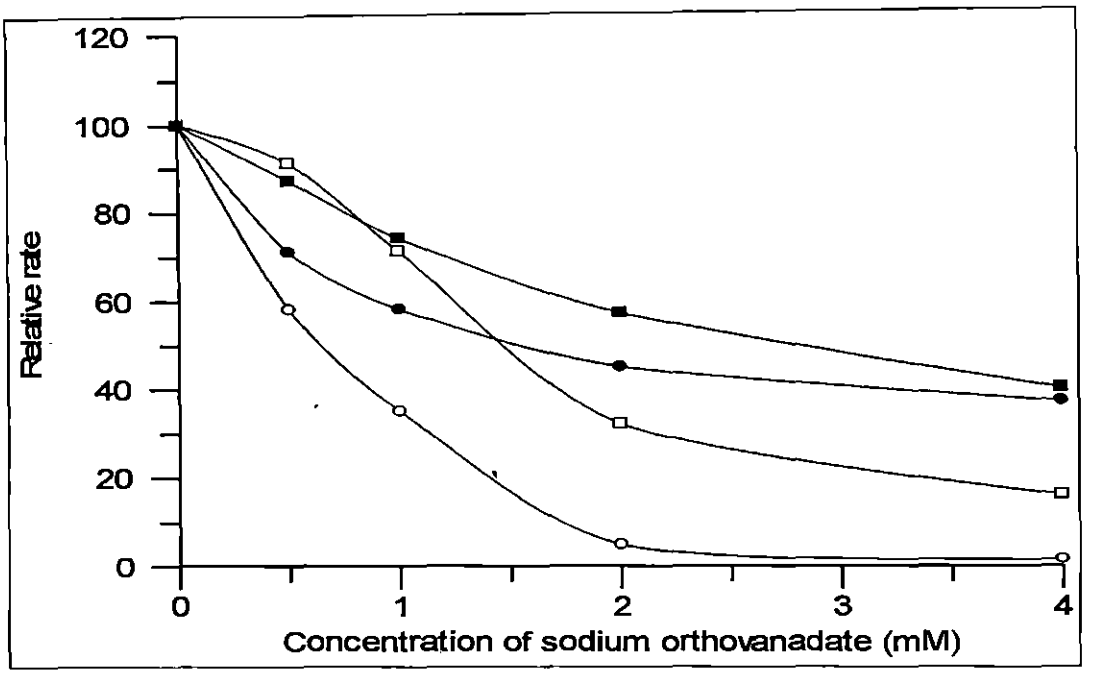


Fig. 6.11. Effect of ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by sodium orthovanadate. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

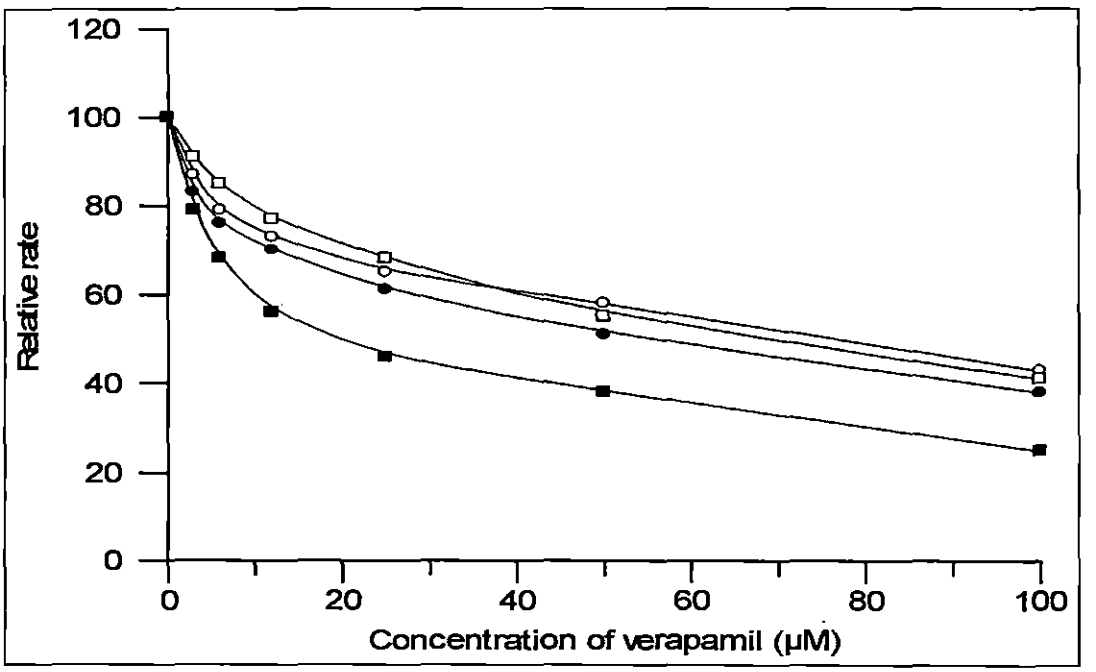


Fig. 6.12. Effect of ALA, ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by verapamil. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

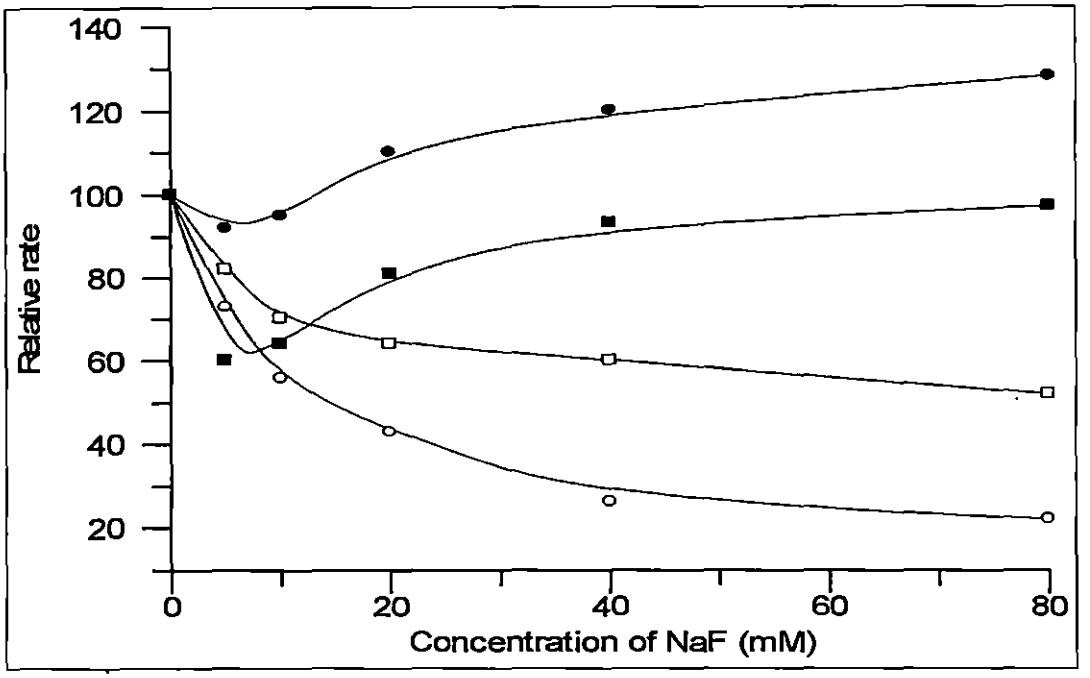


Fig. 6.13. Effect of ALA, ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by sodium fluoride. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

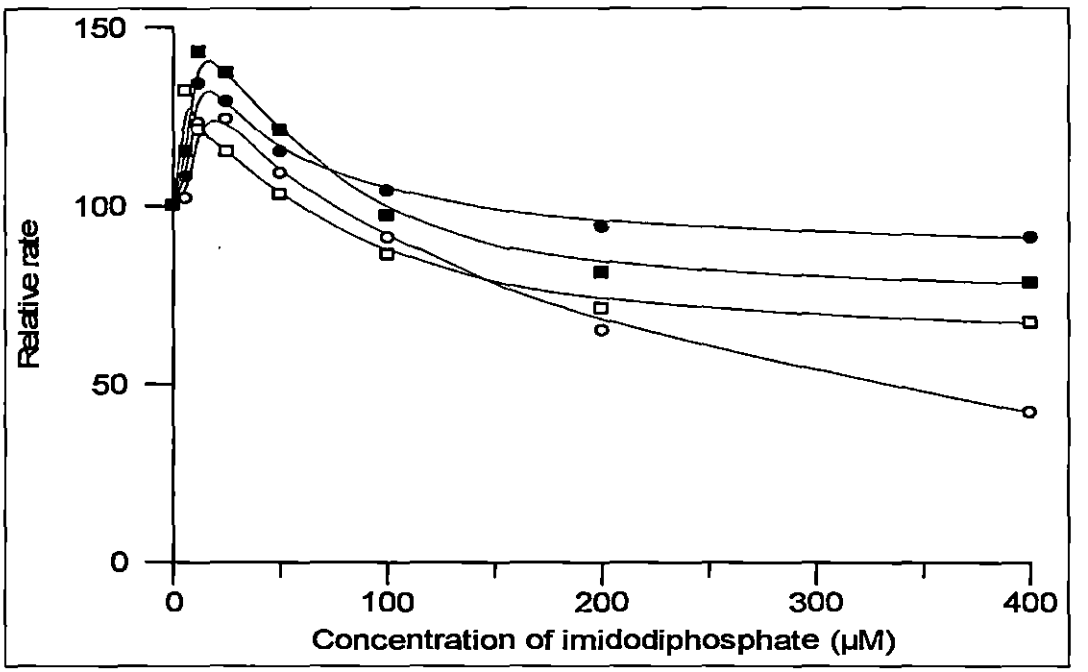


Fig. 6.14. Effect of ALA, ALA, (○); DTNS, (●); NQSA, (□); and $K_3Fe(CN)_6$, (■); reduction inhibition in *L.donovani* promastigotes by imidodiphosphate (IMD). Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

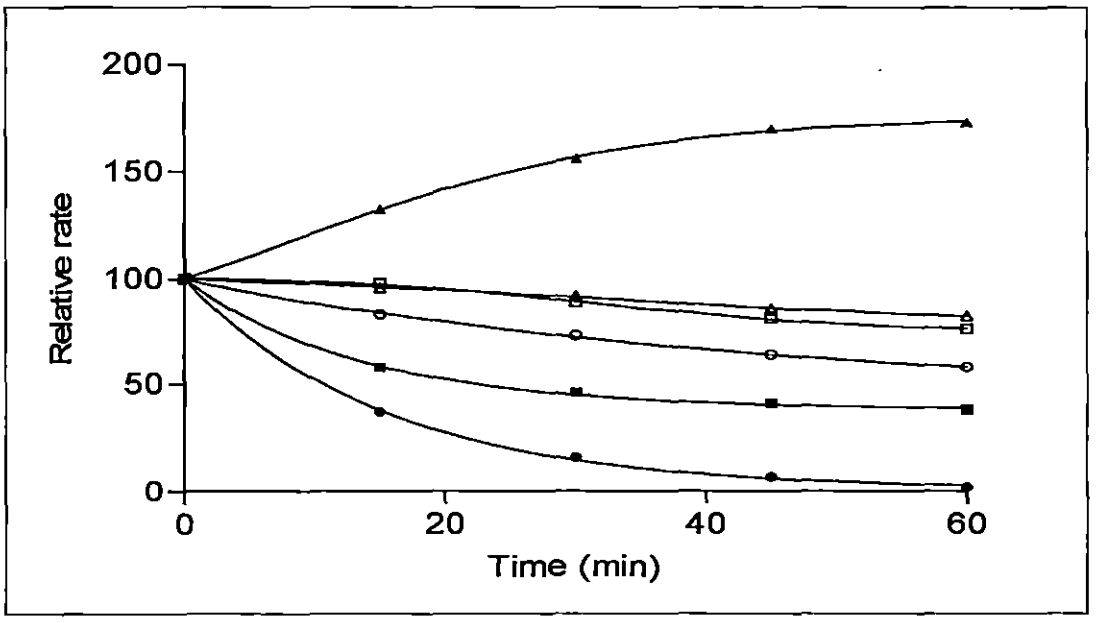


Fig. 6.15. Effect of near-ultraviolet irradiation on oxygen uptake and transplasma membrane electron transport activities of LDC. Cells were either photoinactivated or kept in dark under the conditions described in Materials and methods.(○), oxygen uptake of dark exposed cells; (●), oxygen uptake of UV exposed cells; (□), ALA reduction of dark exposed cells; ■, ALA reduction of UV exposed cells; (△), ferricyanide reduction of dark exposed cells; (▲), ferricyanide reduction of UV exposed cells. Each point represents the average value of four experiments.

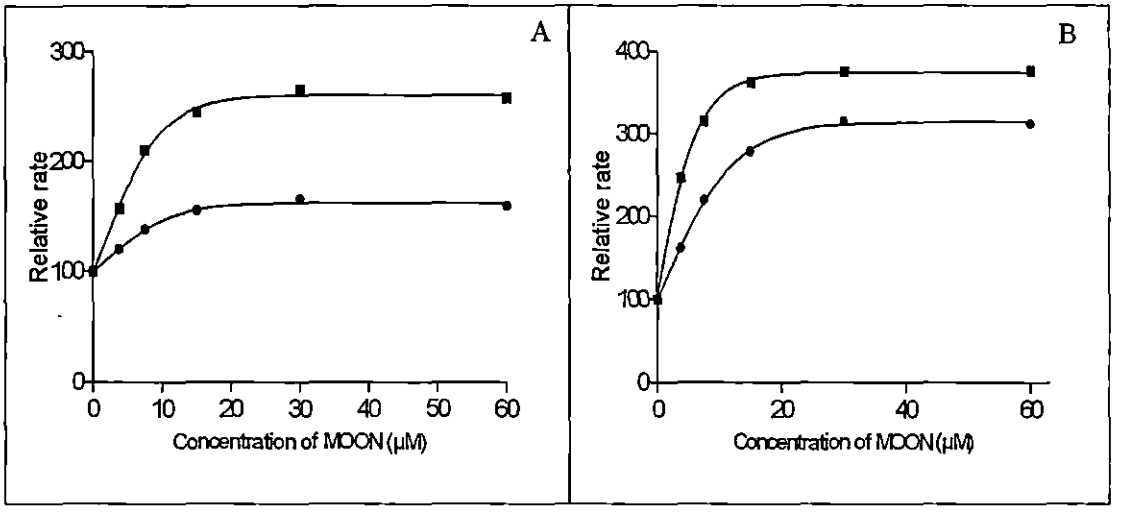
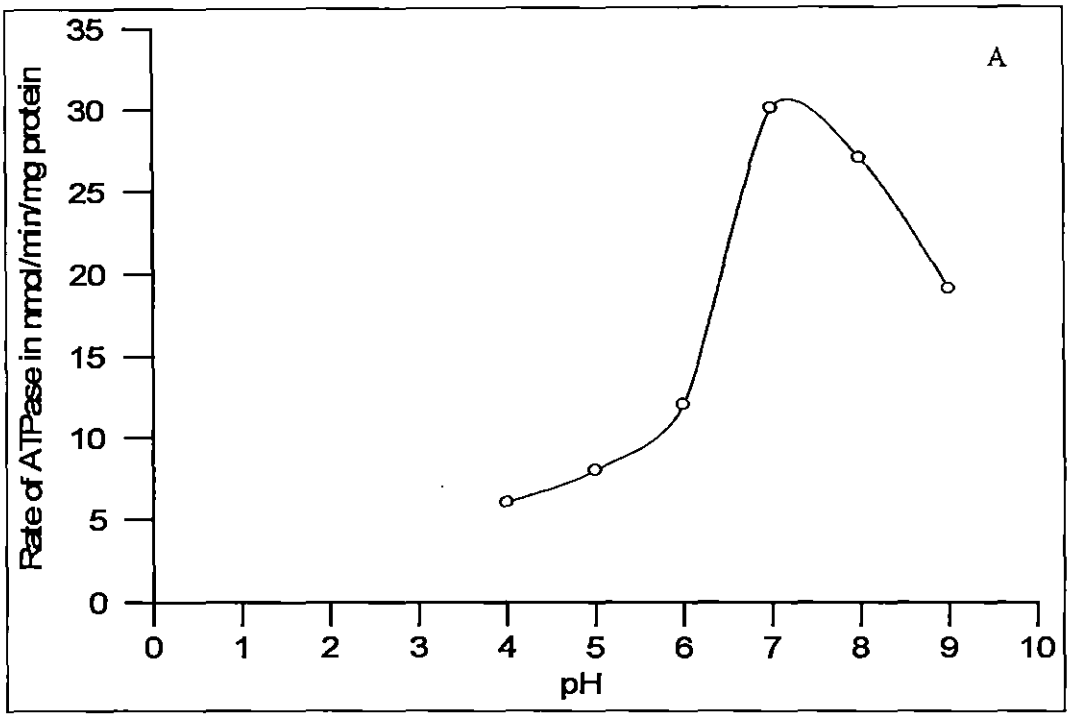


Fig. 6.16. U.V. irradiated LDC was used for various transPMET activities. The LDC was photo inactivated under the conditions described in materials and methods. A: measurement of ALA reduction with MOON, (●); and MOON+octadecylamine, (■). B: measurement of ferricyanide reduction with MOON, (●); and MOON+octadecylamine, (■). Each point represents the average value of four experiments.



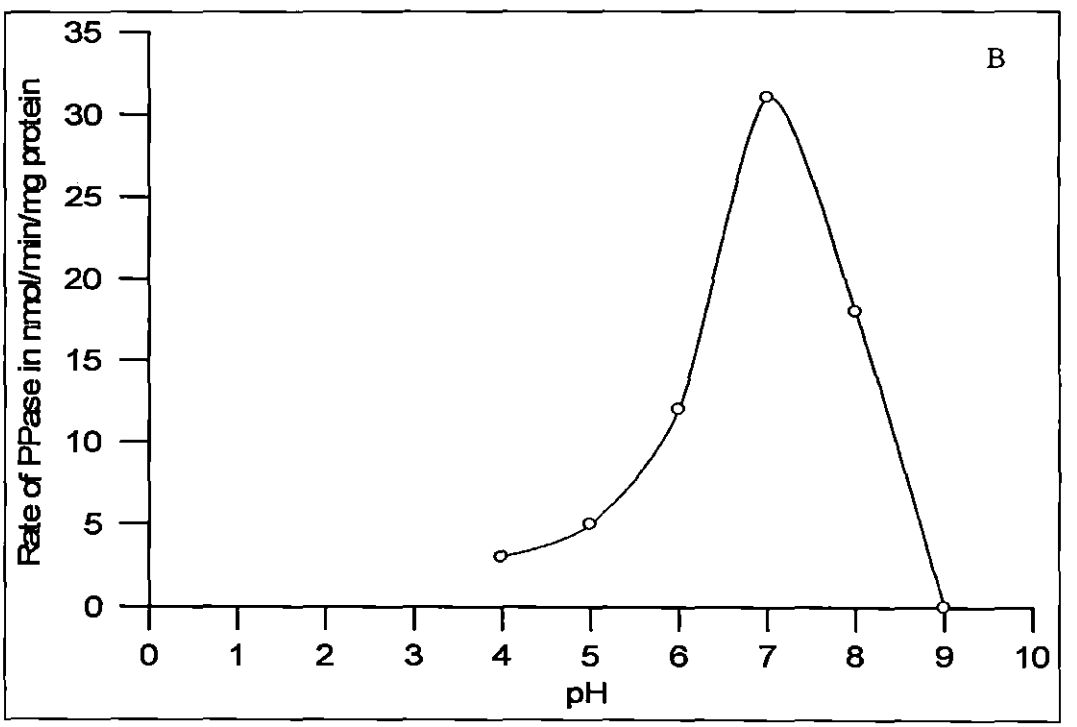


Fig. 6.17. Effect of pH on ATPase (A) and PPase (B) activities in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

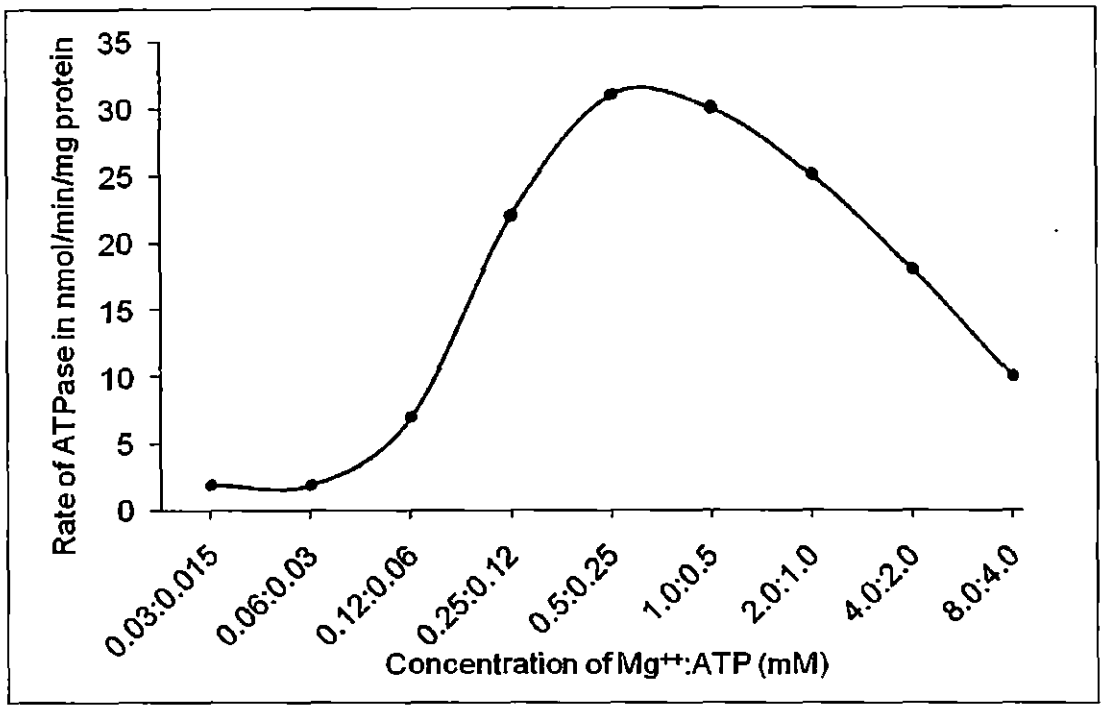


Fig. 6.18. Saturation curve of Mg^{++} and ATP on ATPase activitiy in digitonin permeabilized *L.donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

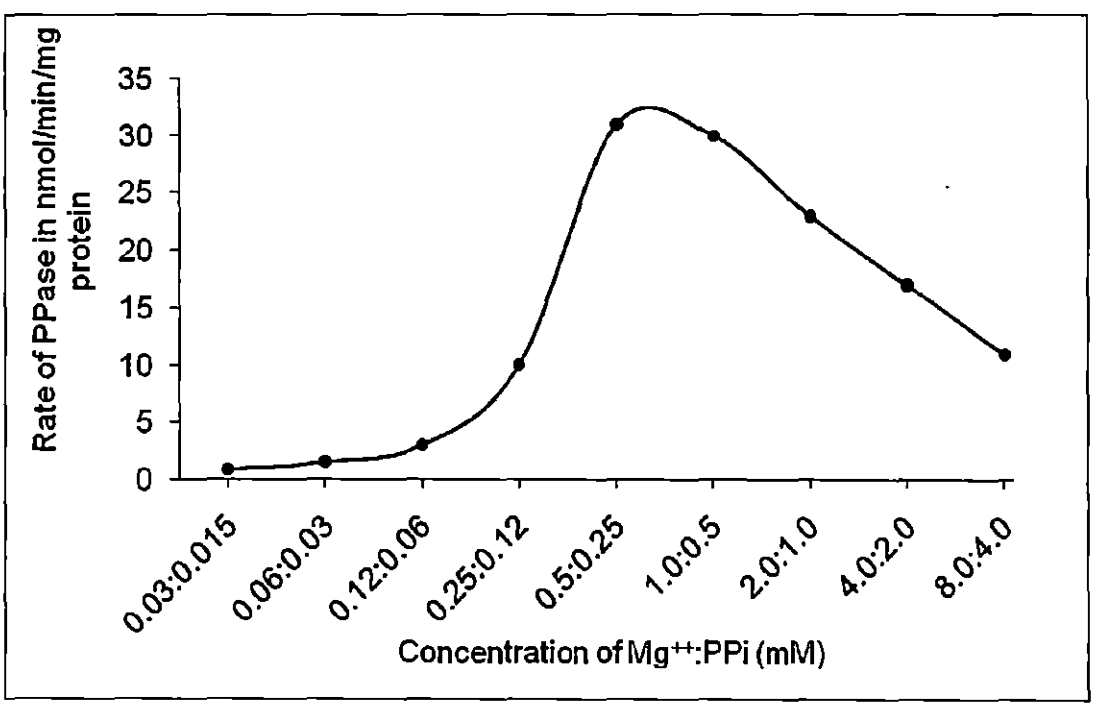


Fig. 6.19. Saturation curve of Mg^{++} and Pyrophosphate on PPase activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

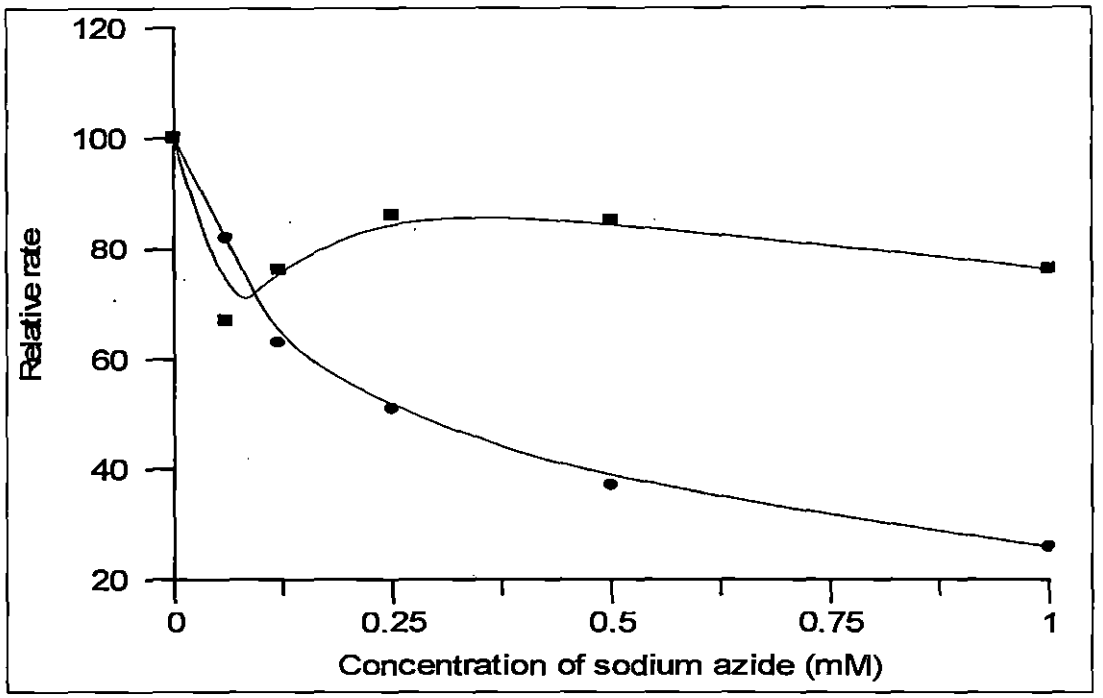


Fig. 6.20. Effect of sodium azide on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

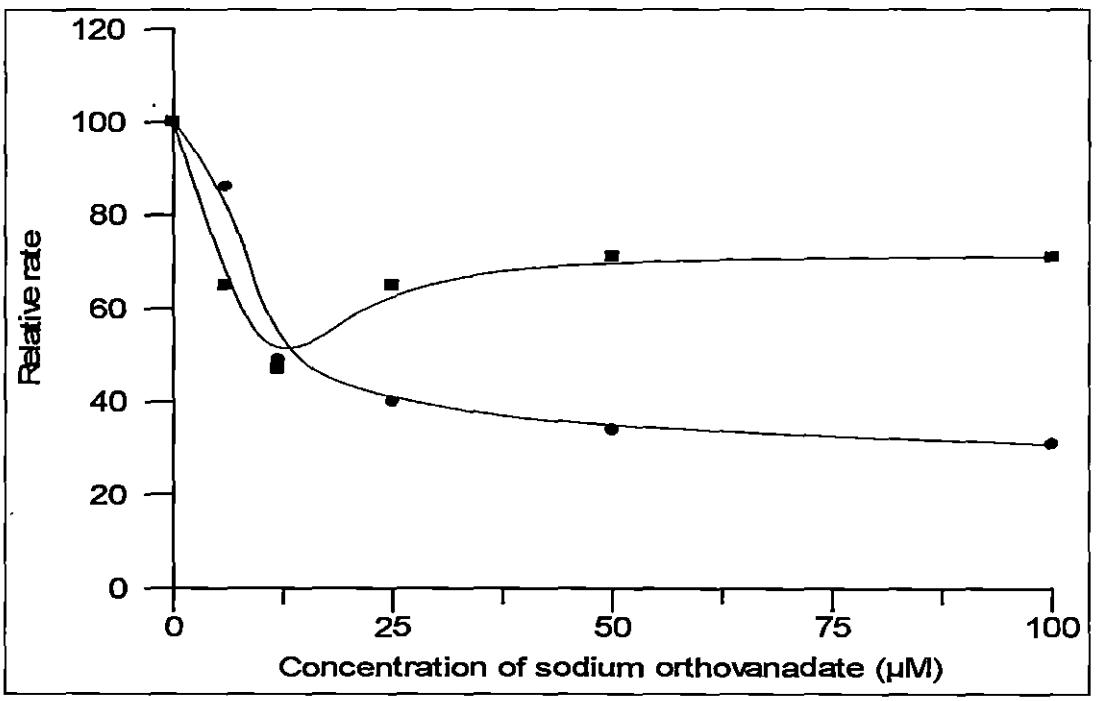


Fig. 6.21. Effect of sodium orthovanadate on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

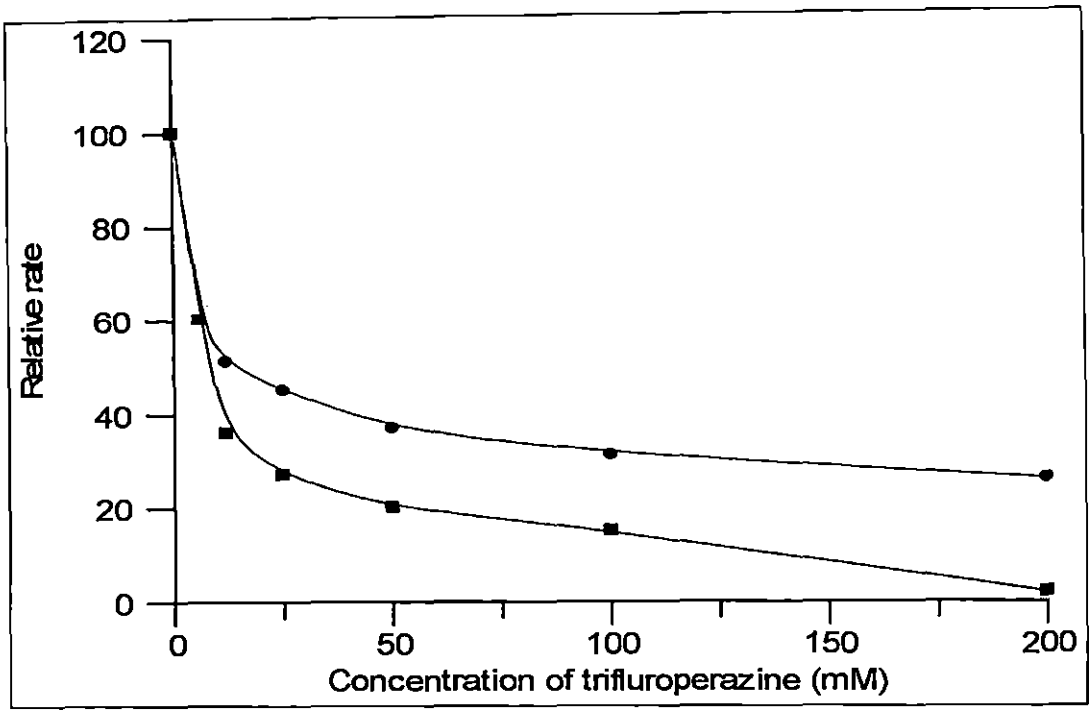


Fig. 6.22. Effect of trifluoperazine on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

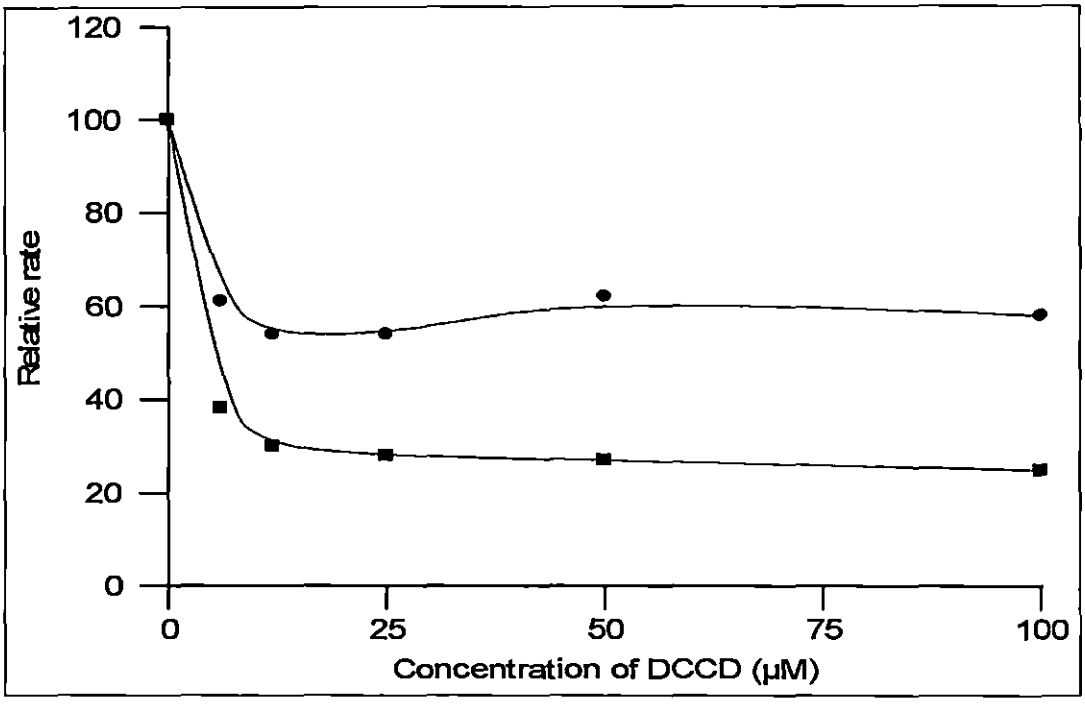


Fig. 6.23. Effect of N, N-dicyclohexyl carbodimide (DCCD) on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

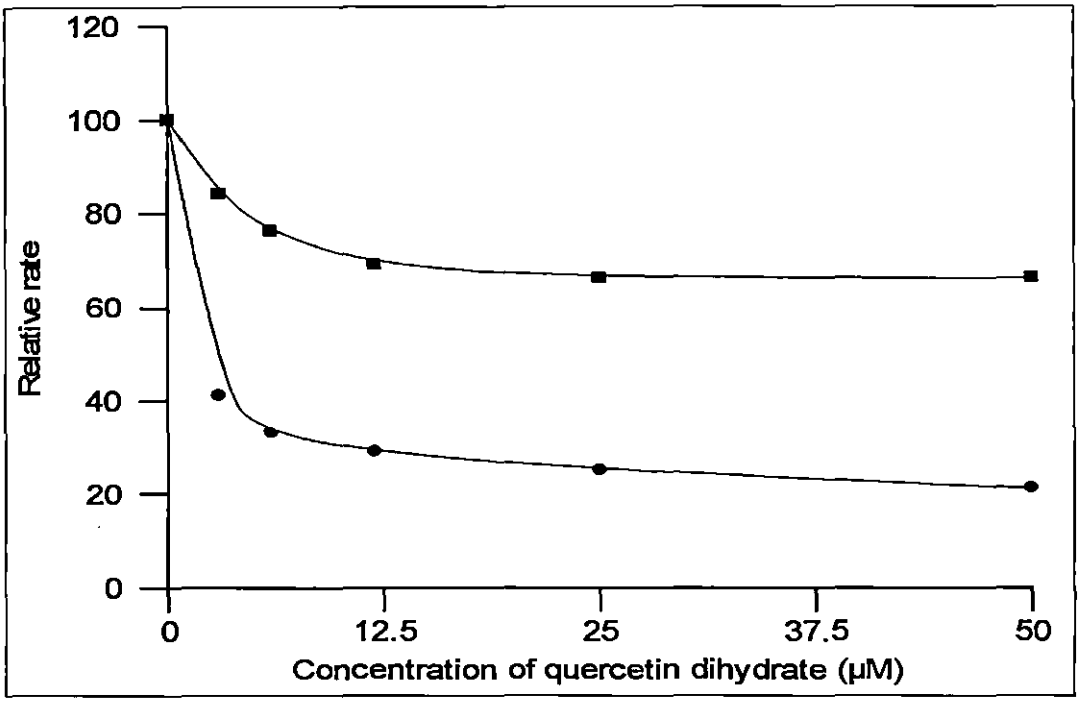


Fig. 6.24. Effect of quercetin on on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

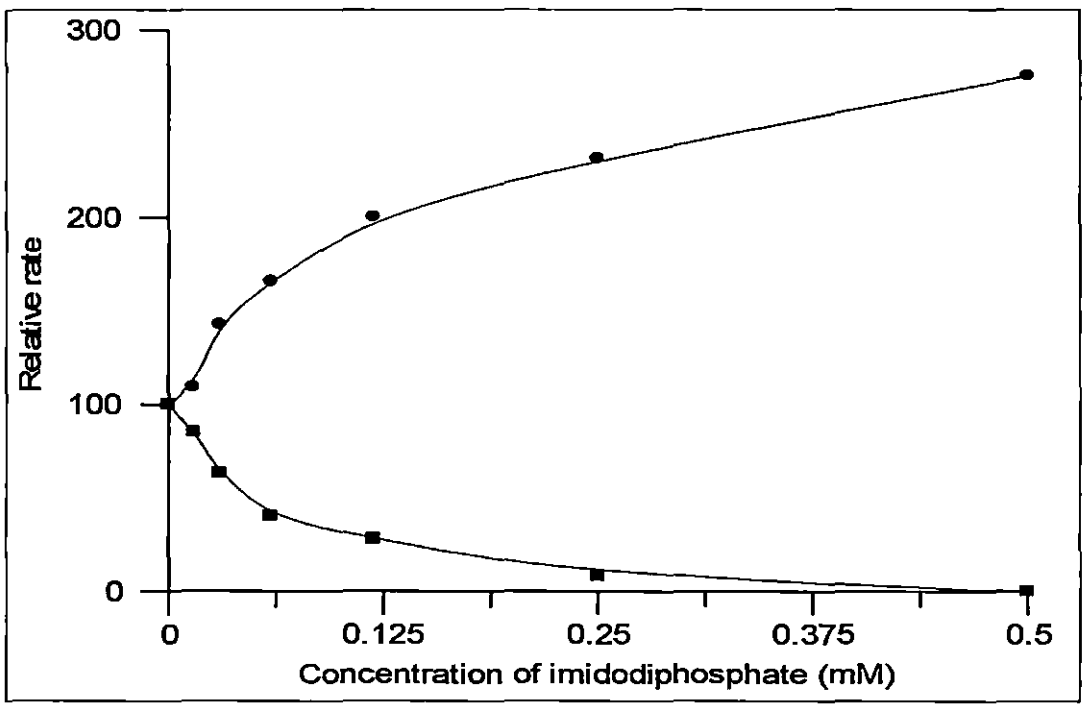


Fig. 6.25. Effect of imidodiphosphate on on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

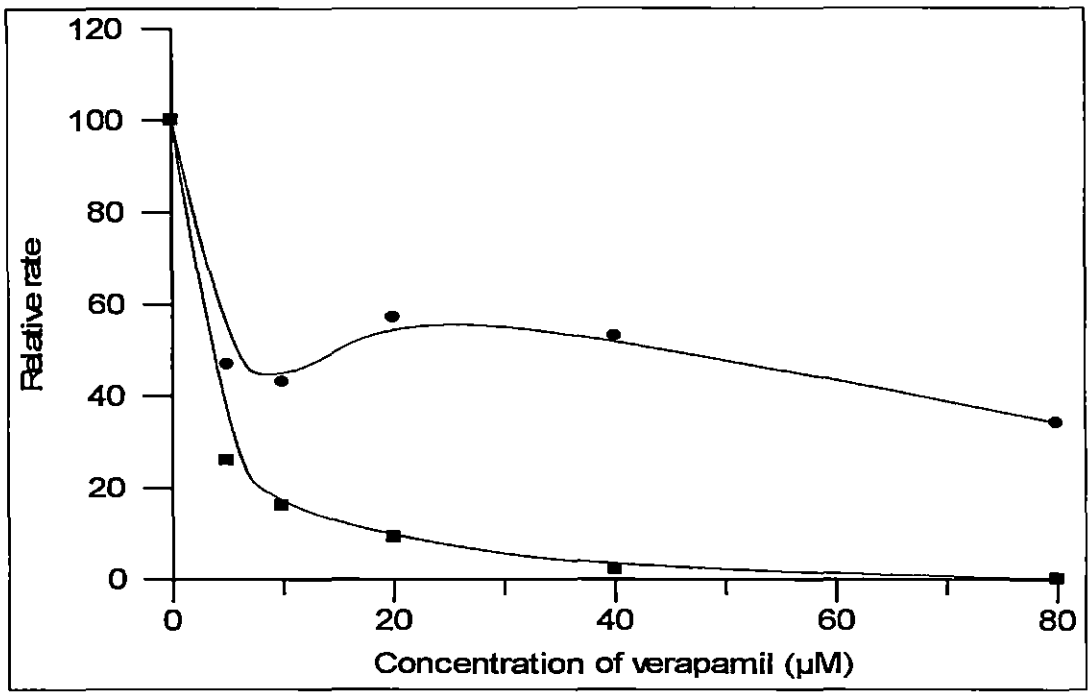


Fig. 6.26. Effect of verapamil on on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

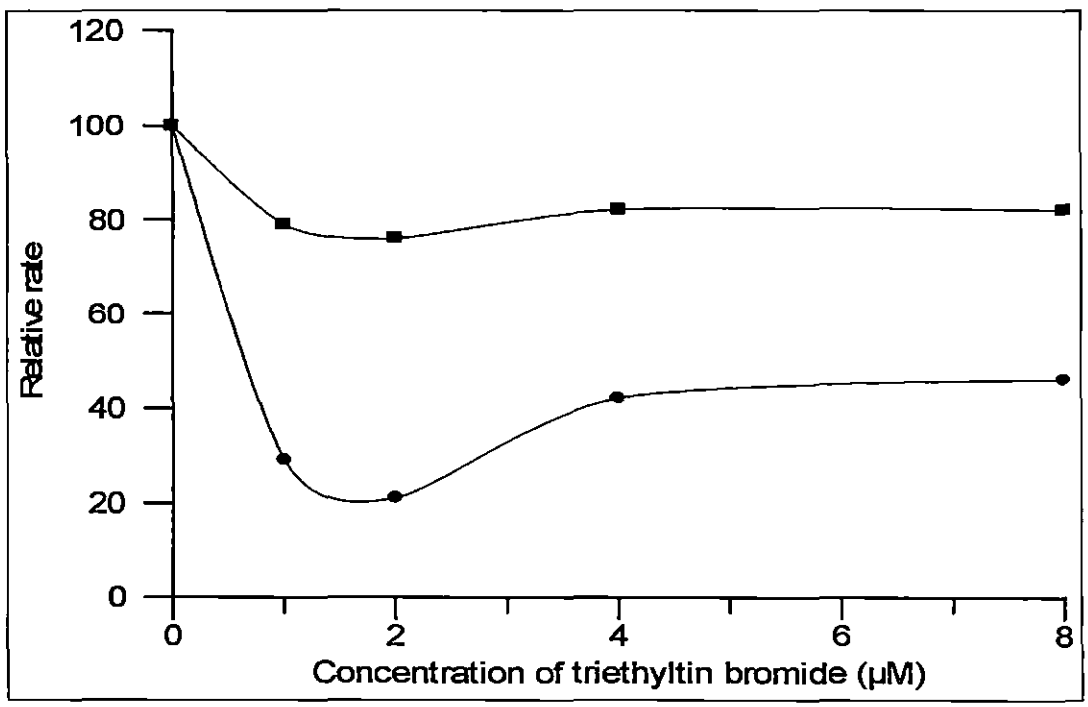


Fig. 6.27. Effect of triethyltin bromide on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

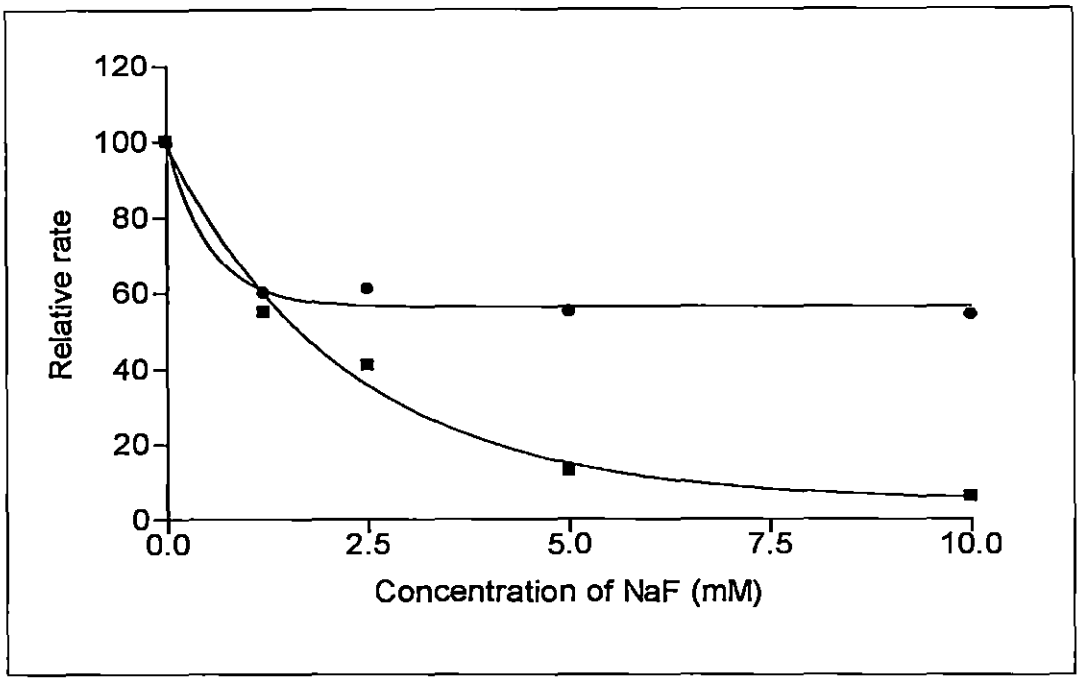


Fig. 6.28. Effect of sodium fluoride on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

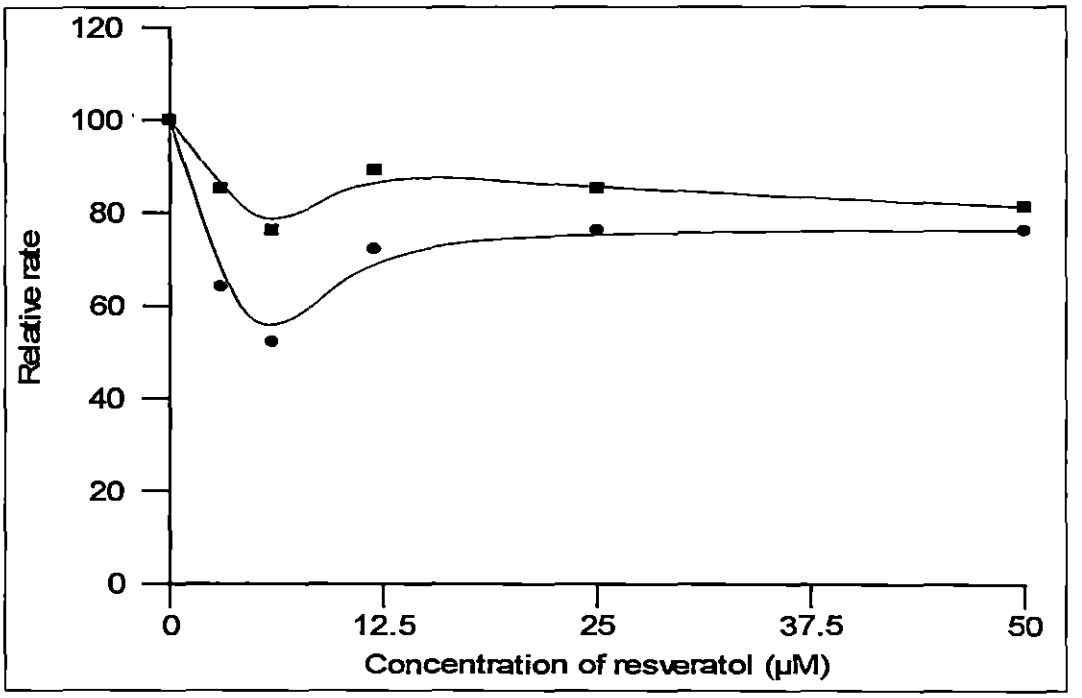


Fig. 6.29. Effect of resveratol on ATPase (●) and PPase (■) activity in digitonin permeabilized *L. donovani* promastigote. Assay was made as described in Materials and methods. Each point represents the mean of four experiments.

Chapter- 7

Discussion and Conclusion

7. DISCUSSION AND CONCLUSION

ALA, NQSA and $K_3Fe(CN)_6$ being impermeable to cell, were assumed to act at the level of the plasma membrane. These compounds act as natural electron acceptor of transplasma membrane electron transport (transPMET). In transPMET the redox enzymes can transfer electrons from cytosolic electron donors to artificial non-permeable electrons acceptors. The transmembrane enzyme(s) can reduce compounds with a negative redox potential down to -290 mV. The release reduced compounds don't affect the transPMET ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction. The high capacity of LDC to transfer electrons from intracellular donors to extracellular electron acceptors like ALA, NQSA, $K_3Fe(CN)_6$ and oxygen is poorly understood [426, 427]. We have synthesized DTNS as impermeable electron acceptor of transPMET system. In this report, we have presented evidence for the presence of a numerous ectoredox enzymes to donate electron to impermeable electron acceptors with different redox potential. It is apparent that impermeable electron acceptor DTNS could be as useful and as specific as ALA, NQSA and $K_3Fe(CN)_6$. We feel that reagents of this type will be important in determining the position and orientation of redox carriers in transPMET system. In both ALA and DTNS, disulfide bridge is reduced after accepting electrons from transPMET system. They differ from each other in respect to the nature of disulfide bridge. In ALA, disulfide bridge is intramolecularly linked with an aliphatic chain whereas in DTNS disulfide bridge is intermolecularly linked with the aromatic ring. Due to these differences DTNS showed higher affinity and reaction rate compared to ALA (Tables 6.1 and 6.2). It is noteworthy that ALA and DTNS showed same pH optimum, which differ little from the pH optimum of NQSA and $K_3Fe(CN)_6$ (Table 6.1).

It is evident from the observation of Table 6.3 that no significant effect on NQSA reduction was found with cells exposed to Na^+, to K^+ or to other cations substituting for Na^+ and K^+. Our data provide evidence that the reduction of ALA and DTNS requires the presence of K^+ for maximal activity, whereas for the reduction of ferricyanide, presence of Na^+ is essential. Stiles et al. [428] have shown that in *Leishmania donovani* surface membrane, P-type ATPases resembling fungal K^+ and Na^+-ATPases are present. The possibility that these K^+ and Na^+-ATPases are coupled with the transPMET system cannot be ruled out, however. Absence of Cl^- anion has stimulating effect for the

reduction of four electron acceptors. It has already been observed that Cl^- transport plays an important role in LDC in putative pH regulatory mechanism [429]. It appears from these observations and also from the tables 6.5- 6.7 that though ALA and DTNS are both disulfide redox reagents, they are not receiving electrons from the same redox site. Similarly, NQSA and $K_3Fe(CN)_6$ differ from each other with respect to the site of redox interaction. Hence, ALA, DTNS, NQSA and $K_3Fe(CN)_6$ may have different sites of redox interaction.

Table 6.4 shows that the four electron acceptors of transPMET system are considerably stimulated by anaerobiosis. Anaerobic stimulation have placed ALA, DTNS in one group and NQSA, $K_3Fe(CN)_6$ to another group. This makes the main transPMET chain into two divisions and signifies the relative importance of the sites of reduction. The major effect under normal conditions appears to be due to oxygen inhibition of the redox sites leading to ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction (Tables 6.4, 6.8). Anaerobic conditions increased ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction so that dihydrolipoic acid, 5-mercapto- 2-nitroaniline-N-sulphonic acid, hydroquinone of NQSA and $K_3Fe(CN)_6$ production increased sharply, respectively, in the incubation mixtures where the O_2 level is being depleted. The stimulation produced by anaerobiosis is probably due to inability in functioning of oxygen redox site which resulted in an increased channeling of electrons into the ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction pathway; however, it is surprising that $K_3Fe(CN)_6$ did not produce O_2 uptake inhibition in LDC whereas ALA, DTNS and NQSA inhibited the O_2 uptake (Table 6.8), an observation concordant with the results of table 6.4. We have observed that incubation of LDC in presence of $K_3Fe(CN)_6$ resulted in increased uptake of 14C-deoxy-d-glucose (data not presented). It is tempting to speculate that increased influx of d-glucose has generated higher level of NADH/NADPH and consequently higher rate of O_2 uptake, which compensated O_2 uptake inhibition by $K_3Fe(CN)_6$.

Antimycin A has been shown to be a potent inhibitor of respiratory chain; the site of action has been placed at the level of Qi site binding and blocking electron transfer from the heme center to ubiquinone [430]. Table 6.5 shows that the ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase system is inhibited significantly in different order by antimycin A. Antimycin A showed complete inhibition of O_2 uptake in LDC (Table 6.8). Thus, on the basis of such observations, it would appear that ALA, DTNS, NQSA, $K_3Fe(CN)_6$ and

oxygen are reduced by the involvement of a b-cytochrome in unrecognized component of the transPMET system. It is obvious from these results that the site of antimycin An inhibition will be before the ALA, DTNS, NQSA, $K_3Fe(CN)_6$ and O_2 redox sites. The effects of HQNO, rotenone and capsaicin reported in tables 6.5 and 6.8 are of interest in view of the above conclusion. HQNO was found to be a potent inhibitor of electron transport. The locus of the action appears to be the same as, or proximate to, that of antimycin A [430]. The inhibition by HQNO of ALA reduction was much less potent than by antimycin A. It is not likely that b-cytochrome involved in ALA reduction is identical with the b-cytochrome of DTNS, NQSA and $K_3Fe(CN)_6$ reductase complex. HQNO inhibition of trans-PMET system has placed ALA and DTNS in one group and NQSA, $K_3Fe(CN)_6$ to another group. Rotenone and capsaicin are among the most widely used inhibitors of mitochondrial and bacterial electron transport because they selectively inhibit the oxidation of NADH-linked substrates [431, 432]. The binding site of rotenone responsible for inhibition of NADH oxidation in complex I of mitochondrial electron transport chain is localized in the O_2 site of the dehydrogenase and not between NADH and flavoprotein [433]. The effect of these inhibitors on the reductase activity is summarized in Tables 6.5 and 6.8. Rotenone was found to be a potent inhibitor of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase activity, but O_2 uptake was only partially inhibited. This observation supports the idea that transmembrane electron transport depends on the production of a cytosolic reducing agent, such as NADH, the oxidation of which liberates electrons which travel to the outside of the cell via a transPMET chain. Partial inhibition of O_2 uptake by rotenone is suggestive to the presence of an O_2 redox site not sensitive to rotenone. Capsaicin, a new naturally occurring inhibitor of proton-pumping NADH quinone oxidoreductases (NDH-1) present in the respiratory chain of various species of bacteria and mitochondria, can be divided into two groups [434, 435]. One group of enzymes that bear the energy-coupling site is designated as NDH-1 and the other group that does not as NDH-2. In response of capsaicin to four electron acceptors they can be divided into highly sensitive NQSA, $K_3Fe(CN)_6$ reductase group and less sensitive ALA, DTNS group (Fig. 6.1.A, Table 6.5). Effect of capsaicin on O_2 uptake inhibition was similar to rotenone (Table 6.8). Here, it is noteworthy that capsaicin do not interrupt rotenone insensitive electron transfer from NADH [436]. The sensitivity to ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction inhibition in LDC by capsaicin supports

the notion of the presence of an energy-coupling site (NDH-1) in transPMET system. This finding correlates the inhibition of plasma membrane NADH oxidase activity Capsaicin caused a dose-dependent decline in LDC growth that closely paralleled its inhibition of reductase activities. These results suggest that transPMET helps to maintain a stable redox environment required for LDC viability.

TTFA is a well-known metal coordinate compound of lipophilic nature [437] and has been shown to be a potent inhibitor for succinate-coenzyme Q reductase of complex II in aerobic tissues such as bovine heart [438, 439]. It is of interest to ascertain the inhibition of transPMET reductase by this compound. As shown in Tables 6.5 and 6.8, about 50% to 85% of reductase activities were inhibited by 1.16 mM to 2 mM TTFA. In contrast, mammalian succinate-coenzyme Q reductase was inhibited about 90% by less than 0.35 mM TTFA [440]. Since TTFA inhibits the reoxidation of the iron–sulphur center (S-3) of bovine complex II [441, 442], the sensitivity of LDC transPMET system towards TTFA may indicate a pathway for electron transfer involving iron– sulphur center. This finding correlates the inhibition of cell surface iron-sulphur center with inhibition of growth by TTFA (Fig. 6.2.B). Effects of mitochondrial complex IV inhibitor cyanide on transPMET reductase system are also included in Tables 6.5 and 6.8. Cyanide showed very strong inhibitory effect of NQSA and $K_3Fe(CN)_6$ reductase system, whereas it showed moderate inhibitory effect on ALA, DTNS and O_2 uptake. The inhibition produced by cyanide is probably due to the inhibition of a heme containing cytochrome. Strikingly, however, cyanide failed to inhibit O_2 uptake completely (Table 6.8) and the extent of inhibition is correlated with the ALA and DTNS reductase inhibition (Table 6.5). Thus, on the basis of such observations, it would appear that NQSA and $K_3Fe(CN)_6$ are reduced by an interaction with cyanide-sensitive cytochrome system and ALA, DTNS reduction inhibition by a cyanide-sensitive factor which forms a separate branch pathway. Cyanide insensitive respiration occurs in various plants and protozoa [443]. An alternative terminal oxidase, cytochrome O, has been found in several kinetoplastid flagellates [444]. The possible explanation of the incomplete inhibition of whole cell respiration is the relatively low sensitivities of the leishmanial enzymes and participation of oxygen in other cellular reactions.

The magnitude of O_2 uptake inhibition by ALA, DTNS and NQSA can be correlated with the anaerobic stimulation of ALA, DTNS and NQSA (Tables 6.4 and 6.8). Anaerobiosis

stimulated ALA, DTNS and NQSA reduction 48%, 44% and 97%, respectively. At the same concentration of ALA, DTNS and NQSA oxygen uptake was inhibited 54%, 52% and 71% respectively. Lack of O_2 uptake inhibition by ferricyanide may be explained by the enhanced rate of glucose uptake in presence of ferricyanide. We, therefore, conclude that transPMET system is intimately linked with the O_2 reduction site as terminal electron acceptor. Tables 6.6 and 6.8 gives the results obtained by adding p-chloromercuribenzene sulfonic acid on ALA, DTNS, NQSA, $K_3Fe(CN)_6$ reductase system and O_2 uptake. The importance of sulfhydryl groups for ALA, DTNS, $K_3Fe(CN)_6$ reductase system and O_2 uptake is shown by the almost complete inhibition produced by nonpermeable PCMBS. In contrast, NQSA reductase system was only partially inhibited by PCMBS. Other sulfhydryl group(s) inhibitors, which can be conveniently discussed in this section, are cadmium chloride and sodium arsenite that blocks the vicinal sulfhydryl groups. Tables 6.6 and 6.8 give the results of the effects of cadmium chloride and sodium arsenite on the transPMET reductase system. It can be seen that cadmium chloride and sodium arsenite can distinguish between the reductase systems and suggest the importance of vicinal sulfhydryl groups for different reductase systems (Table 6.6). These vicinal sulfhydryl groups appear to be involved in disulfide–dithiol interchange activity that intimately involve in redox signaling in biological processes [445, 446].

Another important finding is that valinomycin, which creates a specific K^+ permeability and monensin, which creates a specific Na^+ permeability had marked effects on redox enzymes of transPMET system (Table 6.7). It should also be emphasized that the O_2 uptake by LDC was more sensitive to valinomycin than monensin. Promastigotes in the insect gut and in the blood stream are exposed to an environment whose pH is neutral and K^+ is low. Promastigotes then invade the macrophages via the recruitment of lysosomes, whose environment is acidic (pH 4.5–5.5). The parasite then escapes from the parasitophorus vacuole to the cytosol to multiply as amastigote [85]. In the cytosol of the host cell, the pH is neutral (pH 7.0–7.2), the concentration of K^+ is high (135 mM) and that of Na^+ is low (15 mM). L. donovani likely developed effective mechanisms to efficiently cope with the extremes in environments it experiences. These mechanisms would likely involve ion pumps and carriers located on the parasites plasma membrane. Since both K^+ and Na^+ ion permeability affects the transPMET system, we may presume that transPMET system regulate K^+ and Na^+ ion permeability. Ponte-Sucre et al. have

reported that the K^+ channel blockers 4-aminopyridine and glibenclamide impaired growth and survival of *Leishmania* [447]. When we evaluated the effect of 4-aminopyridine on transPMET, it was observed that 4-aminopyridine strongly inhibited ALA and ferricyanide reduction, whereas DTNS and NQSA reduction was stimulated substantially. Valinomycin-induced respiration inhibition of LDC appears to be due to loss of cytoplasmic K^+. Obliteration of growth was also observed in the presence of K^+ sparing Na^+ channel blocker amiloride (Fig. 6.2.D). The protonophore and uncoupler FCCP inhibits ALA, DTNS and $K_3Fe(CN)_6$ reductase activity. In contrast, NQSA reductase activity was not affected at all (Table 6.7). Since FCCP prevents the formation of pH gradient it appears that ALA, DTNS and $K_3Fe(CN)_6$ reduction by transPMET system is linked with proton translocation.

Tricyclic antipsychotic drug trifluoperazine showed strong inhibition on ALA, DTNS reductase activity and O_2 uptake, whereas it showed moderate inhibition on NQSA reductase activity. In contrast, trifluoperazine showed strong stimulation on $K_3Fe(CN)_6$ reductase activity (Tables 6.5 and 6.8). In light of the current observations, trifluoperazine modulate multidrug resistance by inhibiting P-glycoprotein ATPase [448-450]. In plant mitochondria trifluoperazine, inhibition of electron transport and adenosine triphosphatase activity has been identified [451]. There are a number of reports in the literature of plant-like traits associated with *Leishmania* spp. [452]. Based on these observations, it is tempting to speculate that transPMET system in LDC may be linked with multidrug resistance conferred by glycoprotein. Evidence suggests the presence of a 105-kDa P-type ATPase on *Leishmania donovani* plasma membrane that is mechanistically similar to other P-type enzymes of higher eukaryotes.

Taken in total, these inhibitor/stimulator studies illustrate the presence of different redox sites for four electron acceptors. The possibility that there is more than one site of electron egress to the electron acceptor cannot be ruled out, however. The redox chain appears to be branched at several points, which incorporates b-cytochromes, cyanide sensitive hemoprotein, cyanide insensitive oxygen redox site, NADH linked energy coupling site as judged by capsaicin inhibition, iron–sulphur center and K^+/Na^+ channel. Electron transfer in the transPMET system requires an energized state of the membrane. Capsaicin of the vanilloid type showed its similarity in inhibition with transformed

mammalian cells [453]. Resistance to normal mammalian cell inhibition by capsaicin forms the basis for transPMET as an antileishmanial drug target.

Promastigote form of *L. donovani*, is agglutinated specifically with concanavalin A. Cumulative results demonstrate that ligands similar to α-D-mannosides are constituents of *L. donovani* pellicular membranes [423]. Based on that observation plasma membrane of *L. donovani* promastigote were isolated by concanavalin-A. Peroxidase and oxidase enzymes were selectively labeled with radio labeled iodine (^{125}I) at low temperature. This iodination resulted that more than 90% labeled were recovered in the membrane pellet (pellet I). To remove the bound concanavalin-A, the pellet I was homogenized with α-methyl mannoside. The plasma membrane retained in the supernant was centrifuged to produce the pure plasma membrane (pellet IV). Three enzymes (acid phosphatase, cytochromes c oxidase and NADPH-cytochrome c reductase) were used to assay the purity of plasma membrane fraction. It was concluded that among the three marker enzymes, the acid phosphatase activity was found mostly in plasma membrane fraction.

Hagerhall reported that menaquinone was an obligatory redox mediator of bacterial electron transport [49]. In mammalian system, the localization of ubiquinone-10 in the plasma membrane, the extraction and reactivation experiments of Sun *et. al.* are strong evidences for the implication of ubiquinone-10 as a functional member of the plasma membrane electron-transfer system [11]. Thus this study was undertaken to find out the quinone(s) function in the transplasma membrane electron transport (transPMET) system of *L. donovani* promastigote (Table 4). UV inactivation of napthoquinone co-enzyme resulted in either inhibition or stimulation of reduction rate. Previously oxygen was identified as physiological electron acceptor in the transPMET system of *L. donovani* promastigote [21]. UV exposed *L. donovani* promastigote cells also showed parallel stimulation (Fig. 6) but UV exposed cells inhibited ALA reduction. In conjugation with MOON and octadecylamine more reduction rate for ALA and ferricyanide was observed in UV exposed *L. donovani* cells. HQNO a quinone analog inhibitor showed potent inhibition to the above reduction rates. These observations support the role of redox potential of mobile electron carriers for redox couple in transPMET system. It therefore also suggested that at least some of the electron donor and/or acceptor enzyme complexes are present in the transPMET system.

Most primary ion pumps use the energy provided by the hydrolysis of ATP to energize ion-transport process across cell membranes. Accordingly, 'ATPase' has been found in all organisms studied so far. Several families of ATPase, which can be distinguished by their transport mechanism, their quaternary structure and their sensitivity towards specific inhibitors, have been found in various membranes and cell compartments. Pederson and Carafoli introduced the term P-type, F-type and V-type ATPases [248]. P-type ATPases (P-ATPase) have a phosphorylated transitional stage; F-type ATPases (F-ATPases) are primarily used in ATP synthesis and, therefore, are usually referred to as 'F-ATP synthesis'; V-type ATPase (V-ATPases) are generally and functionally related to F-ATPases but function only in ATP breakdown. To this classification scheme ATP-Binding Cassette (ABC) transporters (or traffic ATPases) have also been added. ABC transporters, which have been found in all living organisms, mediate uptake or efflux of a verity of solutes at the expense of ATP [454].

The use of specific inhibitors has made it possible not only to make quick assignments of unknown ATPases but also to study their functions in the corresponding membrane. Thus, ortho-vanadate is a well-known inhibitor for P-ATPases, ABC-transporters, whereas F-ATPases are inhibited by ventrericidin and DCCD. Bafilomycins are high affinity inhibitors of V-ATPases [455]. This class of antibiotics allows a clear distinction between the three types of ATPases. F-ATPases are not affected, whereas P-ATPases are inhibited in the micro molar and V-ATPases in the nano molar concentration range.

Membrane bound proton translocating inorganic pyrophosphatases (H^+-PPase; EC 3.6.1.1) belong to a category of proton pumps, distinct from F-, P-, and V-ATPases, which utilize pyrophosphate hydrolysis as the driving force for H^+ movement across biological membranes [456]. H^+-PPase have been characterized both at biochemical and genetic levels in various higher plants [457, 458], a few eubacteria [459-461], and in an archaea [462] and some human pathogenic protozoa [463].

Inorganic pyrophosphate was for a long time believed to be merely a byproduct of biosynthetic reactions (synthesis of nucleic acids, coenzymes, proteins, activation of fatty acids and isoprenoid synthesis) subject to immediate hydrolysis by inorganic pyrophosphatases. From the early 1960s, however, data have accumulated suggesting an important bioenergetics and regulatory rate for this compound [464]. In plants [465] and

in some parasitic protozoa, pyrophosphate is used in place of ATP as an energy donor in several reactions, such as the pyrophosphate- phosphofructokinase of *Trichomonas vaginalis* [466], *Entamoeba histolytica* [467], *Giardia lamblia* [468], *Naegleria fowleri* [469], *T. gondii* [470], *Eimeria tenella* [471]; the pyruvate phosphate dikinase of *Giardia lamblia*[472], *Entaamoeba histolytica* [473]; or the phosphoenol pyruvate carboxy-transphosphorylase [474] and the pyrophosphate-acetate kinase [475] of *Entaamoeba histolytica*. It has been postulated that pyrophosphate analogs could be selective inhibitors of some of these parasite enzymes [476].

In this thesis an ATPase and PPase activity has been characterized in permeabilized promastigotes of *L. donovani* .Our results on the general properties of the membrane ATPase with reference to cation requirements, pH optimum, and sensitivity to inhibitors are in agreement with those of Zilberstein and Dwyer [477]. The Mg^{2+}- dependent activities of ATPase and PPase differed little in optimal conditions. ATPase and PPase functioned best at pH 7 (Fig. 6.17). Optimal substrate concentration for ATPase and PPase was 0.5mM ATP and 1mM Mg^{2+}. Strikingly when ATP and Mg^{2+}concentration and PPi and Mg^{2+} concentration was increased at a ratio of 1:2, activities were decreased progressively. Lack of saturation kinetics appears to be due to increasing Mg^{2+} concentration. Increasing Mg^{2+} concentration inhibits ATPase and PPase activity. Stimulation of ATPase activity either by Na^+ or K^+ in comparison to ATPase activity in presence of both Na^+ and K^+ suggest the presence of a Na^+- stimulated and K^+-stimulated ATPase activity and the absence of Na^+, K^+-ATPase activity (Table 6.11).

Our observation was supported by the work of Stiles *et. al.*[428]. Similarly, PPase showed it's to highest activity in presence of K^+. Na^+ appears to be inhibitory for PPase activity (Table 6.11). ATPase and PPase activity was strongly inhibited by –SH group inhibitors Hg^{2+}. Similarly nonpermeable -SH group inhibitor PCMB and APAOS inhibited ATPase and PPase activity moderately (Table 6.13). From this observation it can be concluded that exoplasmic -SH groups of plasma membrane are involved in ATPase and PPase activity. PPase activity was strongly inhibited by fluoride ion, a property found in all types of PPase. Strikingly enough, ATPase activity was also partially inhibited in digitonin permeabilized LDC (Table 6.14). Fluoride is not an inhibitor of ATPase or ATP synthase [478]. Inhibition of ATPase activity by fluoride suggests the involvement of PPase activity in the rate of ATPase. This observation was

further strengthened when imidodiphosphate, a specific inhibitor of PPase, stimulated ATPase activity (Fig. 6.25). Similarly specific F-ATPase inhibitor sodium azide stimulated ATPase activity (Fig. 6.20). At 20μM concentration of DCCD, 80% inhibition of PPase and 40% inhibition of ATPase were achieved (Fig. 6.23), which again supports the involvement of ATPase in PPase activity. DCCD is known to inhibit plasma membrane H^+-PPase activity [479]. Triethyltin bromide an inhibitor of F-ATPase showed profound inhibition on ATPase activity, but little on PPase (Fig. 6.27). Quercetin, an inhibitor of F-ATPase, showed strong inhibition on ATPase activity, but little on PPase activity (Fig. 6.24). Sodium orthovanadate (Fig. 6.21) and verapamil (Fig. 6.26) are inhibitors of P-type ATPases and they showed strong inhibition on ATPase and PPase activity. Similarly trifluoroperazine, an inhibitor of P-type ATPase showed profound inhibition on PPase activity.

From these findings on transPMET system in *Leishmania donovani* promastigotes, it may be concluded that ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase activities are linked with either ATP hydrolysis or ATP synthesis along with either PPi hydrolysis or PPi synthesis. Here it is worth to note that transPMET system in mammalian cells failed to show any ALA, DTNS, and NQSA reductase activity and $K_3Fe(CN)_6$ reductase activity is not coupled with ATPase / ATP synthase activity. *Leishmania* cells differ from the mammalian cells on the above aspects. These differences can be exploited for rational chemotherapy of leishmaniasis.

In conclusion, analysis of their roles in parasite survival and pathogenesis is likely to lead to the discovery of novel targets for antileishmanial chemotherapy.

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List of Publications

List of International Publications

1. Tanmoy Bera, Kuruba Lakshman, D. Ghanteswari, Sabita Pal, D. Sudhahar, Md. Nurul Islam, **Nihar Ranjan Bhuyan**, Pradeep Das. "Characterization of the redox components of transplasma membrane electron transport system from *Leishmania donovani* promastigotes". ***Biochimica et. Biophysica Acta.***, 2005, 1725, 314-326. **(Elsevier publication)**

2. Shibendu Biswas, Rabiul Haque, **Nihar R. Bhuyan**, Tanmoy Bera. "Participation of chlorobiumquinone in the transplasma membrane electron transport system of *Leishmania donovani* promastigote: Effect of near-ultraviolet light on the redox reaction of plasma membrane". ***Biochimica et. Biophysica Acta.***, 2008, 1780, 116-127. **(Elsevier publication)**

3. **Nihar Ranjan Bhuyan,** Md. Nurul Islam, J.P. Mohanty, B.P.Saha, Tanmoy Bera. "An evidence of reduction of the NQSA and DTNS by *Leishmania donovani* promastigote". ***International Journal of Pharmacology and Biological Sciences,*** 2009, 3(1), 71-79.

4. **Nihar Ranjan Bhuyan**, Shib Sankar Sen, J.P. Mohanty, B.P. Saha, Tanmoy Bera. "Characterization of ATPase and PPase activities in *Leishmania donovani* promastigote plasma membrane". ***Research Journal of Biotechnology***. (Accepted, Ref. No. 213/01/2009).

5. S.S. Sen, **N. R. Bhuyan,** K. Lakshman, A.K. Roy, B. Chakraborty, T. Bera. "Membrane bound pyrophoaphatase and P-type ATPase of *Leishmania donovani*: Similarities and differences in inhibitor sensitivities as possible chemotherapeutic targets". ***Biochemistry Moscow*, (In Press).**

6. Shib Sankar Sen, **Nihar Ranjan Bhuyan,** Tanmoy Bera. "Characterization of Plasma Membrane Bound Pyrophosphatase in *Leishmania donovani* promastigote". ***African Health Sciences,*** **(In Press).**

7. S. Dash, L.K. Nath, S. Bhise and **N. R. Bhuyan**. "Antioxidant and antimicrobial activities of *Heracleum nepalense* D.Don root". ***Tropical Journal of Pharmaceutical Research***, 2005, 4(1), 341-347.

8. J.P. Mohanty, L.K.Nath, **N. R. Bhuyan,** S.K.Mohapatra. "Antibacterial spectrum of *Kaempferia rotunda* Linn. and *Eupatorium cannabinum* Linn." ***Advances in Pharmacology and Toxicology***, 2008, 9(1), 45-50.

9. J.P. Mohanty, L.K. Nath, **N. R. Bhuyan** and U.K. Nayak. "A Preliminary Study on Gastric Antiulcer Activity of *Eupatorium Cannabinum* Linn. in rats". ***International Journal of Pharmacology and Biological Sciences,*** 2008, 2(1), 159-164.

10. G. Mariappan, **N. R. Bhuyan,** J.P. Mohanty, B.C. Das. "Synthesis and biological evaluation of some novel chalcone derivatives". ***Asian Journal of chemistry***, 2009, 21.

11. R. Chanda, A. Ghosh, T. Mitra, J. P. Mohanty, **N. R. Bhuyan,** G. Pawankar. "Phytochemical and pharmacological activity of *Aegle marmelos* as potential medicinal plant: An overview". ***The Internet Journal of Pharmacology***, 2008, 6(1), 1-19.

12. R. Chanda, J. P. Mohanty, **N. R. Bhuyan,** T. Mitra, G. Pawankar. "A review on chemical and biological activity of *Tamarindus indica* Linn." ***The Internet Journal of Pharmacology.*** (In Press)

List of National Publications

1. G. Mariappan, **Nihar Bhuyan**, J.P. Mohanty, Subarna Ganguli and D. Dhachinamoorthi. "An overview of the method of positional scanning synthetic combinatorial libraries". ***Indian Journal of Pharmaceutical Sciences***, 2006, 68(4), 420-424.

2. R. Chanda, J.P. Mohanty, **N. R. Bhuyan**, P.K. Kar, L.K. Nath. "Medicinal plants used against gastrointestinal tract disorders by the traditional healers of Sikkim Himalayas". ***Indian Journal of Traditional Knowledge***, 2007, 6(4), 606-610.

3. U. K. Nayak, G. Mariappan, J. P. Mohanty, **N. R. Bhuyan**, B. K. Patro. "Antilipidperoxidation activity of *Curcuma aromatic* Linn". ***Indian Drugs***, 2007, 44(6), 483-485.

4. J.P. Mohanty, L.K.Nath, **N. R. Bhuyan**, G.Mariappan. "Studies on Antioxidant Potential of *Kaempferia rotunda* Linn." ***Indian Journal of Pharmaceutical Sciences***, 2008, 70(3), 362-364.

5. S. Das, **N. R. Bhuyan**, R. Chanda, J.P. Mohanty. "Preparation and evaluation of sustain release enteric coated mucoadhesive tablets of omeprazole for local action". ***Research Journal of Pharmacy and Technology***, 2008, 1(3), 166-170.

List of Presentations in conferences

1. **N.R. Bhuyan**, T. Bera, B.P. Saha, J.P. Mohanty. "Plants and their constituents used to prevent leishmaniasis". National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.

2. **Nihar Ranjan Bhuyan**, B.P. Saha, Sourav Saha, T. Bera. "Effect of Imidodiphosphate on plasma membrane of *Leishmania donovani* Promatigote UR6". 60th Indian Pharmaceutical Congress (IPC), New Delhi, India, 12-14th December, 2008.

3. D. Sudhahar, Tanmoy Bera, K. Lakshman, **Nihar Bhuyan**, U. Mondal, "Antileishmanial activity of some naphthoquinone derivatives". 56th Indian Pharmaceutical Congress (IPC), Kolkata, India, 2-4th December, 2004.

4. S. Dash, S. Bhise, **Nihar Bhuyan**, L.K. Nath, P. Kar, S. Mahapatra. "Evaluation of Antioxidant and Immunostimulatory potentiality of *Dactylorhiza hatogirea* (D.Don) Soo: An Ethnomedicine". 57th Indian Pharmaceutical Congress (IPC), Hyderabad, India, 2-4th December, 2005.

5. R. Chanda, J.P. Mohanty, **N.R. Bhuyan**, A. Bakshi, S. Ganguli. "Market Survey of Sellings of Pharmaceutical Products in India". 58th Indian Pharmaceutical Congress (IPC), Mumbai, India, 1-3rd December, 2006.

6. J.P. Mohanty, **Nihar Bhuyan**, R. Chanda, Pinak Paul. "Antiulcer activity of *Kaempferia rotunda* Linn. of Sikkim Himalaya Region". 59th Indian Pharmaceutical Congress (IPC), Varanashi, India, 20-23rd December, 2007.

7. J.P. Mohanty, **N.R. Bhuyan**, L.K. Nath, K. Gautaman. "Studies on Analgesic and antipyretic of *Kaempferia rotunda* Linn". National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.
8. G. Mariappan, **N.R. Bhuyan**, Tarun Dhara, Ayan Dasgupta. "Synthesis and anti-inflammatory evaluation of some novel pyrazoline derivatives". 60th Indian Pharmaceutical Congress (IPC), New Delhi, India, 12-14th December, 2008.
9. J.P. Mohanty, R. Chanda, **N.R. Bhuyan**, S.S. John. "Ethno medicinal studies of plants by the Tribals of Sikkim and Darjeeling Himalaya against Poliomyelitis of children". 60th Indian Pharmaceutical Congress (IPC), New Delhi, India, 12-14th December, 2008.
10. J.P. Mohanty, **N.R. Bhuyan**, Trishna Das, Sivani Agarwal. "Studies on ethnomedicinal plants of North East region against wound". International Herbal Conference, Bangalore, India, 26-28th February, 2009.
11. J.P. Mohanty, **N.R. Bhuyan**, T. Uriah, G.Mariappan. "Ethno medicine used by tribal of Sikkim Himalayan as Aphrodisiac". National Seminar, GRYIP, Borawon, Khargone, MP, 30-31st March, 2009.