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.

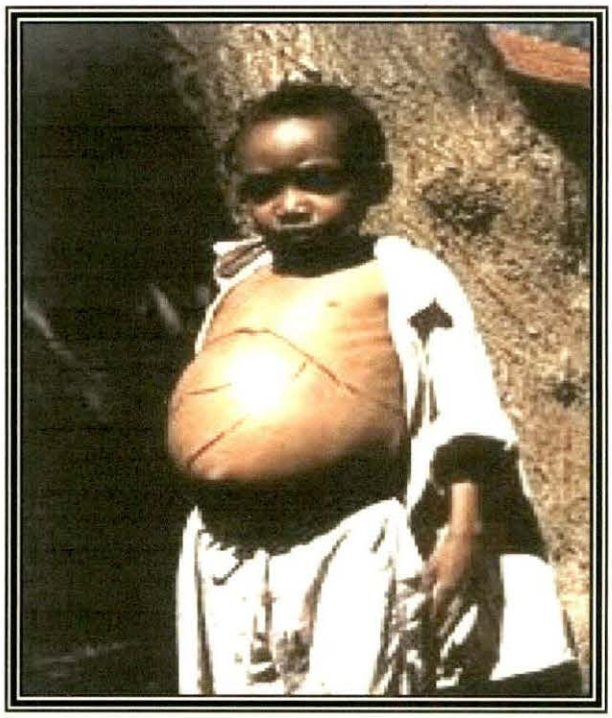

Fig.2.2.1. Person suffering from visceral leishmaniasis

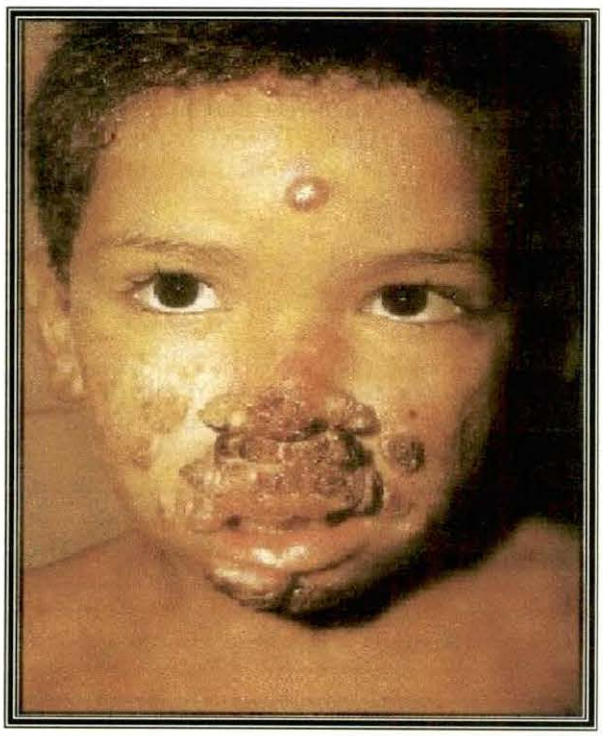

Fig.2.2.2. Mucoceutaneous leishmaniasis resulting in a total disfigurement of the face

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.

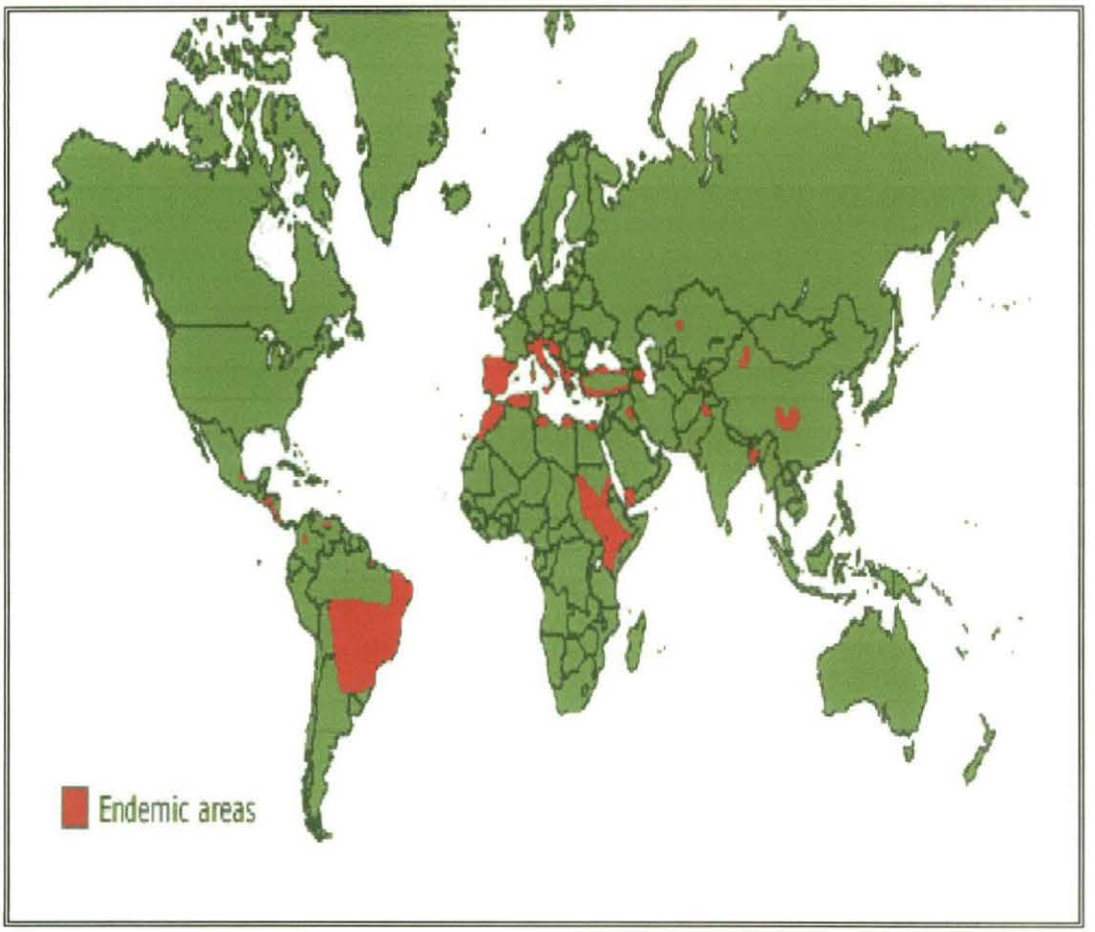

Fig 2.3. Geographycal distribution of Leishmaniasis World wide

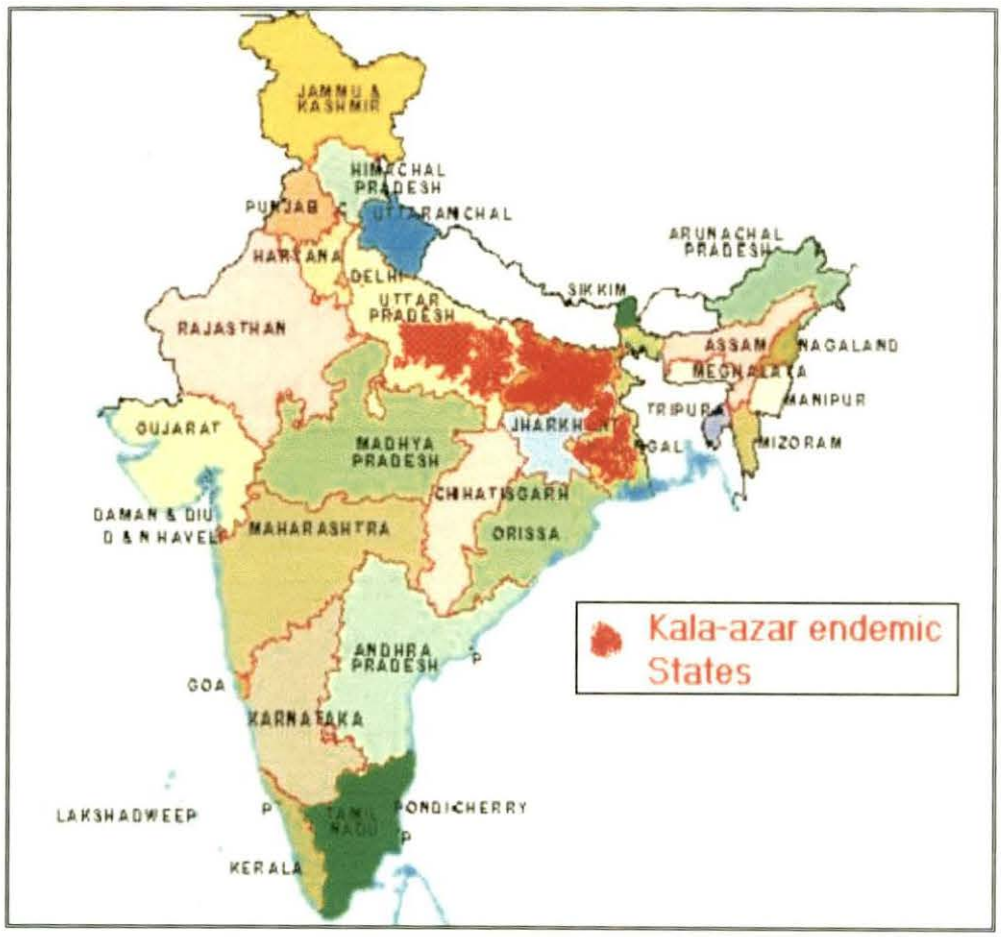

Fig.2.4. Geographycal distribution of Leishmaniasis in Indian region

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].

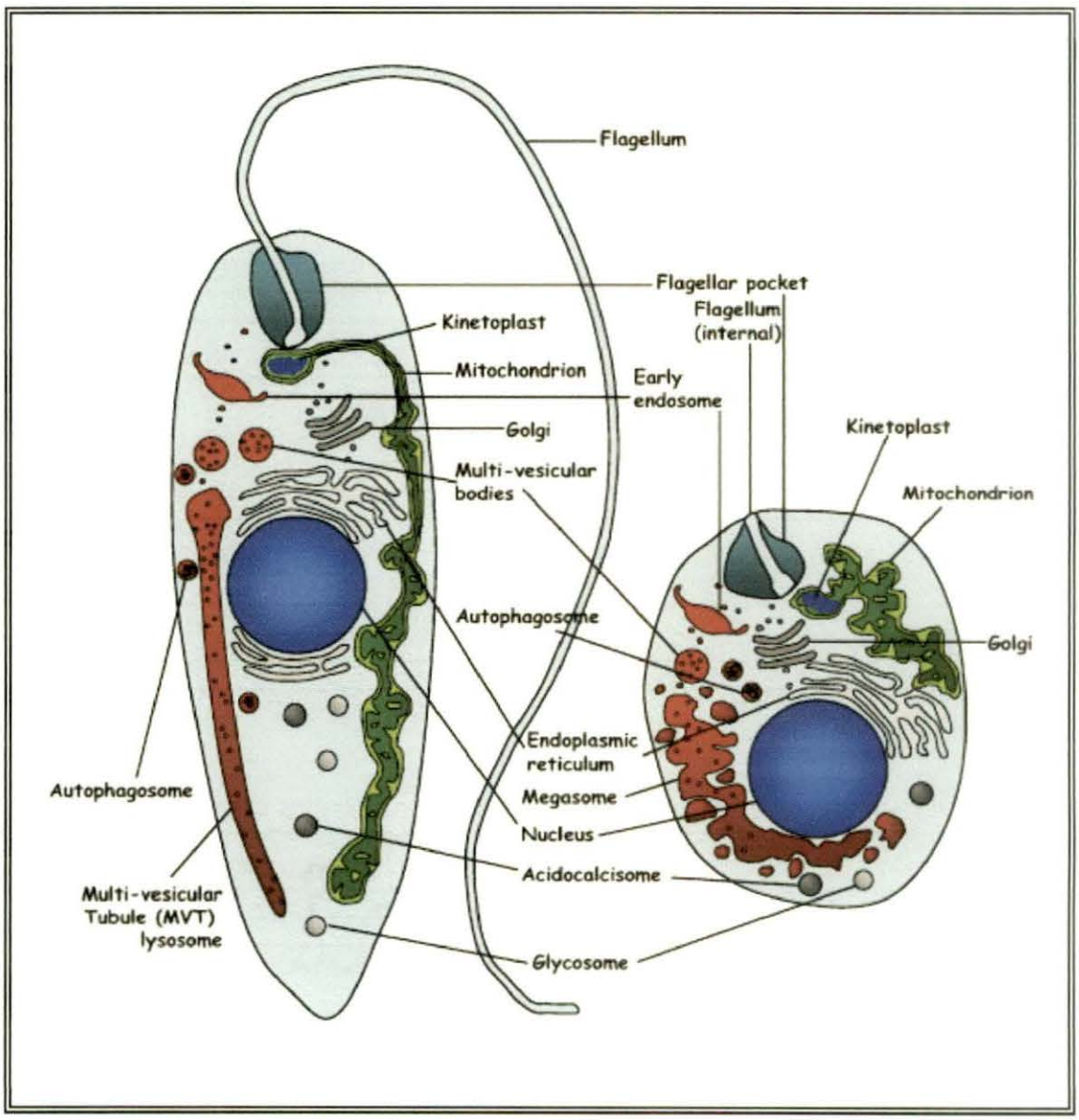

Fig. 2.5. A schematic representation of the fine structure of a promastigote (left) and an amastigote (right)

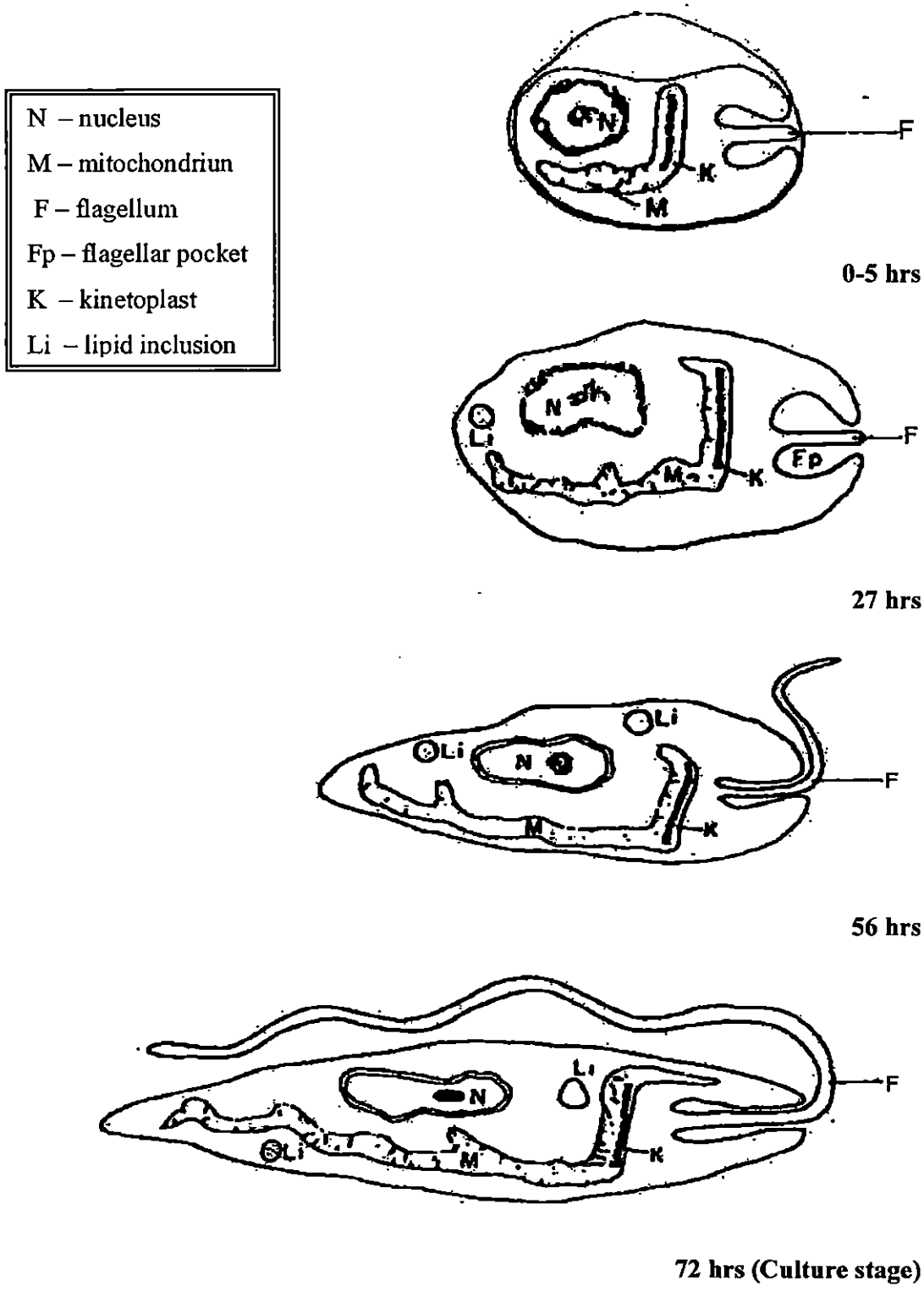

Fig. 2.6. Schematic diagram of the fine structural changes of *L. donovani* in the course of amastigote to promastigote transformation

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*.

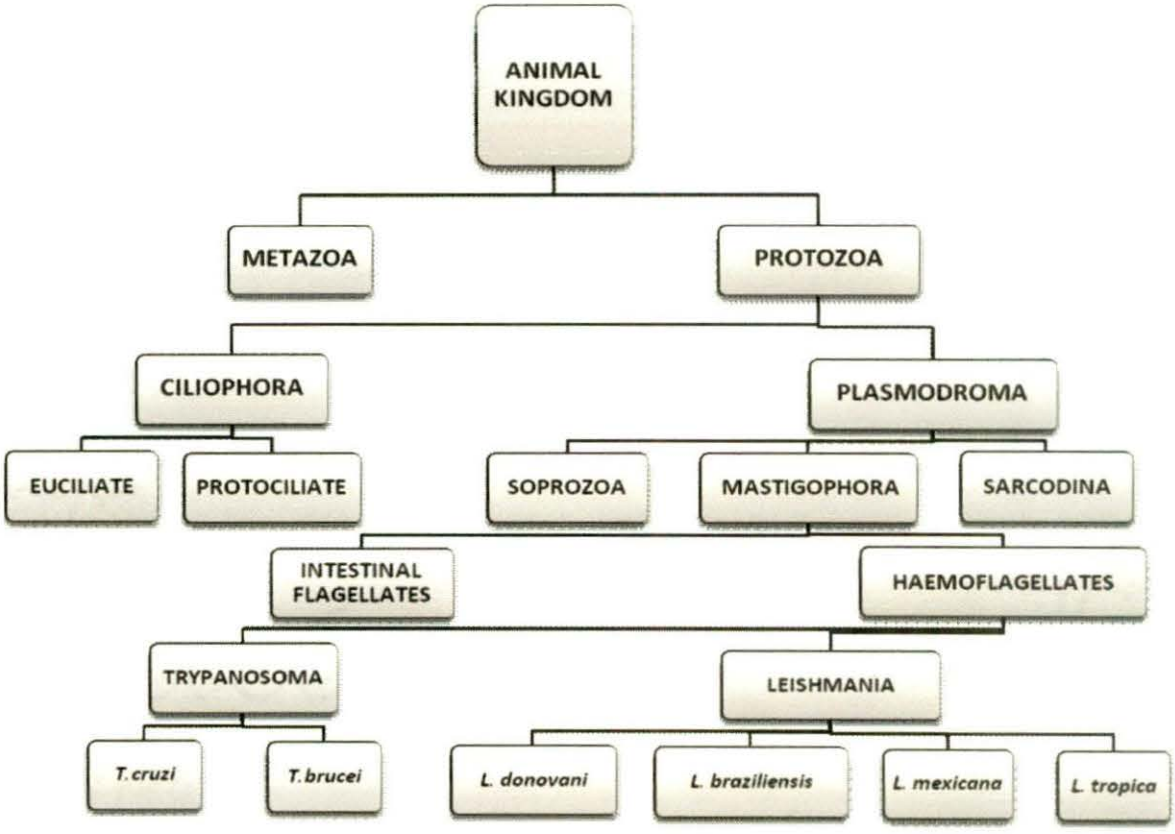

Fig. 2.7. Classification of protozoa

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).

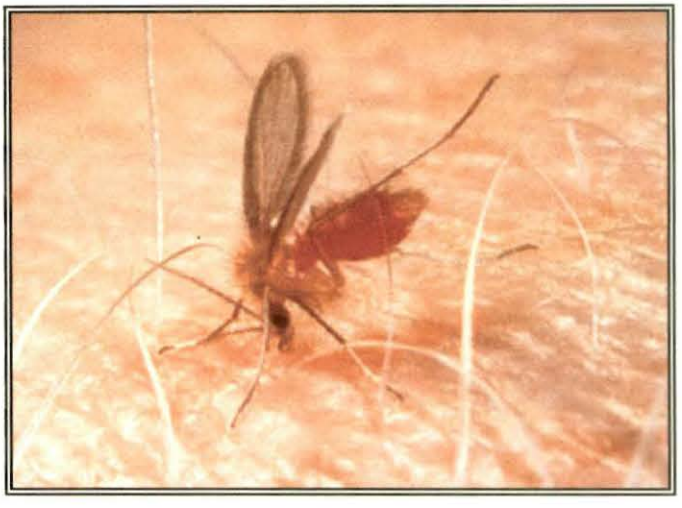

Fig. 2.8. Phebotomus sand fly

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.

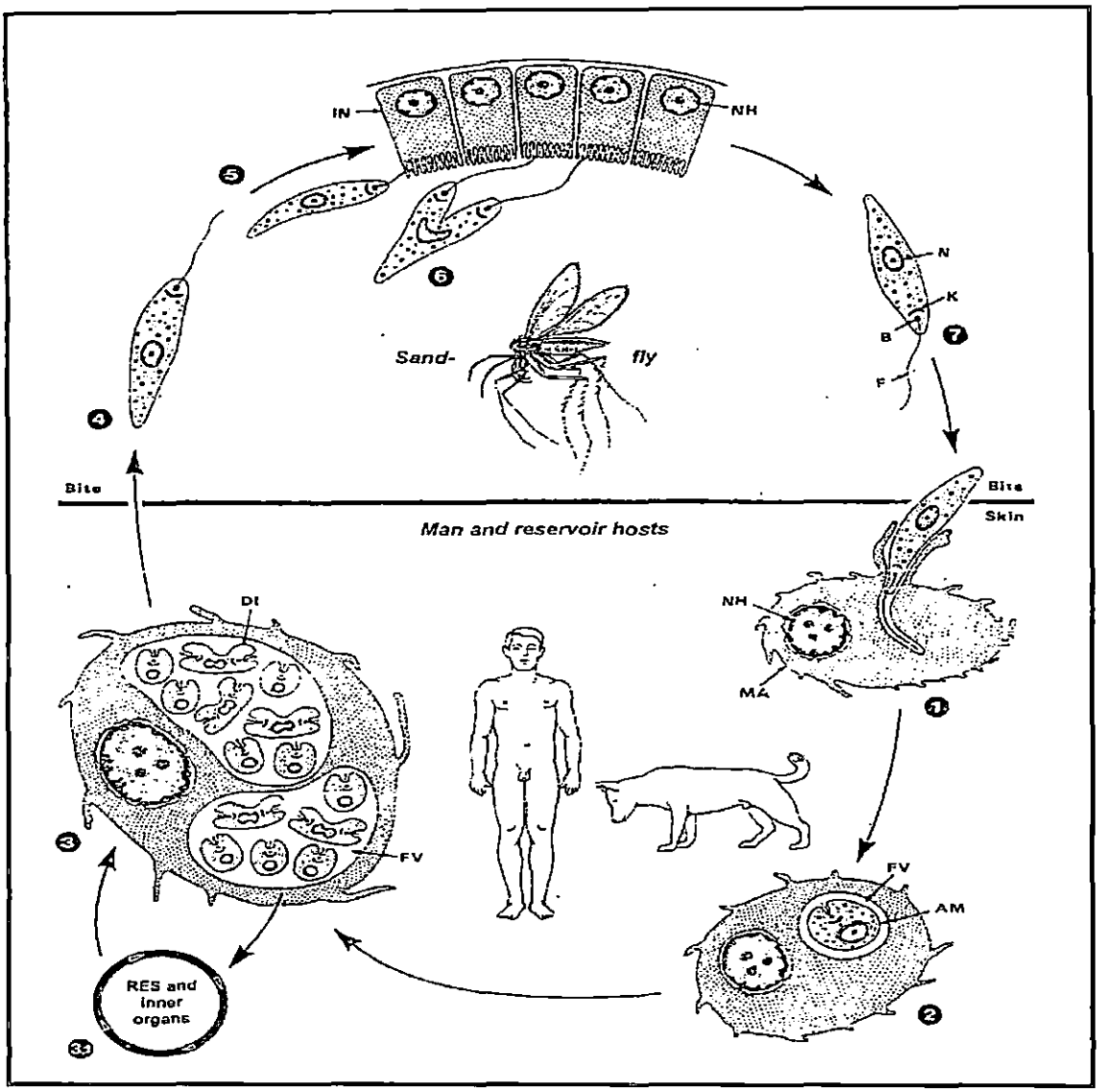

Fig. 2.9. Life cycle of *Leishmania* spp.

(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.

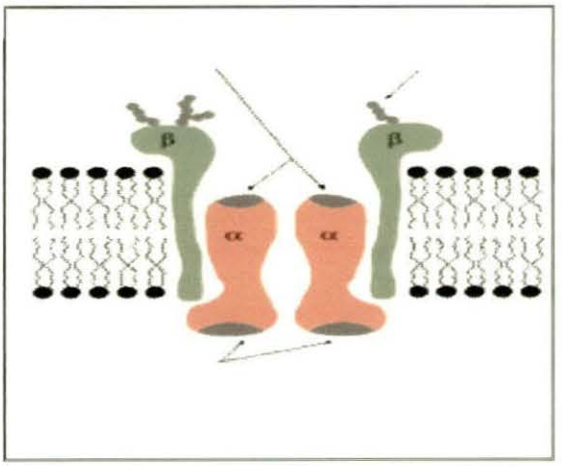

Fig. 2.10. Alpha- Beta subunits

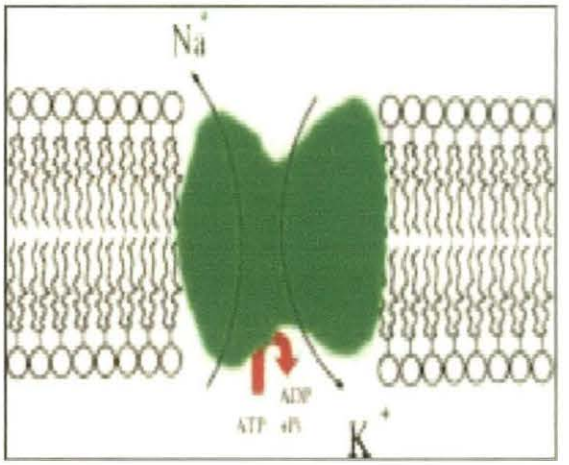

Fig. 2.11. Flow of ions

- **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**

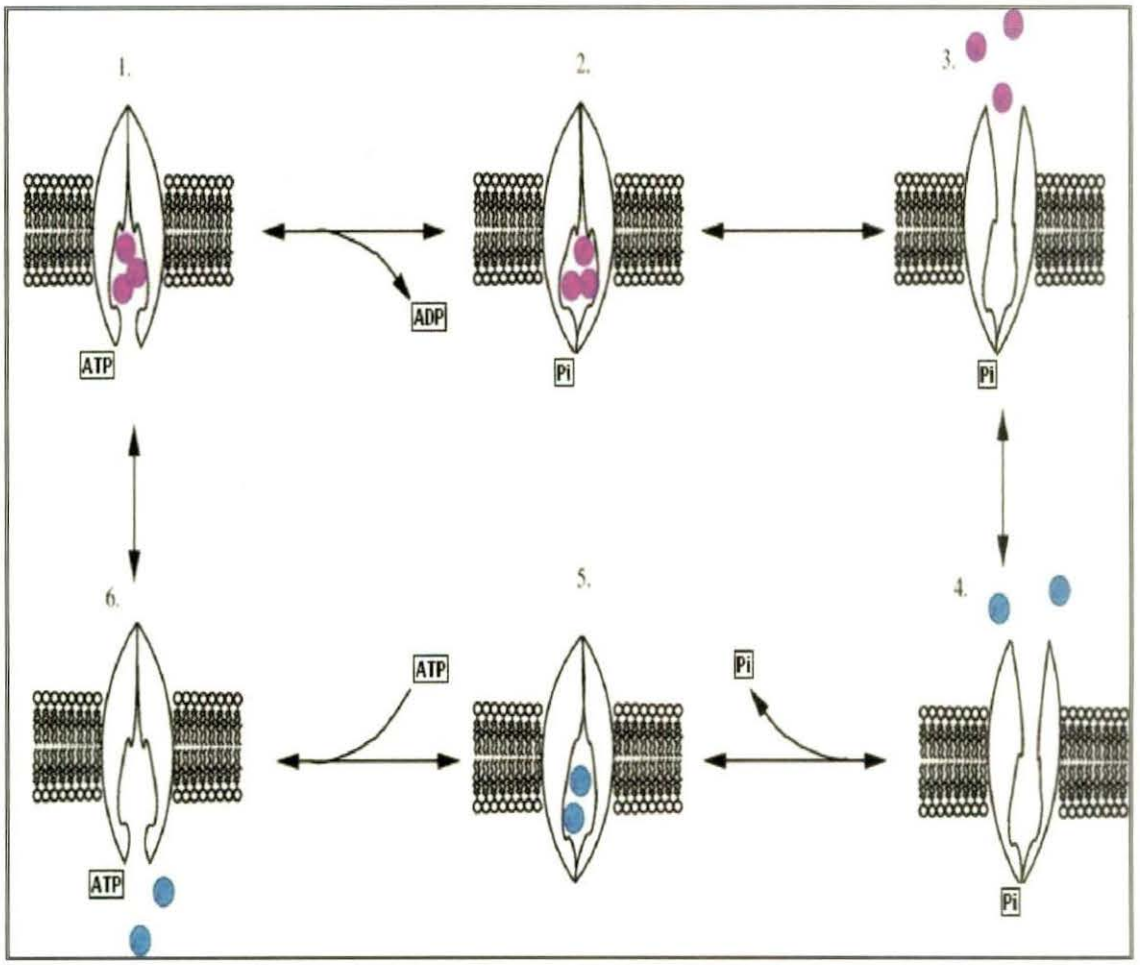

Fig. 2.12. Mechanism of Na^+/K^+ transport in ATPase system

- 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.