BIOCHEMISTRY OF MICROBES

Parasite biochemistry

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CONTENTS

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Filariasis

Introduction

Parasitic infections of one kind or the other have been estimated to affect about 3 billion people in the world; of which about 250 million people are infected with filarial parasites. As compared to developed world, where there has been considerable progress in combating major diseases, parasitic infections have remained major obstacles for economic progress and a better life in developing countries. Helminth parasites represent major cause of human misery because ascarid, hookworm and filarial infections are ubiquitous in developing nations and cause malnutrition, disfiguration and disability. Although these infections do not cause acute mortality, they sap the vitality of nations already plagued by overpopulation, food shortages and poor hygiene and health. The magnitude of suffering is so enormous that WHO felt compelled to include three helminthic diseases in its special programme.

The protozoa represent the first ladder of eukaryotic evaluation while the helminthes are a few more steps ahead. As compared to their hosts, these organisms have very primitive level of structural and biochemical refinement and some of them survive in very selective ecological niches and have to adapt their life cycles with the functioning of alternate host systems. However, they have proved to be the most formidable enemies of humanity and have defied all efforts to vanquish them. They have developed intricate and impressive molecular mechanisms to counter host defenses and to exploit their metabolic machinery and regulatory molecules for their own proliferation. In many cases, the parasites inhabit in tissues which are impregnable to the host defenses or cannot be attacked without damage to the host itself. Very few effective drugs are available, many of them have limited action on only one of the several developmental stages, and some of them have severe and even fatal side reactions. Much, therefore, remains to be desired in the chemotherapy of helminth and especially filarial parasites. Although considerable research have been done in the field of morphology, life cycle and taxonomy of filarial parasites, comparatively little attention has been paid to the physiology and metabolism of these parasites and their effect on the host.

Distribution and life cycle

Filariasis represents a class of diseases caused by “Thread like” worms of the super family filariodae of Phylum nematode. Filarial infection is wide spread in India and prevalent in Assam, Kerala, Andhra Pradesh, Madhya Pradesh, Orissa, Uttar Pradesh and West Bengal. A recent report states that 304 million people in India alone are living in endemic areas of filariasis and hence are exposed to the grave risk of contacting the disease. An estimated population of 22 million is known to harbour circulating microfilariae and further 16 million people suffer from filarial manifestations like elephantiasis of limbs, genitals and hydrocoele etc. Further, due to rapid industrialization and ensuing migration of people from one place to another the disease has radiated to areas where it did not exist before.

Many species of filarial parasites are known, each relatively specific for its host. *Wuchereria bancrofti, Brugia malayi, Onchocerca volvulus, Dipetalanema perstans, D. streptocerca, Loa loa* and *Mansonella ozzardi* are the species responsible for producing infestations in man (Manson-Bahr and Apted, 1982). In India the causative organisms in human are *W. bancrofti* and *B. malayi*. In animals the disease is caused due to *Setaria cervi* (cattle), *Dirofilaria immitis* (dog) *D. uniformis* (rabbit), *Litomosoides carinii* (cotton rat) and *Chandlerella hawkingi* (jungle crow).

There are three distinct phases in the life cycle of filarial parasites viz., microfilariae (mf.), infective larvae (L_a) and adult worms. The adult parasites reside in connective tissues, muscles, circulatory or lymphatic system of the host. The microfilariae (Sheathed/unsheathed) are released into peripheral blood of the host, have a life span of 14-70 days and exhibit nocturnal/diurnal periodicity. All the filarial parasites of man are vector-borne, transmitted by mosquitoes, biting midges, tabanids and black flies. Arthropods such as fleas, ticks, and mites transmit some filarial infections of animals. In India, *Mansonia* species of mosquito propogate *B. malayi,* while *Culex pipens* is responsible for transmission of *W. bancrofti*. While circulating in peripheral blood some mf are taken up through the bite of insect vector and they undergo further development to the infective larval stage within 15 days. When the blood sucking arthropode takes its next meal, the L_3 larvae (3rd stage larvae) are transmitted to the recipient host through the skin and the cycle is completed (Fig. 1).

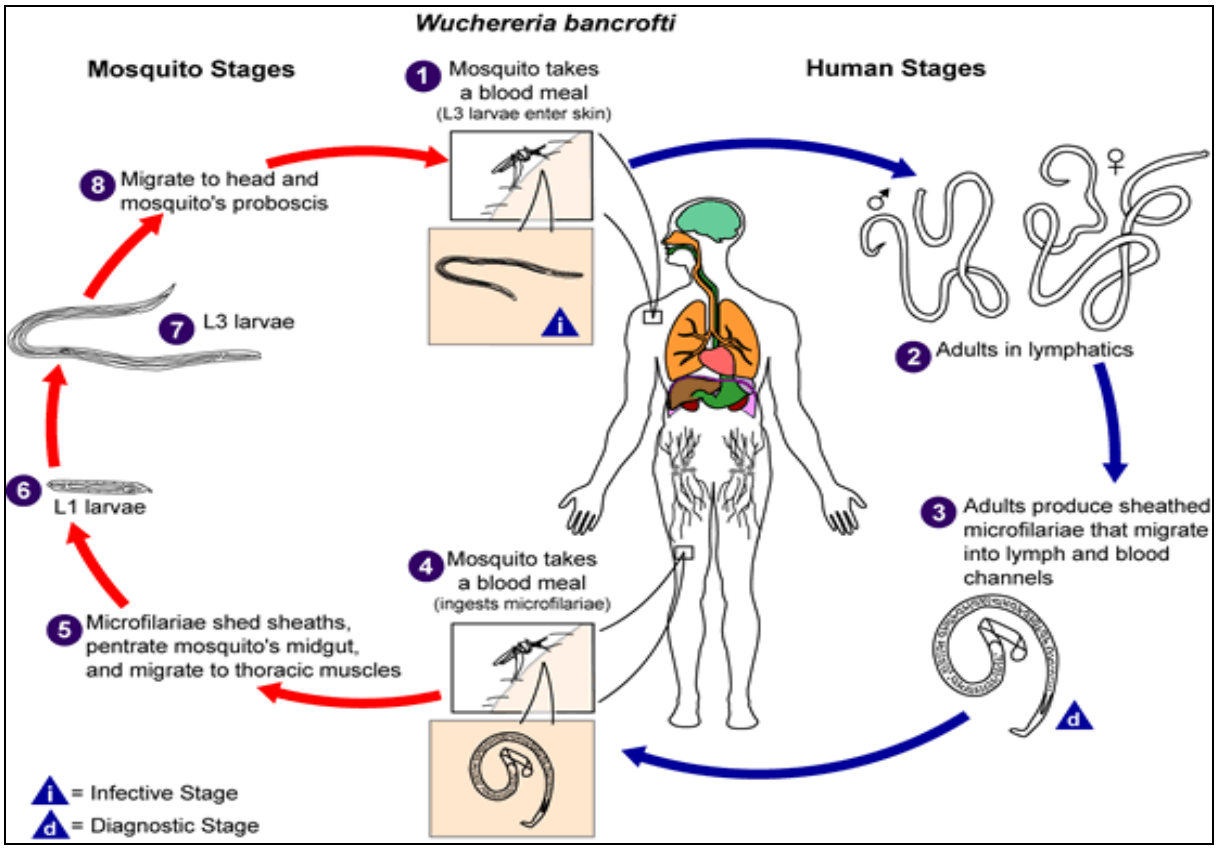

Fig. 1: Life cycle of Filarial Parasite

Surface structure and composition

Cuticle, which forms the nematode surface, differs from the plasma membrane or tegumental surfaces of other helminthes. Glycoproteins have been identified as a structural component of cuticle in *D. immitis, B. pahangi* and *B. malayi*. Complex oligosaccharides and their conjugates with protein and lipids present on the parasite's surface play a significant role in determining its antigenicity and host immune responses.

The sheath and the epicuticle of *B. pahangi* stain positively with concavalin A (Con A) and sheath of mf also shows activity of acid phosphatase, 5'-nucleotidase and peroxidases; the

enzymes were located in the cortex and basal layers of cuticle. Presence of N-acetylglucosamine, glucose and mannose on mature mf and sialic acid, galactose and N-acetylglucosamine on sheath of immature mf has been demonstrated. The mf directly isolated from the blood of infected cats were found immunochemically to carry serum proteins on their sheath but not on the cuticle. Treatment of *D. immitis* mf (unsheathed species) with proteases, neuraminidases, DEC or EDTA also failed to expose any lectin binding sites. Infective larvae and adult worms of *B. malayi* did not bind any of the lectins tested but mf bound WGA in a specific and saturable marner giving evidence for the presence of exposed N-acetylglucosamine.

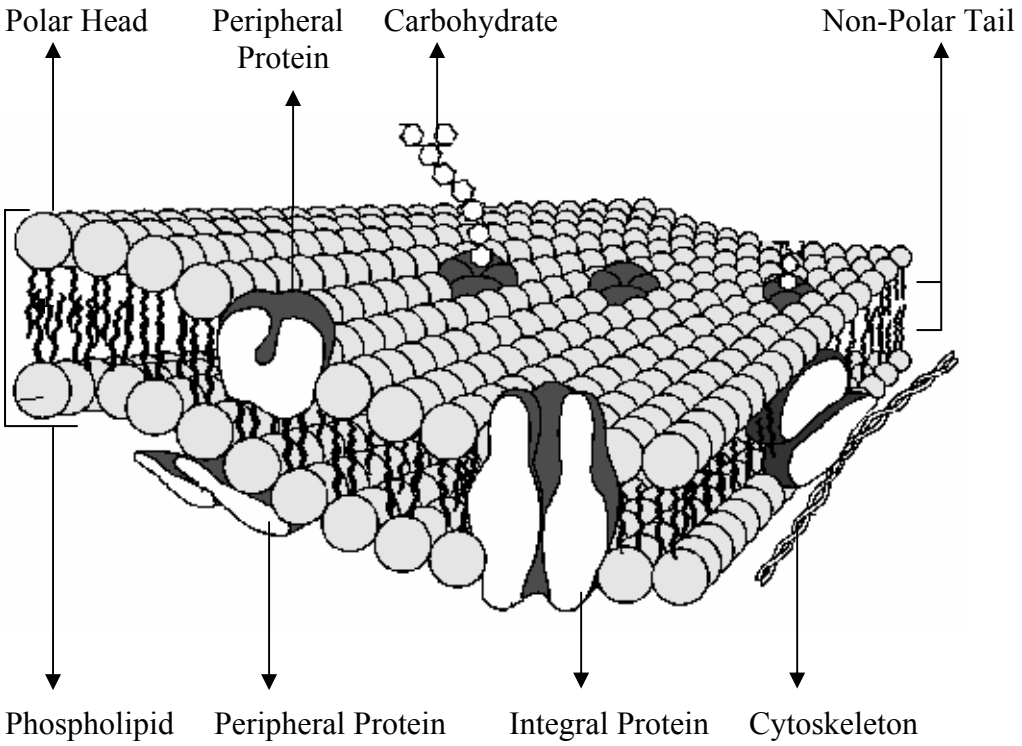

Fig. 2: Structure of cell membrane

The changes in the surface structures of mf related with developmental status as well habitat of mf may be involved in host recognition and immune respone. The sheath carbohydrates has been suggested as a component in the molecular trigger including sheathment in mosquito and proteases in mosquito midgut may play a crucial role in exsheathment, recognition by specific receptors in mosquito and also for the further development of parasite.

Chemical analysis of filarids and their developmental stages

Glycogen is the main reserve food in parasitic nematodes for providing energy under adverse conditions. Helminth parasites inhabiting the intestine and living in an oxygen deficient atmosphere usually have a high content of glycogen. However, tissue parasites such as filarial do not generally store glycogen. Filarial parasites like *C. hawkingi* and *L. carinii*, which thrive in trachea, lung, heart and pleural cavity have a continuous supply of food material even when the host is starved and hence it is not necessary for them to store these macromolecules.

Carbohydrates are the major energy source for helminth parasites and are used as essential constituents of the media employed to maintain them *in vitro*. In some other parasites amino acids and lipids may also be important in providing energy as evident from their conversion to carbohydrate. Adult *L. carinii* can synthesise glycogen from exogenous glucose or mannose.

Filarial parasites utilize glucose as an energy source *in vivo* as well as the constituent of maintenance medium under *in vitro* conditions. Incubation of *L. carinii* and *S. cervi* adults with glucose, mannose, fructose and galactose produced lactic and pyruvic acids; glucose and mannose were utilized at faster rate than galactose and fructose. Galactose and fructose could not serve as the carbon source for *in vitro* maintenance of filarial parasites. Considerable variation has been observed in the rate of carbohydrate consumption by *L. carinii*, *D. uniformis* and *C. hawkingi, D. immitis* and *C. hawkingi.* The filarial parasites convert 50-60% added glucose to lactic acid but small amounts of succinate were also produced in the medium. *L. carinii* has an aerobic requirement and fermentation end products consisted of CO_2, acetate and lactate. *D. viteae* and *B. pahangi* were found to be homolactate fermenters under *in vitro* conditions and obtained their metabolic energy by anaerobic metabolism. As compared to *L. carinii* these two filarial parasites had no oxygen requirement for either their survival or motility.

Lipid metabolism

Few reports are available regarding the lipid composition of filarial parasites. Total lipids accounted for 9 and 12% of dry weight in adult and mf of *S. cervi*. However, 31-34% lipids have been reported in *C. hawkingi*. *D. immitis* has been reported to contain 2.1% lipid on wet weight basis. Phospholipids constituted the major portion of total lipids.

Most of the phospholipids have been demonstrated in various filarial parasites. Phosphatidylinositol (PI), diphosphatidylglycerol, cerebrosides, plasmalogens, lysophosphatidyl choline (LPC), sphingolipids and traces of phosphatidic acid have all been found in adult filarial parasites. All major classes of neutrallipids viz., triacylglycerols, diacylglycerols, sterols, sterol esters, hydrocarbons and traces of free fatty acids have been demonstrated in various filarial parasites. Adult female *D. immitis* has been shown to synthesize PE by three pathways viz., (a) via phosphorylethanolamine, CDP ethanolamine, diacylglycerol, (b) decarboxylation of phosphatidyl serine and (c) exchange of ethanolamine for choline or serine in PC or PS. The latter two pathways present in particulate fraction of worm homogenates have demonstrated that adult *D. immitis* can form PC by way of phosphorylcholine, cytidine diphosphocholine (CDP-choline) and 1,2-diacylglycerol as well as by way of S-adenosyl methionine-mediated methylation of PE. PS was synthesized by calcium-stimulated enzyme catalyzed exchange of L-serine for the base components of preformed phospholipids.

Filarial parasites like other nematodes, are unable to synthesize sequalene or sterols *de novo*, however, ubiquinone 9 and short and long chain isoprenoid alcohols are formed from mevalonate. In short chain isoprenoid alcohol fraction geranyl geraniol constituted the main fraction while, long chain isoprenoid alcohol (dolichol) consisted of dolichol 18(C_{90}), 19(C_{95}), 20(C_{100}), 21(C_{105}) and dolichol 22(C_{110}). These dolichols and other isoprenols are responsible for the enzymatic transfer of sugar groups of glycoproteins and proteoglycans.

The occurrence and uptake of retinol and retinoic acid and formation of retinol from β-carotene has been demonstrated in several helminth parasites. The adult *B. pahangi* catalysed conversion of retinol to retinyl phosphomannose and a possible role for retinyl phosphate has been suggested in filarial glycoprotein synthesis. Specific retinol binding proteins have been detected in parasite and inhibitors of retinol binding or retinol analogs could have possible chemotherapeutic significance. Menaquinones are involved in oxidative pathways of parasites but neither *B. pahangi* nor *D. immitis* could convert menadione (Vitamin K_3) to menaquinone (Vitamin K_2).

Protein metabolism

Protein constitutes over 63 and 57% of the dry weight of adult and mf of filarial parasites. Most of amino acids are present in bound form and only traces of free amino acids could be detected in these filarial parasites. Cysteic acid, ornithine, hydroxyproline, methionine and alanine were detected in *C. hawkingi* but these amino acids could not be detected in *S. cervi* isolated glycol and lipoprotein fractions from *L. carinii*.

The enzyme responsible for the interconversion of serine and glycine has been demonstrated in adult *B. pahangi* and *D. immitis*. These two parasites are however able to convert methionine to cysteine via 5-adenosylmethionine, 5-adenosylhomocysteine, homocysteine and cystathione.

Folate metabolism

The role of folic and folinic acids in growth and reproduction of nematodes has been demonstrated in axenic cultures. The folate antagonists aminopterin and amethopterin were found to be inhibitory to nematodes in axenic cultures. There is indirect evidence that adult filariae do not synthesize dihydrofolate, but require a source of preformed folate. Folic acid is not taken up by adults, juveniles, L_a or mf of filarial parasites but they can convert N^5-methyltetrahydrofolate to N^5, N^{10}-methylene tetrahydrofolate, N^5,N^{10}-methyltetrahydrofolate, N^5-formyltetrahydrofolate and N^{10}-formyltetrahydro-folate.

Adult *B. pahangi* and *D. immitis* contain most of the enzymes involved in folate metabolism viz., N^5,N^{10}-methylenetetrahydrofolate reductase, serine hydroxymethyl transferase, N^5N^{10}-methylenetetrahydrofolate dehydrogenase, N^{10}-formyltetrahydrofolate synthetase, N^{10}-formyltetrahydrofolate dehydrogenase (NADP dependent/independent), N^5N^{10}-methylenetetrahydrofolate cyclohydrolase, N^5-formyltetrahydrofolate cyclodehydrase, a complex containing N^5-formiminotetrahydrofolate cyclodeaminase and formiminoglutamate, tetrahydrofolate N^5-formiminotransferase, N^5-formyl, N^{10}-formyltetra-hydrofolate mutase. Adult *B. pahangi* and *D. immitis,* unlike mammalian cells, convert N^5-methyl tetrahydrofolate directly to N^5N^{10}-methylenetetrahydrofolate and other folate cofactors indicating qualitative differences between the folate metabolism of the host and parasite. The enzyme responsible for this step N^5,N^{10}-methylene tetrahydrofolate reductase is a flavoprotein that operates in these parasites preferentially in the reverse direction. In vertebrates, this enzyme catalyses the irreversible formation of N^5-methyltetrahydrofolate which serves as methyl donar in the synthesis of methionine from homocysteine. Dihydrofolate reductases have been demonstrated in *D. immitis, L. carinii, D. viteae* and *O. volvulus*. Presence of serine hydroxymethyltransferase and thymidylate synthetase has been demonstrated in adult stage of *B. pahangi* and *D. immitis*. Methionine synthetase is present in mammalian cells but is absent in filariae.

Dihydrofolate reductase activity was not detected in mf of *B. pahangi* and *D. immitis*. The vector, *Aedes aegypti* has a full complement of folate cofactors and enzymes and during infection with *B. malayi* the enzymes involved in the synthesis of N^5-methyltetrahydrofolate and methionine are increased, possibly due to depletion of these materials by the filarial parasite.

Nucleic acid metabolism

Only limited data are available about nucleic acid and nucleotide composition and metabolism of filariids. DNA comprises 0.3 and 1.44% while RNA 0.6 & 1.29% of the dry weight of adult and microfilarial stage of filarial parasite. Large uptake of adenosine was demonstrated *in vitro* by adult male and female of *D. immitis* but no uptake of thymidine occurred under similar conditions. The 5'-nucleotidase of *O. volvulus* and *D. immitis* exhibited broad pH optima and specificity towards AMP. The enzymes from both parasites were inhibited by amoscanate derivative (CGP 8065) and could be involved in antifilarial action of this compound. Incorporation of uridine and uracil into nucleic acids has been reported in adult *D. immitis* while mf of *D. immitis* incorporated uridine uracil , adenine and adenosine into RNA. Orotic acid derivatives have been reported to be incorporated into RNA by *D. immitis*. These results suggest that mf could synthesise pyrimidines but not purines and filarial parasites possess salvage pathways for both purines and pyrimidines. Two pathways are involved in the synthesis of purine nucleotides by most parasites - a) the salvage pathway involving the utilization of preformed purine bases and b) the *de novo* pathway involving the synthesis of purine nucleotides from simpler precursors e.g., glycine and formate. *B. pahangi* utilizes former pathway and *de novo* purine synthesis does not occur in filarial parasites.

Microfilariae of *S. cervi* and *L. carinii* transport glucose from the incubation medium and *D. immitis* and *B. pahangi* are able to utilize glucose, amino acids, RNA, glycine, uracil, adenine, hypoxanthine from the medium. The mf possess nonfunctional gut, hence uptake takes place through cuticle. The gut is probably nonfunctional in 3rd stage larvae of filarial worms, while it becomes functional in 4th stage larvae. Little information is available regarding the uptake of nutrients by the infective larvae of filarial worms. Autoradiographic studies have demonstrated the transport of adenine, amino acids and uridine by developing larvae of *B. pahangi* and *B. patei*. The incorporation of labeled phosphate by developing larvae of *S. cervi* and *W. bancrofti*.

Enzymes of carbohydrate metabolism

Helminth parasites derive energy for their survival mainly through the degradation of carbohydrate. The nature of the metabolic processes by which nematode parasites obtain energy has been examined by many investigators and glycogen is considered as the chief energy reserve. The anaerobic breakdown of glycogen to lactate via hexose phosphates and triose phosphates follows a course which is superficially similar to that in yeast and mammalian muscles. Evidence for the functioning of different metabolic pathways in parasites has been adduced mainly by the demonstration of the enzymatic steps or the identification of the intermediates of the pathway. The wide distribution of many glycolytic enzymes and the demonstration of phosphorylated glycolytic intermediates within the bodies of numerous parasites clearly indicate the operation of typical glycolytic sequences until phosphoenol pyruvate or pyruvate is reached, in many cases only a few enzymes have been explored. The operation of a full glycolytic pathway for conversion of glucose to lactic acid

has been demonstrated in *Dracunculus insignis*, *D. uniformis, C. hawkingi, L. carinii* and *D. immitis. S. cervi* appeared to be equipped with most of the enzymes of glycolytic and oxidative pathways. The enzymes of Embden-Meyerhof scheme were localized mainly in soluble fraction, showing resemblance with the mammalian system.

Malate dehydrogenase (MDH) is the most active enzyme in *S. cervi* adults although significantly high activities of lactate dehydrogenase (LDH), fumarase, glucose phosphate isomerase (GPI) phosphoglucomutase, glyceraldehydes-3-phosphate dehydrogenase, FDP-aldolase, phosphopyruvate hydratase, PEP carboxykinase (PEP-CK) and pyruvate kinase (PK) were also detected. Phosphofructokinase (PFK), glucokinase, malic enzyme and fructose diphosphatase are less active. Diaphorase activity was not detected in the system. Hexokinase of *S. cervi* was found to be specific for glucose but the corresponding enzyme from *L. carinii*, *C. hawkingi* and *A. galli* were of non-specific type. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the enzymes of pentose phosphate pathways could not be detected in measurable amounts in *C. hawkingi* and *L. carinii* but *S. cervi* contained significant quantities of these enzymes. Hexokinase from adult *D. immitis* phosphorylated glucose, fructose, mannose and glucosamine, while the hexokinase from *L. carinii* and *C. hawkingi* phosphorylated glucose, mannose, galactose and fructose. Three isoenzymes of hexokinase have been observed in *D. immitis*; the enzyme was inhibited by Glucose-6-phosphate and it could also use glycerol as a glycolytic substrate (Fig. 3).

Under aerobic conditions *L. carinii* produces acetate which is derived from decarboxylation of pyruvate, probably via pyruvate dehydrogenase. This enzyme is present in significant amount in *L. carinii* but low activity is observed in *B. pahangi* and *D. viteae*. Since the discovery of PEP-oxaloacetate pathway in invertebrates, its operation has been studied in several helminth parasites. Balance between PK and PEPCK and their affinity for the substrate (PEP) are the factors that determine whether metabolic products are channeled to succinate or lactate. *S. cervi* which converts only 25% carbohydrate (glucose) to lactic acid and possessing low levels of PK and LDH and high activities of PEPCK and MDH has a functional PEP-succinate pathway. On the other hand typical lactic acid producers like *C. hawkingi* converting 80 to 90% glucose into lactic acid resemble vertebrate tissues in possessing high levels of PK and LDH and low levels of PEPCK and MDH. These parasites have a metabolic pathway leading to lactate accumulation while values less than one suggest the operation of carbon dioxide fixing pathway and probable production of succinate. Both mf and adult forms of *S. cervi* have a PK/PEPCK ratio less than one. However, in adult forms of *D. immitis*, *C. hawkingi* and *L. carinii* this ratio was higher than one supporting the preferential formation of lactate. Thus, *S. cervi* differs metabolically from other filarial prasites viz., *L. carinii* and *C. hawkingi* and resembles more closely the intestinal parasites with regard to the activities of PEP-metabolising enzymes (Fig. 4).

The occurrence of G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase in mf indicates the possible utilization of pentose phosphate pathway which may be related to the need for large amount of ribose. Similarly the presence of glucose-6-phosphatase in mf suggests that gluconeogenic processes may also occur in this life stage.

Few enzymes of glycolytic and PEP – succinate pathway have been purified and characterized in order to elucidate their regulatory roles. Partial purification and characterization of glucose phosphate isomerase (GPI) from *S. cervi* adult suggested the possible existence of three isoenzymic forms differing from each other on the basis of K_m values. Partially purified GPI was stable upto 50^oC, optimally active at pH 8.6, had no metal ion requirement, inactivated by Mn^{++} and Co^{++} and possessed functional-SH groups.

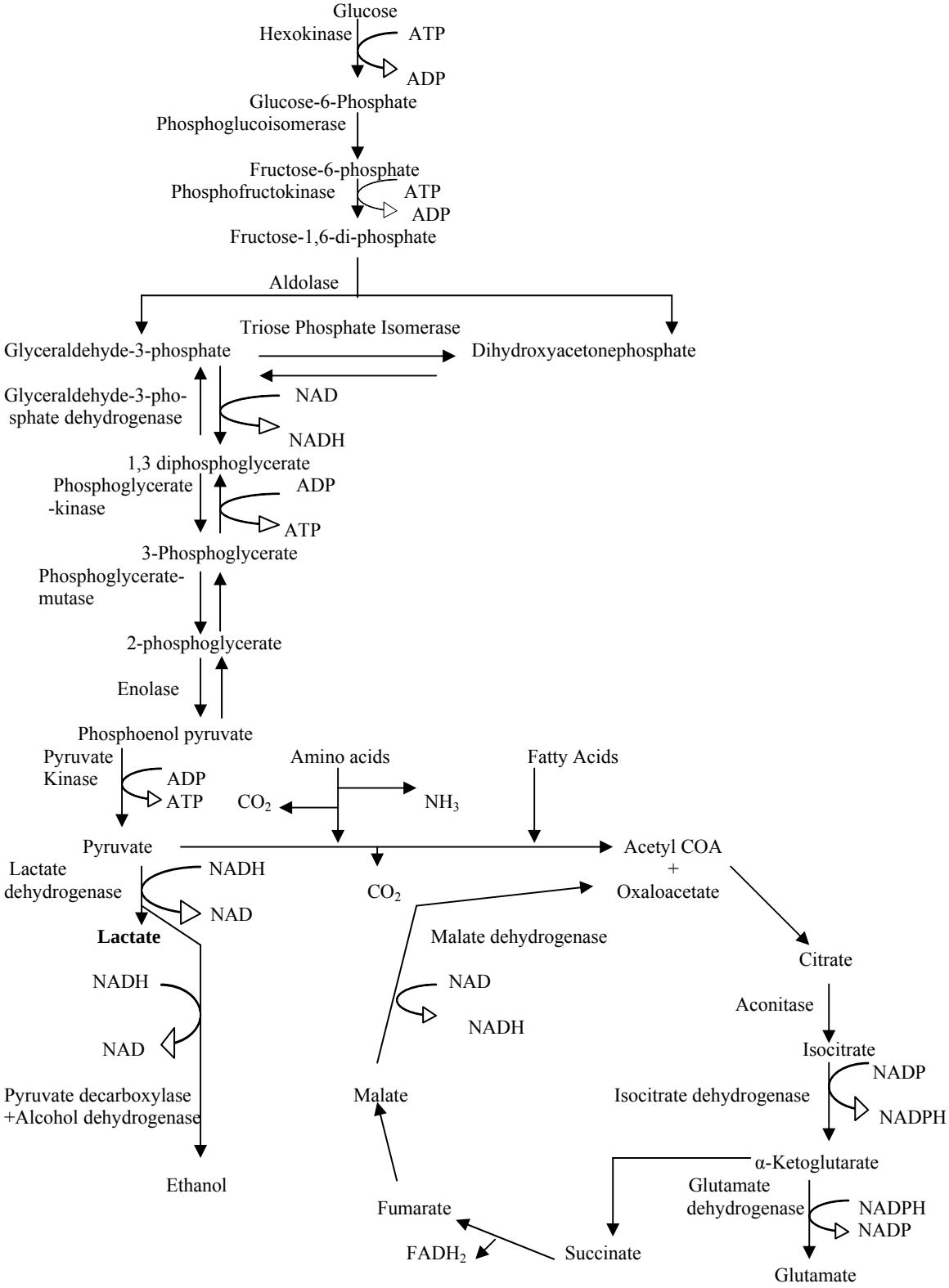

Fig. 3: The pathways of carbohydrate metabolism in parasites

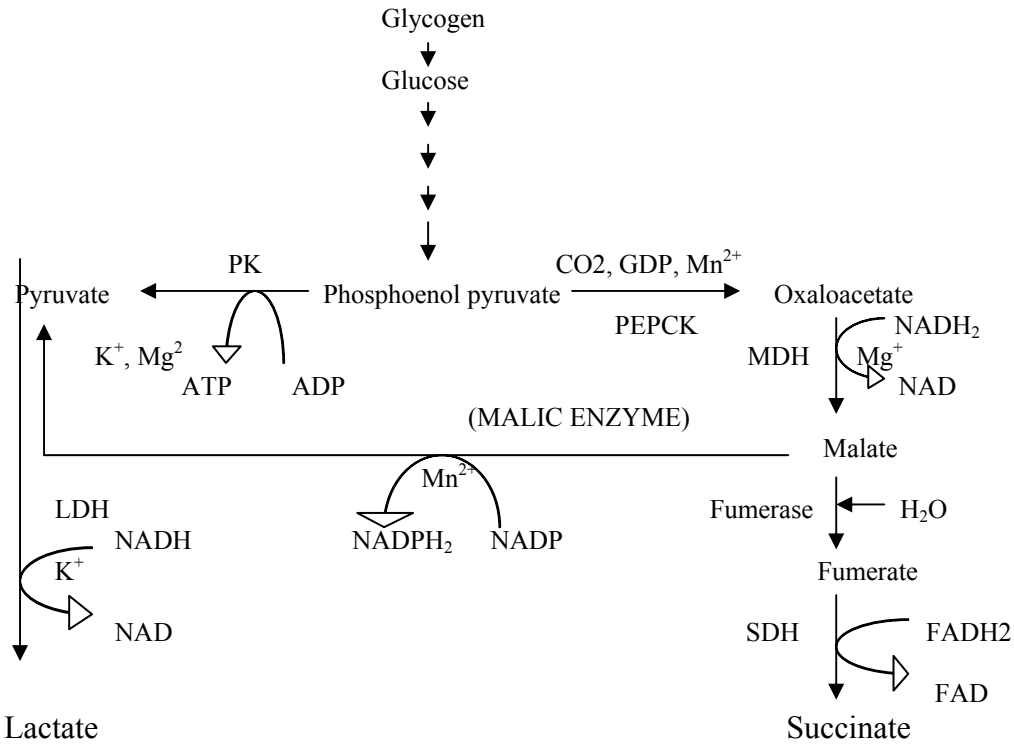

Fig. 4: Phosphoenolpyruvate- succinate pathway in filarial parasites
PK = Pyruvate kinase; PEPCK= Phosphoenol pyruvate carboxykinase; MDH = Malate dehydrogenase; LDH = Lactate dehydrogenase; SDH = Succinate dehydrogenase

Phosphofructokinase (PfK), a key enzyme of glycolysis has been partially characterized. PfK from *S. cervi* females could be stabilized using 2 mM F-6-P and purified enzyme was optimally active at pH 7.4, had a K_m value of 1.05 x 10^{-3}M and contained SH groups at the active site of enzyme molecule Mg^{++} and NH4 stimulated the enzyme while Mn^{++} and Cu^{++} were potent inhibitors. PfK was activated by AMP while GDP, FDP and ATP exerted significant inhibitory effect. Suramin inhibited the enzyme at very low concentration with a K_i value of 3.5 μM. FDP-aldolase has been separated into two isoenzymic forms. The optimum pH was observed to be 8.6 and the enzyme was not metal activated and could be classified as aldolase I. K_m values for isoenzyme I and II had been reported to be 0.11 mM and 0.4 mM respectively.

Pyruvate kinase (PK), a regulatory enzyme of carbohydrate metabolism has been purified and its kinetic properties were studied. Unlike most regulatory enzymes, PK from *S. cervi* showed normal Michaelis Menton kinetics towards both the substrates i.e., PEP and ADP. On the basis of the effect of various activators, inhibitors and metal ions it can be suggested that the activity of *S. cervi* PK is controlled by the variation in the intracellular concentration of PEP, ATP, malate and certain metal ions. PEP-carboxykinase (PEPCK) of *S. cervi* has also been partially purified and characterized, PEPCK was stable upto 50°C with pH optimum of 6.0 Mn^{++}, was more effective promoter of the reaction than Mg^{++}. The enzyme was activated at higher concentration of PEP, its substrate, FDP stabilized the enzyme while ATP exerted significant inhibitory effect.

Lactate and malate dehydrogenases are associated with the key role of reoxidation of NADH permitting continuous operation of metabolic cycles. This process is critical for helminthes because they thrive in anaerobic habitats and these enzymes from filarial parasites have been

used as target for chemotherapy of *Onchocerca* and *D. immitis* infections. LDH has been purified from *D. immitis, O. volvulus* and *S. cervi*. MDH has also been purified and characterized from *S. cervi* and *O. volvulus*. Both LDH and MDH needed functional SH groups for the enzyme activity. The K_m values for oxaloacetate and NADH were found to differ significantly for MDH from various helminth parasites. Polycations viz., protamine, histone and spermine were found to be strong activators of the *S. cervi* MDH. These polycations also protected the enzyme from dilution inactivation effect. Suramin, aurin tricarboxylic acid and dextran sulfate strongly inhibited the soluble enzymes. Aurin tricarboxylic acid was found to be potent inhibitor for MDH and LDH inhibiting them by 88% at 0.025 μg and 52% at 2.5 μg concentration respectively. A few Leo compounds viz., polyphloretin phosphate (PPP), polyestradiol phosphate (PEP), polydiethylstilbesterol phosphate (PSP), polybisfenol A phosphate and polymethylene salicylic acid were discovered as new inhibitors for LDH and MDH.

Malate and lactate dehydrogenases of *S. cervi* were immobilized on insoluble matrix (alkylamine and arylamine) glass beads. The insolubilised enzyme exhibited changes in its kinetic characteristic and other properties. The matrix bound enzyme had higher efficiency as evidenced by greater temperature stability, longer half-life and capability of being repeatedly used as compared to soluble enzyme. K_m (app) for NADH was increased upon insolubilisation and the immobilized enzymes were very weakly inhibited by the inhibitors of soluble enzyme. The process of immobilization altered the conformation of the enzyme and disposition of its various groups and sites in such a way that they were not accessible or sensitive to the action of potential anionic inhibitors. Immobilization of parasitic enzymes may be of great significance in understanding the mode of action of various antifilarial drugs under *in vivo* conditions.

The LDH and MDH of *O. volvulus* were inhibited by suramin at 1 μM concentration implicating them as targets for antifilarial drug action. The LDH iso-enzymes from *O. gibsoni, B. pahangi* and *D. viteae* have been purfied by affinity chromatography on oxamate-sepharose column and characterized. Each of the parasites had only a single LDH isozyme. The enzyme from *O. volvulus, O. gibsoni* and *D. viteae* had similar electrophoretic mobility intermediate between bovine LDH and LDH_4, while *B. pahangi* LDH had different electrophoretic mobility.

The TCA cycle is of great importance of many aerobic organisms as energy yield through this pathway is much greater than from glycolysis. Most of the helminthes have been found to be aerobic fermentors and sensitive to deficiency of oxygen. They consume oxygen and have been shown to survive longer when traces of oxygen are present. Oxygen uptake has been measured in mf and adults of *D. immitis* and in adult stage of *D. uniformis* and *L. carinii*. The nature of the terminal oxidase in filarial worms is uncertain. *S. cervi* contains most of the enzymes of TCA cycle, however, the levels of the enzymes were considerably lower when compared to those in mammalian system. Fairly normal looking cristate mitochondria have been reported in *B. pahangi* and *L. carinii* adults as well as adults and mf of *D. immitis*. However, neither cytochrome nor cytochrome oxidase could be detected in *B. pahangi* or *D. viteae*.

Cytochrome C (C and C1) and low level of cytochrome a have also been reported in adult *D. immitis*. It has been suggested that *D. immitis* contains a branched cytochrome chain and O-type cytochrome may serve as alternative oxidase. *S. cervi* adult females contain cytochrome b_5 but inspite of several attempts presence of cytochrome P_{450} could not be established in this

filarial parasite. Cytochrome P_{450} was also not detected in *O. gibsoni* and *D. viteae* and involvement of classical and alternate electron transport system in their metabolism. An anaerobic system for production of ATP has been demonstrated in cuticle hypodermis muscle system and is insensitive to cyanide but 2,4-dinitrophenol (DNP) and antifilarials inhibited ATP formation.

Enzymes of amino acid metabolism

The presence of serine hydroxymethyl transferase was detected in adult *B. pahangi* and *D. immitis*. Neither methionine synthase nor betaine: homocysteine transmethylase could be detected in adult *B. pahangi* and exogenous methionine is required by these two parasites. Glutamate dehydrogenase which plays an important role in deamination of amino acid and formation of α-amino nitrogen group from ammonia has been demonstrated in mf of *D. immitis* and adult of *S. cervi*. Presence of alanine amino transferase (GOT), glutamate pyruvate transferase (GPT), serine dehydratase, threonine dehydratase and arginase were found in adult *S. cervi*. 1-Alanine aminotransferase was more active than aspartate-aminotransferase showing maximal activity at pH 8.0 and 8.5 respectively and K_m of 13 mM and 9mM respectively. SH blocking reagents markedly inhibited the enzyme and metal ions with the exception of Mg^{++} had no effect.

Hydrolytic enzymes

Acid phosphatase activity has been demonstrated in adult female of *D. immitis*. The enzyme showing optimal activity at pH 3.8-5.8 was inhibited by tartarate. Reproductive organs and body wall of *D. immitis* exhibited high activity. Presence of two non-specific acid phosphomonoesterases had been reported in *S. cervi*, $MgCl_2$ activated the enzyme while NaF and tartaric acid inhibited it. Infection of mosquitoes by *W. bancrofti, B. malayi, B. pahangi* and *D. immitis* were accompanied with changes in the activity of acid sulfatases that were characteristic of the parasite as well as the developmental phase.

N-acetyl-β-D-glucosaminidase, β-galactosidase, β-glucosidase, β-glucuronidase, acid phosphatase, alkaline phosphatase, acid ribonuclease, acid deoxyribonuclease and cathepsin were found to be present in adult and microfilarial stages of *S. cervi* female worms. The distribution pattern of these enzymes also differed in various body parts of the parasite.

Protein kinases

Parasites modify their metabolism and make varied adaptations for their survival within the host as well as during their development. These adaptations involve regulation of various metabolic pathways. Transcuticular uptake of cAMP has been observed in *L. carinii* and *D. viteae* which was inhibited by lectins suggesting involvement of surface sugar molecules in transport mechanism. Phosphorylated proteins play major role in the control of diverse biological processes and glycogen and energy metabolism may be regulated by cAMP and also by protein kinases. The occurrence of cAMP dependent and independent protein kinases has been reported in *O. volvulus*, *B. malayi, D. viteae, L. carinii* and *S. cervi* and in their developmental stages. Protein kinases of *B. malayi, D. viteae, S. cervi* and *L. carinii* phosphorylated wide variety of exogenous proteins and peptides. The functions of these protein kinases in the metabolism of filarial worms and the possible involvement in differentiation and development have not yet been investigated. The endogenous substrates

for these protein kinases are still unknown in the case of filariids. Beside the involvement of protein kinase in regulation of glycogen metabolism, they activate phosphofructokinase of *A. suum* while pyruvate dehydrogenase is inactivated by cAMP dependent protein kinase.

Neurotransmitters and their metabolism

Various biogenic amines viz., Norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT) and histamine (Hm) have been reported to be present in mf and adults of *L. carinii*, *D. viteae* and *S. cervi*. The parasite can survive in their natural habitat due to their ability to remain *in situ* when exposed to peristaltic movement in the case of intestinal parasites or movement of blood or lymph in systemic parasites. Neurotransmitters play an important role in the regulation of motility and metabolism of the parasites. Sensory receptors provide the organism with information concerning environment of the host. Acetylcholine, serotonin, epinephrine and dopamine have been implicated as a putative neurohormonal transmitters in parasitic worms. Dopamine was not detectable in mf of *L. carinii* while the content of other amines were 10-20 times higher in mf as compared to adults on wet weight basis. Neurotransmitters are taken up into nerve endings by the process of reuptake resulting in the termination of its effect. This process is energy dependent and sensitive to metabolic inhibitors, temperature etc. Presence of such uptake mechanism has been reported in a few parasitic worms. *L. carinii* was found to possess both high and low affinity uptake mechanism for 5-HT. Initially the 5-HT incorporation was rapid and uptake mechanism operated against a concentration gradient. Two distinct receptors seem to have been detected in *L. carinii* for 5-HT; K_m values for high and low affinity system were 1.9 μM and 10 μM respectively. The uptake mechanism was found to be temperature dependent at 25^oC the incorporation was 3 times lower as compared to 37^oC. Although biosynthesis of 5-HT in mammalian brain has been well established, evidence for *de novo* synthesis of 5-HT in *L. carinii* could not be presented. Incubation of worms with tryptophan and pargyline for different time intervals in the medium resulted in no significant increase of the amine level probably due to saturation of tryptophan hydroxylase with endogenous tryptophan. Centperazine significantly inhibited the 5-HT uptake as compared to DEC. Presence of low concentration of 5-HT and lack of tryptophan hydroxylase in *L. carinii* indicate that the parasite must have obtained their 5-HT from the host. The high affinity uptake mechanism might be responsible for the supply of this neurotransmitter. Monoamine oxidase (MAO), responsible for the catabolism of neuroamines was present in both mf and adults of *S. cervi*. The enzyme, mainly localized in mitochondria was found to be more active in female worms as compared to male worms and mf. Spectrofluorometric studies of purified MAO revealed the presence of FAD. *S. cervi* MAO can be differentiated from the host enzyme (bovine) on the basis of substrate specificity, pH optima and K_m value. Presence of dopamine-β-hydroxylase in mf and adults of *S. cervi* has also been shown.

Acetylcholinesterase (AchE), having a role in neuromuscular transmission, was detected both in mf and adults of *S. cervi*. Microfilariae contained ten times more activity of AchE as compared to adult. *S. cervi* enzyme did not show any activity with butyrylthiocholine suggesting the absence of pseudocholinesterase. Both adult and mf of *S. cervi* released significant amount of AchE during *in vitro* incubation at 37^oC in a defined medium. Centperazine, DEC and levamisole strongly inhibited the released enzyme.

Polyamine metabolism

Octopamine (OA) is the only amine with no apparent vital function in mammals. OA plays an important role in the regulation of a number of key processes in nematodes, including pharyngeal pumping, locomotion and egg laying. Among filariids it has been identified in *O. volvulus* and *B. pahangi*. The polyamines putrescine, spermidine and spermine are found in all living organisms and are involved in growth, differentiation and macromolecular synthesis. Polyamine determinations of filarial worms *O.volvulus, D.immitis, Brugia patei, S.cervi* and *L.carinii* have demonstrated that these parasites contain high levels of spermidine and spermine but low levels of putrescine and N-acetylated polyamines. The enzymes of polyamine biosynthesis viz. ornithine decarboxylase (ODC), S-adenosyl metheonine decarboxylase (SAMDC) and arginine decarboxylase (ADC) were either very low or absent in filarial parasites. More ever uptake of polyamines from the incubation medium as well as interconversion and excretion of putrescine and N1- acetylputrescine has been found in filariids. There is evidence for the existence of complete reverse pathway generating putrescine from spermidine and spermine respectively in filarial worms. The presence of considerable levels of polyamine oxidase, an important enzyme of the reverse pathway of polyamines, indicates a strong point in favour of salvage pathway for polyamines in helminthes. S-adenosyl –methionine decarboxylase (SAMDC) a key regulatory enzyme of the polyamine biosynthesis is considered as a potentially important target for chemotherapy of filarial infection. Various inhibitors of SAMDC like Berenil and aromatic methyl glyoxal bis (guanil hydrazone) analogues might have potential as drug candidates against filarial worms. The *in vitro* treatment of adult filariae with polyamine analogues and inhibitors of enzymes involved in the polyamine biosynthesis were effective in killing the parasites (Fig. 5).

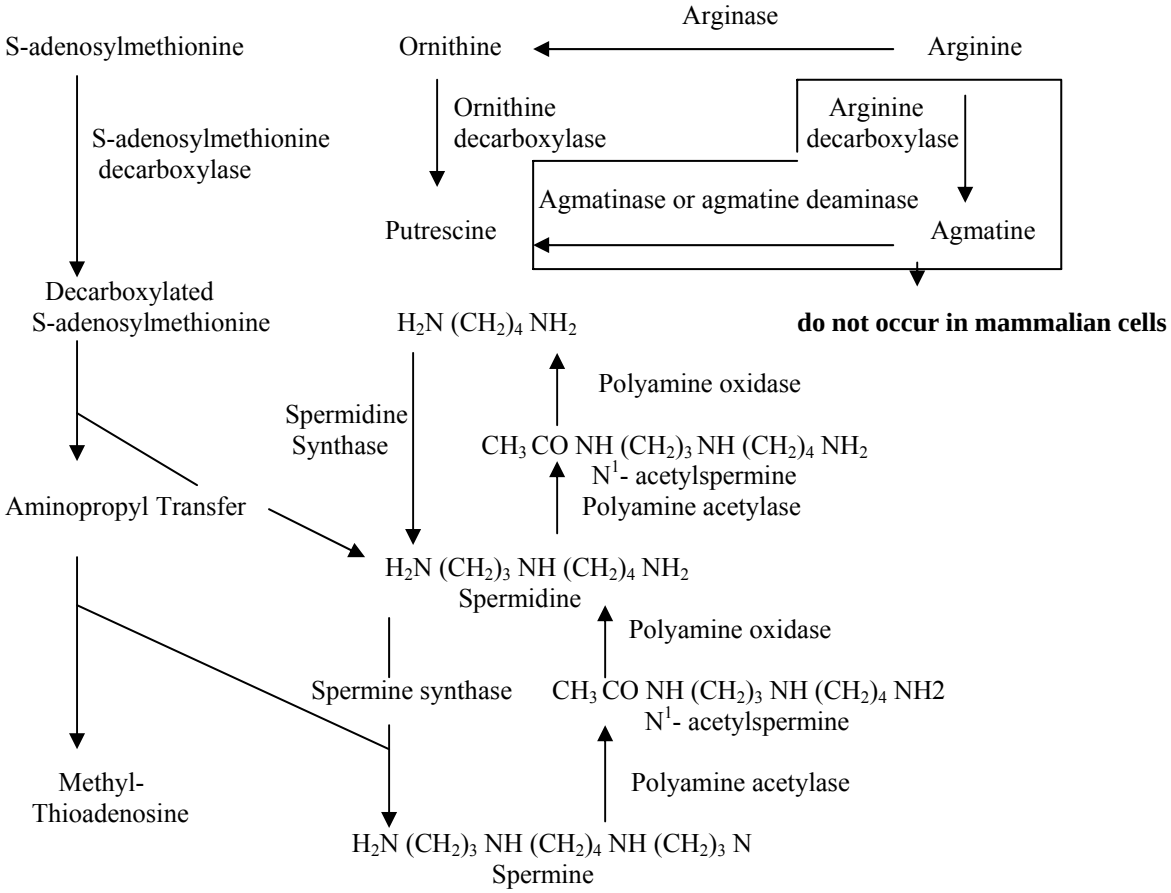

Fig. 5: Polyamine biosynthetic and interconversion pathways in filarial parasites

DNA topoisomerases in filarial parasites

DNA topoisomerases are cellular enzymes and are intricately involved in maintaining the topological structure of DNA, transcription and mitosis. Among the various enzymes identified for drug development against parasitic disease, DNA topoisomerase II has been picked up as a novel target for antifilarial drug development due to several reasons. Eukaryotic DNA topoisomerase I has been identified as the primary target for the antineoplastic alkaloid camptothecin, whereas DNA topoisomerase II is the target for many anticancer agents including both non intercalating (VM-26) and interacalating (m-AMSA) compounds. Antibacterial agents coumarins and quinolones, are inhibitors of DNA-gyrase. The studies have shown that filarial parasites *Brugia malayi*, *Acanthocheilonema viteae* and *Setaria cervi* adults and microfilariae contained ATP-independent (topoisomerase I) and ATP-dependent (Topoisomerase II) activities. The activities were localized in nuclear fraction and distribution pattern differed between adults and mf stages of filarial parasites. *B. malayi* and *S. cervi* topoisomerase II differed significantly from its human homologue in its kinetic properties. The DNA topoisomerase inhibitors exerted significant effect and antifilarial compounds suramin and ivermectin proved to be strong inhibitors of the parasitic enzyme suggesting the potentials of the enzyme as drug target and designing of novel compounds against adult parasite.

Cellular and molecular targets for antifilarial drugs

The metabolic difference between the parasite and host as well as between various species of parasite can yield information regarding the mechanisms for multiplication and survival of parasite as well as the disease process. The aim of specific chemotherapy is the removal of invading organism without injury to the host. In order to achieve this one must define the biochemical structure and metabolic pathways of the parasite and its various developmental stages and synthesize selective reagent(s) which can inhibit the developmental of the parasite without affecting the host. The identification of sensitive molecular targets can provide a more rational approach for the chemotherapy of parasitic diseases. Benzimidazole derivatives (mebendazole and flubendazole) have wide spectrum of anthelminthic action. Mebendazole has micro- as well as macrofilaricidal action on *L. carinii* in cotton rats, while flubendazole has been reported to have chemoprophylactic action against *B. pahangi* in cats. Flubendazole is more effective against *O. gibsoni* than mebendazole, while the latter drug has proved effective against adults as well as mf of *O. volvulus*. The primary site of action of mebendaeole is by inhibiting microtubule assembly. Benzimidazole derivatives also affect the enzymes of glycolysis and PEP-succinate pathway in helminth parasites. The enzymatic reduction of fumarate to succinate catalysed by fumarate reductase (FR) has received much attention as a possible site for anthelmintic action. Thiabendazole inhibited FR system in susceptible strains of *Haemonchus contortus* only while cambendazole inhibited FR system of even resistant strain of this parasite. Thus, FR system which functions as a respiratory chain in many helminthes also appears to be a likely target for action of other broad spectrum antihelmintics viz., thiabendazole, cambendazole, 1-tetramisole, morantel tartarate and disophenol. Table 1 shows the molecular targets of antifilarials drugs.

The microfilaricidal action of DEC is mediated through immunological system, antibodies, complement and specially eosinophils appears to be the key mediators. DEC enhanced cell adhesion and cell clump formation with entangled mf of *W. bancrofti* in presence of immune serum and leucocytes. Higher concentration of DEC inhibited adhesion. DEC-N-oxide, a major metabolite of DEC, exhibited effects similar to DEC but no clump formation was

observed. DEC is effective against mf of *O. volvulus* but not adult worms. Severe immunological side reactions of allergic nature are encountered in some individuals on administration of DEC.

Table 1: Possible mode of action of antifilarial compounds

S. No.	Antifilarial compound	Possible mode of action
1.	Diethylcarbamazine (DEC)	Neuromuscular system, cuticular surface, carbohydrate and folate metabolism, host-immune factors.
2.	Suramin	Carbohydrate and folate metabolism, protein kinases, intestinal epithelium, LDH, MDH.
3.	Ivermectin	Neuromuscular system, host immune factors.
4.	Benzimidazoles	Assembly of microtubules.
5.	Isothiocyanates and Derivatives	Cuticular surface, carbohydrate metabolism, cyclic AMP phosphodiesterase, 5'-nucleotidase, aminoacyl-tRNA synthetase.
6.	Levamisole	Neuromuscular system, carbohydrate metabolism.
7.	Arsenicals	Carbohydrate metabolism, intestinal epithelium, glutathione metabolism.
8.	Antimonials	Carbohydrate metabolism.
9.	Benzthiazoles	Glutathione and related metabolism.

Centperazine, DEC, levamisole and CDRI compound 72/70 significantly inhibited glucose utilization and synthesis of glycogen in *S. cervi*. These drugs also inhibited glucokinase thereby decreasing the utilization of exogenous glucose. Protein synthesizing capacity and release of mf from adult females was severely affected by these drugs. Both DEC and centperazine inhibited fumarate reductase, PEPCK, succinate dehydrogenase as well as protein and RNA synthesizing capacity of *S. cervi* mf and female worms.

Incorporation of glucose, valine and synthesis of glycogen and protein in adult *L. carinii* was significantly altered by centperazine, DEC and compound 72/70. Centperzine was most effective in altering the metabolic activity. Decrease in the release of mf in the incubation medium after 4 hr treatment by above drugs suggested that these filaricides may also affect the reproductive system of *L. carinii* and *S. cervi*. Centperazine and DEC also inhibited 5-HT uptake by *L. carinii* adults.

Most antihelmintic agents act directly or indirectly by inhibiting either neuromuscular transmission or energy generation. Hence for, rational understanding of the drugs action and design, knowledge about energy generating pathway is essential. Enzymes of *S. cervi* mf have also been shown to be affected by antifilarial agents. LDH and MDH from *S. cervi*, *O. volvulus* and LDH of *D. immitis* are effectively inhibited by suramin. NADP-dependent malic enzyme from both these parasites are specifically inhibited by suramin. Aurin tricarboxylic acid and leo compounds were found to be potent inhibitors of LDH and MDH from *S. cervi*. It has been suggested that inhibition of LDH and MDH in filarial parasites will inhibit the

reoxidation of NADH generated by glyceraldehyde-3-phosphate dehydrogenase leading to eventual blockage of glycolysis in the parasite.

Amoscanate, a potential filaricide inhibits ^{3}H labeled glucose uptake and transport in *B. pahangi* and *L. carinii*. It also inhibited cAMP phosphodiesterase in *O. volvulus* and *S. mansoni* and aminoacyl-tRNA synthetase complex in *A. summ.*

The information on the biochemistry of filarial parasites reveals the fascinating mosaic of biochemical reactions employed by the organisms for their survival and adaptations to different hosts, different issues with differing structure and chemical composition, defence parameters as well as pH and redox potentials. The metabolic reactions of parasite differ considerably from the respective hosts in the gross pathway as well as in molecular and biochemical properties. The parasites differ considerably from each other again reaffirming the suggestion. Each organism must be examined as a biochemical entity before any reasonable understanding of helminth metabolism can be attained. All the parasites examined so far employ predominantly anaerobic metabolism of carbohydrates as the major energy yielding pathway. However, these parasites also utilize limited amount of oxygen if available but do not have ability to bring about complete oxidation of substrates to carbon dioxide. The biochemical insufficiency of these parasites is manifested in formation of lactate and some other organic acids as end products of metabolism. Electron transport chains are rudimentary, catalyze only limited terminal oxidation with meager generation of energy. Energy yielding biochemical pathways, nerve transmission and neuromuscular conduction regulating parasitic motility represent the targets for action of many antifilarials. Folate, nucleic acid, polyamine metabolism and other biochemical pathways are being explored as alternate target for chemotherapy. Microfilariae have proved to be the most vulnerable target so far. However, microfilaricides yield only transient cure. Vulnerable targets of adult parasites are badly needed to design macrofilaricidal drugs for any lasting solution to filariasis. Recent studies on molecular biology of the simple nematode *Caenorhabditis elegans* also rise hope for similar breakthrough in molecular biology of filariids and other helminthes which can be exploited for control of helminth infections. The study of parasitic helminthes, in view of their public nuisance, would generate knowledge of comparative biochemistry as well as the origin of regulatory and defence mechanisms, which have seen perfection in higher forms of life. This information in turn can be meaningfully utilized for understanding the host invader interaction and managing it in favour of mammalian host.

Malaria

Malaria still remains one of the most important parasitic diseases of the developing world although it is known to humankind since ancient times in different forms. It kills approximately 1-3 million people and causes disease in 300-500 million people annually. Pregnant women are the main adult risk group in most endemic areas of the world. The malaria parasite is a protozoan species, and four distinct species; *P. falciparum, P. vivax, P. malariae* and *P. ovale are* causative agent in man. Some other related species including *P. berghei* and *P. yeolii* are specific to other group of the mammalian class. *P. falciparum* is the cause of malignant tertian or *falciparum* malaria, which has a substantial mortality if it is untreated especially in the first or an early attack.

Pathogenesis and life cycle of the malarial parasite

The female Anopheles mosquito, injects sporozoites into human host at the time of blood suck. The sporozoites migrate to the liver and invade hepatocytes within 1 h. where they complete the pre-erythrocytic and exo-erythrocytic stages of their life cycle leading to hepatic schizogony. After 5-7 days, the infected hepatocytes rupture and release thousands of merozoites, which invade erythrocytes and start the erythrocytic phase. The parasite develops and replicates within the erythrocytes and after 24-26 hr the trophozoite adhere to the endothelium of small blood vessels. The trophozoites grow to the schizonts (erythrocytic schizogony) and after 48 hr rupture the erythrocytes and release their progeny (16-32 merozoites per schizont) in the blood (Fig 6). An unidentified malaria toxin is released on rupture of schizont-erythrocyte resulting in cytokine response, which leads to clinical manifestations of the typical malaria including high fever, chills, prostration and anemia. The pathogenicity of the parasite results due to its rapid rate of asexual reproduction in the host and its ability to sequester in small blood vessels.

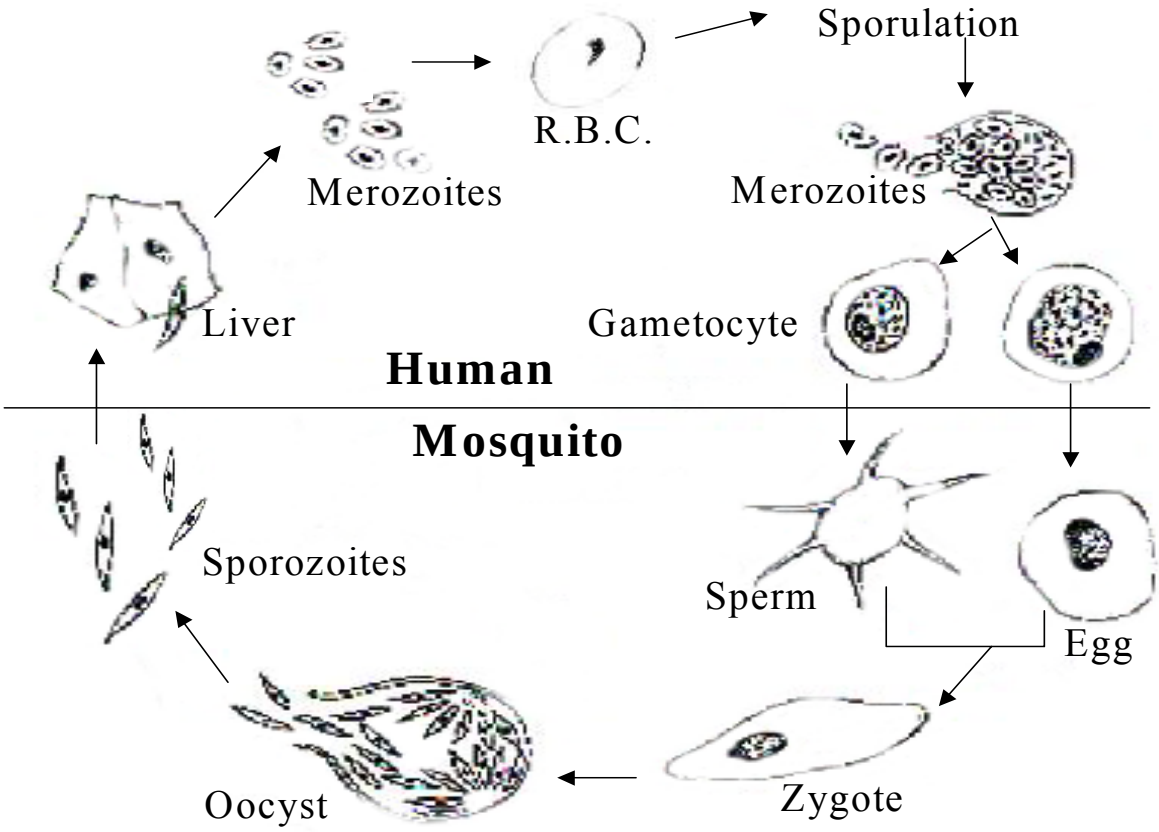

Fig.6: Life cycle of malarial parasite

Both the traditional and current approaches have been used to control malaria. The use of impregnated bed nets with residual pyrethroids, e.g. perimethrin and deltamethrin, is likely to increase, once their value in reducing malarial morbidity is more widely established. The current approaches to curtail this disease include the vector control, immunotherapy, vaccination and the chemotherapy.

Vector control can be achieved either by minimizing the contact of human host and mosquito host impossible or killing the mosquitoes by insecticides. Vector control may be broadly divided into three main categories (a) reducing vector density (b) interrupting their life cycle, and (c) creating a barrier between the human host and the vector, i.e. simply preventing the mosquito bite. The environmental modification/ manipulation and changes in the biosystem are solutions to control vector density. Interruptions in the life cycle of vector mosquito leading to its eradication include destroying their breeding sites and resting areas and more specifically by use of organisms feeding on vector larvae. Further, artificial barrier between the vector and the host can be met by using the insecticides, repellents, protective clothings and the bed nets.

Vaccination in malaria represents one of the most important approaches that would provide a cost-effective intervention in addition to currently available malaria control strategies. During the past decade understanding the immune mechanism involved in the protection against this disease has made significant progress and many vaccine candidate antigens and their genes have been identified. An ideal malaria vaccine encompasses mainly three essential characteristics (a) it should incorporate antigenic characteristics of multiple stages of *P falciparum's* life cycle, (b) it should be multivalent containing multiple epitopes restricted by different MHC molecules, which would help in overcoming the genetic restriction and allelic and antigenic variations, and (c) it should induce more than one type of immune response, comprising both cell-mediated and humoral immunity. Such a multi-component vaccine would increase the probability of a more sustainable and effective host response. Most of the vaccine trials are directed against liver stages or sporozoites, and these vaccines included completely synthetic peptides, conjugates of synthetic peptides with proteins such as tetanus-toxoid to provide Helper T-cell, recombinant malarial proteins, particle-forming recombinant chimeric constructs recombinant viruses, and bacteria and DNA-based vaccines. Asexual blood stage vaccine trials have used either synthetic peptide conjugates or recombinant proteins. Some of the recently developed vaccines against falciparum malaria are:

SPf66 is the first recognized malarial vaccine developed from three merozoite-derived proteins by joining them with sequences derived from the repeat domain of the circumsporozoite (CS) protein of *Pl. falciparum*. SPf66 was confirmed to be safe and immunogenic. CSP is a circumsporozoite protein (CSP) incorporating the recombinant (Asn-Ala-Asn-Pro15-Asn-Val-Asp-Pro)-2-Leu arg (R32LR) covalently linked to purified *Pseudomonas aeruginosa* toxin A9. Furthermore, DNA vaccines against malaria are known to have CSP sequencing genes (Fig.7).

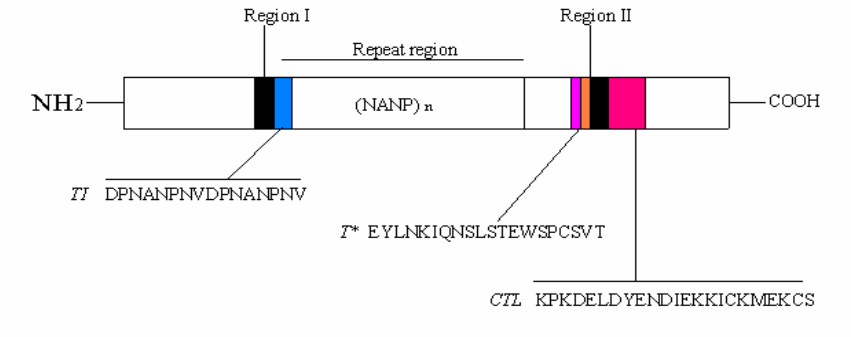

Fig. 7: CS protein of *P. falciparum*

The multistage vaccine NYVAC-Pf7 is a single NYVAC genome containing genes encoding seven antigens from *Plasmodium falciparum*. Out of these seven antigens, two are derived from the sporozoite stage of the parasite life cycle (CSP and sporozoite surface protein 2 (PfSSP2), one from the liver stage (liver stage antigen 1 (LSA), three from the blood stage (merozoite surface protein 1 (MSP1), serine repeat antigen (SERA), and AMA-1, and one from the sexual stage (25-kDa sexual-stage antigen (Pfs25).

NANP consists of 19 repeats of the sporozoite surface protein (NANP) and the schizonts export antigen 5.1. However, this vaccine has limitation of containing no immunodominant T-cell epitopes.The circumsporozoite surface protein of the sporozoite stage of *Plasmodium falciparum* RTS elicits antibodies that are capable of preventing sporozoites from invading hepatocytes, and a cellular response that is capable of eliminating infected hepatocytes. Pfs vaccine is a sexual-stage falciparum surface antigen and can elicit antibodies, which block the infectivity of gametes to mosquitoes.

DNA based vaccines are the newest technology that may hold the key to control many infectious diseases including malaria. DNA vaccine is a source of a stable and long-lived protein vaccine which can induce both antibody and cell mediated immune responses to a wide variety of antigens.

Immunity development against malaria is a very complex phenomenon in individuals living in areas of high endeminity where the population naturally acquires varying protective immunity against the disease. Clinical studies have demonstrated that experimental vaccination of humans with attenuated sporozoites can induce effective protection against a subsequent challenge. Animal studies of malaria vaccination clearly demonstrated the potential for the induction of protective immunity; following active immunization using different *Plasmodial* components for e.g. immunization with *P. knowlesi* can induce immunity, which has been found to be superior to the immunity developed from natural infections in humans.

Genetic mapping of *P. falciparum* has revealed that the parasite contains 14 chromosomes and approximately 5300 genes responsible for protein synthesis. Two third of the total genes are unique to the parasite. Furthermore about 208 genes are known to be responsible for the evasion of parasite from host immune response.

Biochemistry of parasite

A fundamental reason for studying the biochemistry of malaria parasites is to uncover those metabolic differences between the host and parasite that might be exploited in the design of drugs specifically targeted to *Plasmodium*, as well to provide an understanding of the mode of action of existing antimalarials. The tools of molecular biology have provided the possibility for cloning, sequencing, and expressing the plasmodial genes of various metabolic pathways.

The intraerythrocytic stages of malaria store no energy reserves in the form of glycogen or lipids; consequently, the glucose present in the blood plasma serves as the directly utilizable energy source. Glucose is rapidly taken up by parasitized erythrocytes where it is metabolized, human erythrocytes (10^9) consumed approximately 5 μmol of glucose per 24 hr, whereas a similar number of infected red cells from an *in vitro* culture of *Plasmodium falciparum* used around 150 μmol in the same time period. The amount of glucose consumed

by a single infected red cell could be 100 times greater, than that of the uninfected red blood cell. The precise amount of glucose utilized, is dependent on the number of parasites present, the stage of parasite development (e.g., Schizonts or rings), and the experimental conditions (i.e. pH, temperature, initial concentration of glucose, medium composition etc). The increase in glucose consumption by an infected red blood cell could be due to the stimulation or deregulation of the enzymes of the host cell. Studies have shown that almost all the increase in utilization is the result of glycolytic enzymes synthesized by the parasite. These enzymes operate at an accelerated rate and with a lower pH optimum than those of the host red blood cell. Almost all of the glucose used by the malaria-infected red cell passes through the anaerobic Embden-Meyerhoff-Parnas (EMP) pathway.

Glycolytic enzymes

Many of the key enzymes of glycolysis occur as isoenzymes, that is, multiple molecular forms of the enzyme having different affinities (K_m) for the substrate, different maximum activity (V_{max}). The enzymes of the EMP pathway viz., hexokinase, phosphoglucose isomerase (PGI), phosphofructokinase (Pfk), aldolase, triose phosphate isomerase (TPI), phosphoglycerate kinase, phosphoglucomutase, enolase and lactate dehydrogenase have been identified in *P falciparum* infected cells and several avian and rodent parasites. In malarial parasites diphosphoglycerate mutase required for synthesis of 2, 3 - diphosphoglycerate is absent (Fig. 8).

Plasmodial-specific hexokinase activity has been identified in extracts of rodent, avian and *P. falciparum*-infected red cells. In *P. falciparum* infected erythrocytes there was a 25-fold increase in hexokinase activity when compared to that of uninfected red cells, and the plasmodial enzyme has a lower K_m for glucose. In *P. falciparum* the gene for hexokinase located on chromosome 8, shows 26% homology with human hexokinase and has a molecular size of 54 kDa. The gene of PGI from *P. falciparum* is located on chromosome 14 and the plasmodial enzyme has a molecular size of 66 kDa, showing 34% homology to that from human tissues and has the highest degree of similarity in the region of the active sites. As in mammalian cells, PFK is major regulatory enzyme in malarial parasites. *P. berghei* PFK has been studied in detail, and it differs in kinetic properties from other eukaryotes. The *P. berghei* and *P. falciparum* genes for aldolase have been cloned, sequenced, and expressed. The two isoenzymes of *P. berghei* aldolase are virtually identical to the enzyme expressed in sporozoites and asexual stages of *P. falciparum*. Amino acid sequences in the active site of the malarial enzyme is similar to the enzymes from vertebrate tissues, *Drosophila, Trypanosoma brucei* . The plasmodial enzyme, however, is unique in several respects; it lacks a conventional AUG initiation codon and contains two tandem lysine residues close to the conserved tyrosine at the carboxy terminus. The TPI gene from *P. falciparum*, is located on chromosome 14 has a single intron and showed 42 to 45% homology with enzymes from other sources. The plasmodial enzyme has a molecular size of 28 kDa. Phosphoglycerate kinase having two isoenzymes has been purified from *P. falciparum*, and it is distinct from the host enzyme in its isoelectric point, K_m, V_{max} and immunologic epitopes. The gene for phosphoglycerate kinase is located on chromosome 9 and shows 60% homology to enzymes from other sources. *P. falciparum* infected red cells showed 15 times higher activity of enolase as compared to that of uninfected red cells. The gene for this enzyme is located on chromosome 10 of *P. falciparum*, and enzyme has a molecular size of 49 kDa and shows 60 to 70% homology to enolase from other eukaryotes.

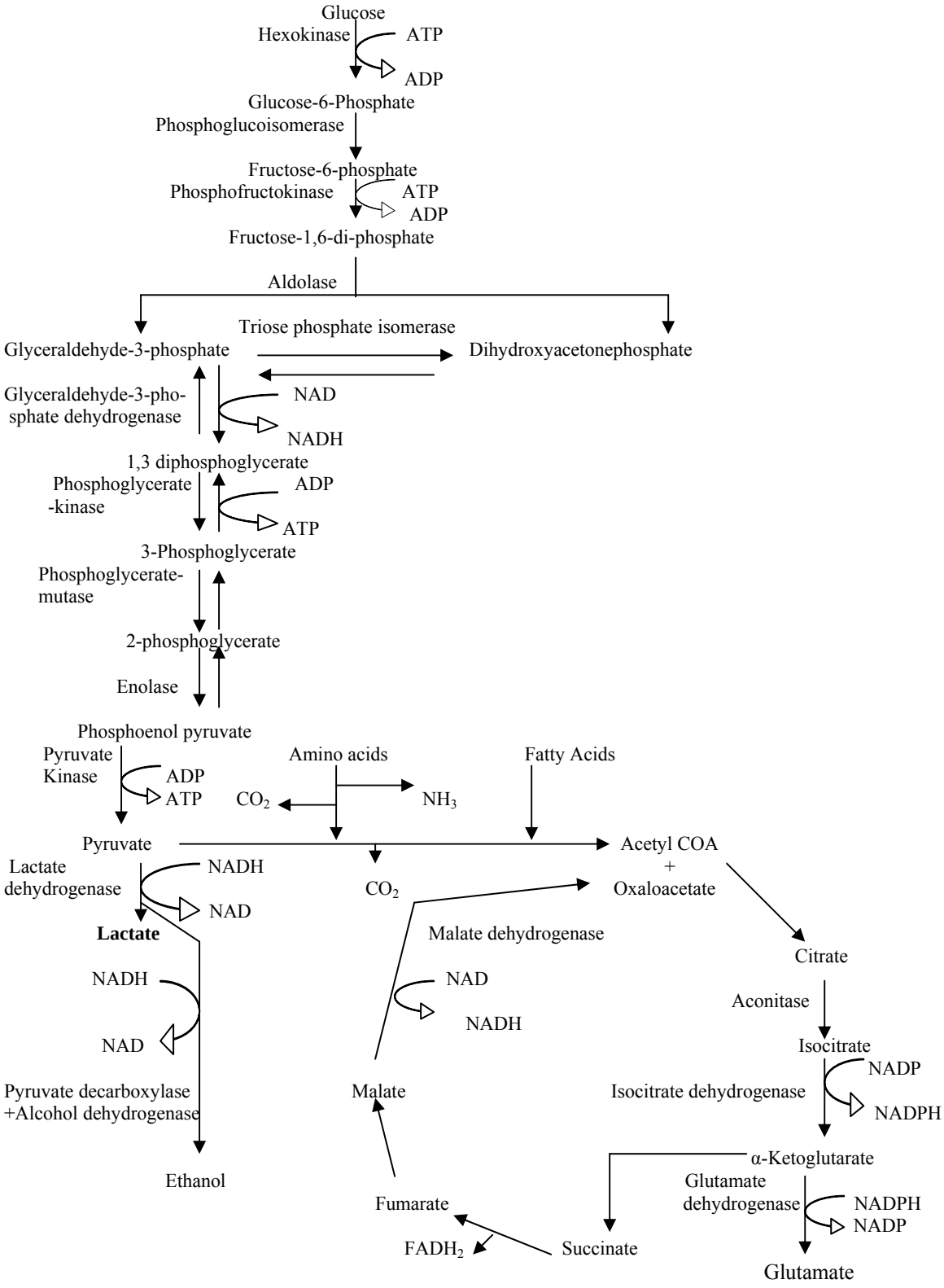

Fig. 8: The pathways of carbohydrate metabolism in malarial parasites

Malaria parasites, require the regeneration of NAD for the continued flux of glucose through LDH, the first plasmodial enzymes shown to be electrophoretically and kinetically distinct from that of the host. Unlike the LDH of other organisms, the plasmodial enzyme is not

inhibited by high levels of pyruvate. Presumably, this is an adaptation for activity in the anaerobic potentially pyruvate rich environment found within the erythrocyte. *In vitro* the plasmodial LDH has been shown to be 500 times more active with 3-acetylpyridine adenine dinucleotide, an analog of NAD, than with the natural cofactor NAD. The LDH of *P. falciparum* has the same molecular mass (35 kDa) as the enzyme from various prokaryotes and eukaryotes. The gene for *P. falciparum* LDH is localized on chromosome13 and shows considerable homology (50-57%) to LDH from other sources. The crystal structure of parasite LDH showed that the plasmodial enzyme departs from the mammalian LDH in several structural features, and these are presumably responsible for the differing biochemical properties.

Citric acid cycle and electron transport

It is has been shown that bird malaria parasites posses a functional citric acid cycle, as compared to mammalian malaria parasites. The major differences observed are: (i) the cristate structure of the mitochondrion of intraerythrocytic avian malaria parasites, (ii) stimulation of oxygen uptake in the presence of pyruvate, lactate, and citric acid cycle intermediates and (iii) identification of citric acid cycle enzymes, intermediates, and end product only in avian malaria parasites as compared to mammalian malaria parasites.

Mitochondria

The avian malaria parasites *P. fallax P. cathemerium*, and *P. lophurae* and the rodent malaria parasite *P. yoelii* have been reported to contain cristate mitochondria while the murine malaria parasite *P. berghei* and monkey malarial parasite *P.knowlesi* are reported to have acristate mitochondria. *P.berghii* and *P.gallinaceum* mitochondria have been shown to contain cytochrome oxidase and NADP/NADPH dehydrogenases activities. The oxidation of α-glycerophosphate, succinate, proline and dihydroorotase by malarial parasites indicated the presence of classical electron transport system. The respiratory chain does not have rotenone sensitive NADH-ubiquinone reductase, but NADH fumerate reductase activity was present. *P.gallinacem* has been reported to utilize all the intermediates of citric acid cycle except citrate and cis-aconitase and malate (a succinic dehydrogenase inhibitor) significantly reduced pyruvate utilization and resulted in accumulation of succinate indicating presence of citric acid cycle. *P. lophurae* and *P. gallinaceum* contain succinate dehydrogenase activity, however, the only enzyme associated with the citric acid cycle and consistently identified in rodent, bird, and human malarias is malic dehydrogenase (MDH). The MDH of *P. lophurae* and *P. falciparum* is, however, extra mitochondrial (i.e., soluble) and therefore, is not a citric acid cycle enzyme. It may play a role in the reoxidation of NADH for glycolysis, and in this way it could serve a role similar to that of LDH. Glutamic dehydrogenase (NADP specific) is not a citric acid cycle enzyme and has been found in all malarias. This enzyme serves as a specific marker for the presence of soluble parasite (but not membrane) proteins. A mitochondrial glutamic dehydrogenase was not measurable in isolated mitochondria of *P. yoelii.*

The identification of end products (succinate, carbon dioxide) and specific enzymes (isocitrate, succinate, and malate dehydrogenases) does not constitute conclusive evidence of the existence of a functional citric acid cycle in the avian species of *Plasmodium*. These enzymes and substrates identified in parasite may not be indicative of a citric acid cycle but may be involved in pyrimidine biosynthesis.

The favoring effect of oxygen on the *in vitro* growth of *P. lophurae, P. knowlesi* and *P. falciparum* suggests that the parasites require low level of oxygen. *P. yoelii* and *P. falciparum* mitochondria have been shown to contain cytochromes aa_3, b, C and C_1. The hydroxynaphthoquinone, atovaquone, has been shown to interfere with the function of the cytochrome bc_1 complex. The intraerythrocytic stages of parasite depend mainly on glycolysis for their energy production, but partial mitochondrial function are critical for the survival of the parasite, since inhibitors of electron transport and mitochondrial protein synthesis act as antimalarial agents.

The oxygen requirement of malaria parasites is markedly lower than that required to support mammalian cells (~11 to 15%), and the plasmodia do not show more rapid utilization of substrate anaerobic ally than aerobically. Hence in Plasmodium species oxygen utilization may not be coupled with energy generation; instead, the oxygen requirement of the parasite may be linked to the *de novo* biosynthesis of pyrimidines.

Pentose phosphate pathway

Glucose is also metabolized by the pentose phosphate pathway (PPP), or the hexose monophosphate shunt (HMPS) in malarial parasites. The PPP serves to convert glucose-6-phosphate (glucose-6-PO_4) to ribose-5-phosphate, which in some cells can then be used in nucleotide biosynthesis. NADPH, a major component of this pathway serves as a hydrogen donor in reductive biosynthesis and also plays a role in defense against oxidative stress. The pentose pathway can be divided into oxidative and non-oxidative pathway. In the oxidative cycle glucose 6-PO_4 is converted into ribulose-5-phosphate with the concomitant production of two molecules of NADPH per molecule of glucose 6-PO_4 consumed and in the non-oxidative cycle various pentose phosphate isomerases generate substrates for transketolase and transaldolase which transfer 2 and 3 carbon fragments, respectively, between a variety of substrates to form glycolytic intermediates such as fructose-6-phosphate and glyceraldehydes-3-phosphate. The nonoxidative cycle may also use fructose-6-phosphate and glyceraldehydes-3-phosphate from glycolysis to generate other phosphorylated sugars. In P. *falciparum* pentoses for nucleic acid synthesis are mainly derived from the condensation of fructose-6-phosphate and glyceraldehyde-3-phosphate mediated by transketolase and Transaldolase (Fig. 9).

G6PDH has been characterized electrophoretically and kinetically in *P. berghei* and *P. falciparum*. The *P. falciparum* enzyme has a molecular size of 450 kDa and the gene for this enzyme is located on chromosome14. The N terminus region contains a 286 amino acid extension particularly rich in hydrophilic and charged residues as compared to human G6PDH. The *P. berghei, P. knowlesi* and *P. gallinaceum* enzyme contain several isoenzymic forms. In *P. berghei, P. knowlesi* and *P. lophurae*, the end products of CO_2 fixation are alanine, malate, citrate, aspartate and glutamate, with α-ketoglutarate and oxaloacetate (OAA) formed as intermediates. In P. *berghei* the enzymes of CO_2 fixation, phosphoenolpyruvate carboxylaseand phosphoenolpyruvate carboxykinase, have been identified. The phosphoenolpyruvate carboxylase appears to be unique, since it has not been found in any other animal tissue.

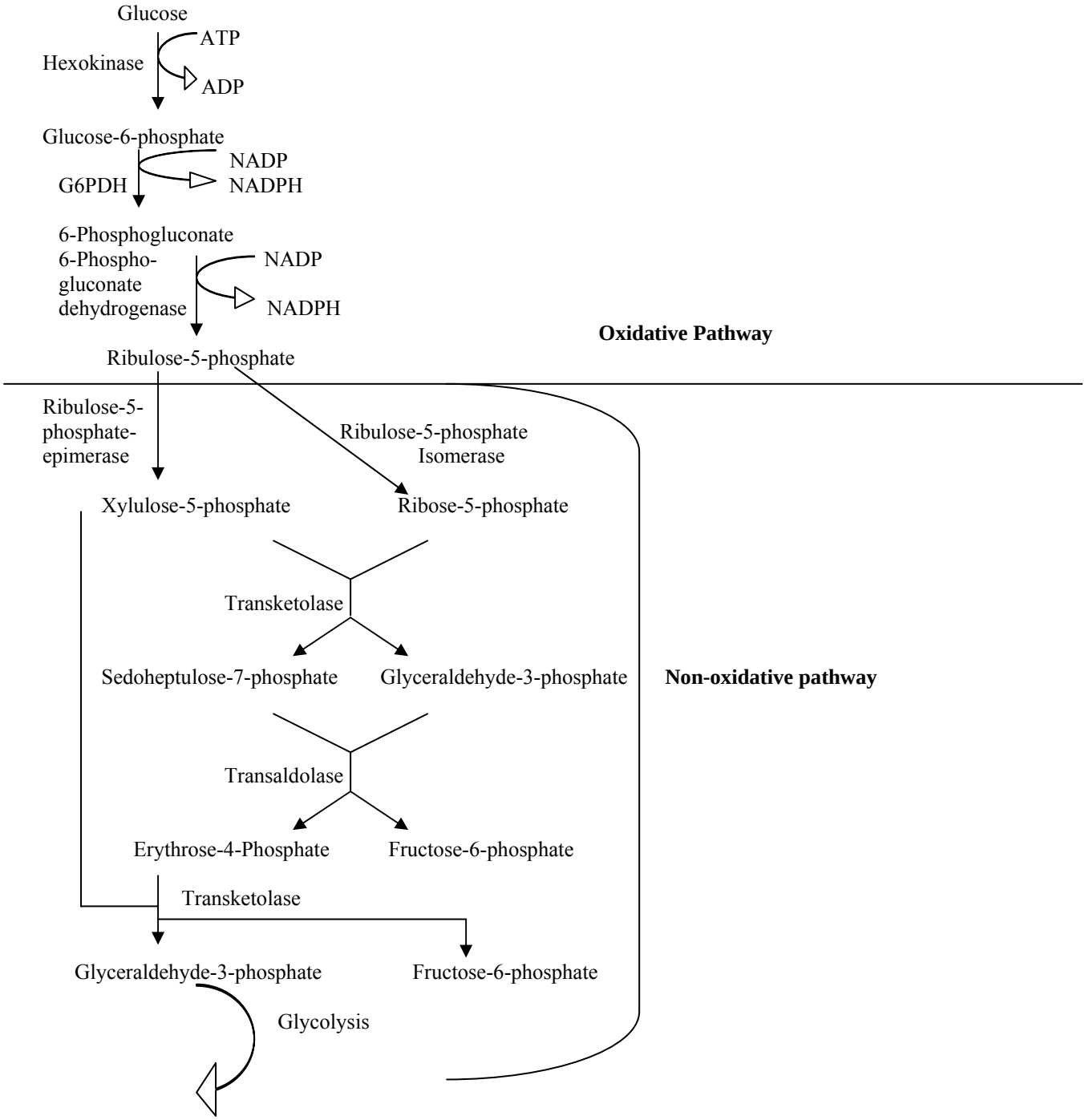

Fig. 9: Pentose phosphate pathway / Hexose Monophosphate Shunt (HMPS) in malarial parasites

Pyridine nucleotides

NAD and NADP play key roles in the EMP and pentose phosphate pathways. NADPH may provide reducing equivalents for the reduction of oxidized glutathione. The increased levels of pyridine nucleotides appear to be due to parasite synthesis from both nicotinamide and nicotinic acid, since nicotinic acid phosphoribosyltransferase and nicotinamide phosphoribosyltransferase and nicotinamide deamidase are all elevated in infected erythrocytes. Although the NAD antagonists, 6-aminonicotinic acid and 6-aminonicotinamide

and 2-acetylpyridine, all reduced the NAD level in infected cells, parasite growth was unaffected.

Although glucose metabolism might seem to be an unlikely target for chemotherapy since this metabolic route is fundamentally similar in all living cells, the fact remains that the plasmodial enzymes can be quite distinct from those of the host. Therefore, the detailed characterization of these enzymes could provide a rational basis for the design of parasite-specific drugs.

Hemoglobin processing and the metabolism of amino acids, heme, and iron

Malaria parasites residing in erythrocytes are protected from immunologic attack but the ready supply of nutrients circulating in the bloodstream is also inhibited. The parasites have evolved novel transport mechanisms to take up circulating metabolites; in addition, they have developed the means of acquiring nutrients from the erythrocyte cytosol. Malarial parasites take up and degrade large quantities of hemoglobin and transport it to an acidic food vacuole where the components of hemoglobin are separated and globin is hydrolyzed. Hemoglobin digestion provides most of the amino acids required for parasite protein synthesis and parasite iron. However, the processing of large numbers of hemoglobin molecules yields significant concentrations of free heme, which must be modified or sequestered to avoid parasite toxicity. The mechanisms by which malaria parasites hydrolyze globin to free amino acids, acquire iron and convert free heme to the malarial pigment hemozoin is not well understood although significant advances in these areas have been made in recent years. These processes are targets for a number of existing antimalarial compounds and understanding the pathways for hemoglobin processing would help in the design of new chemotheuraptic targets.

Sources of parasite amino acids

Malarial parasites, having limited ability to synthesize amino acids can metabolize glucose, pyruvate, or acetate and CO_2 into glutamate, aspartate, alanine, and leucine. Glutamate dehydrogenase activity responsible for the synthesis of glutamate has been identified in a number of parasite species, including *Plasmodium falciparum*. Additional amino acids can be synthesized when specific precursors are available In *Plasmodium knowlesi* and *P. falciparum* methionine is synthesized from homocysteine and serine (in part to recycle S-adenosyl methionine) and cysteine can be synthesized from glutathione or methionine in *P. knowlesi.*

Parasites grown *in vitro* in a minimal medium required supplementation with seven amino acids (cysteine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine) to achieve growth rates. The specific mechanisms of transport of amino acids in malarial parasites are uncertain. Most of the amino acids needed by the malarial parasites are provided by the hydrolysis of globin, certain amino acids (in particular isoleucine and methionine) are supplied by uptake mechanism and only small quantities of a few amino acids synthesized de novo by the parasite. In malarial parasites iron is required for the synthesis of iron-containing proteins viz, ribonucleotide reductase, superoxide dismutase and cytochromes and for de novo heme synthesis. Potential sources of parasite iron include plasma transferrin bound or free iron, erythrocyte iron, erythrocyte and hemoglobin. Available data provide no firm evidence that plasma or erythrocyte iron is used by malaria parasites. It seems plausible that the extensive processing of hemoglobin that takes place in erythrocytic parasites satisfies the iron needs of parasites. Nonetheless, the hypothesis that

parasite iron is supplied by heme has not been rigorously tested, and so the relative roles of plasma, the erythrocyte, hemoglobin and other sources in supplying iron for parasite metabolic needs are unknown.

Degradation of hemoglobin

P. falciparum food vacuole, with sufficiently acidic pH denatures hemoglobin upon delivery and releases heme, but this denaturation is very slow at the pH of the food vacuole, as demonstrated by spectrophotometric methods. Oxidation of heme iron from the ferrous to ferric state and parasite protease activity, are required to efficiently separate heme from globin in the food vacuole.

The *P. falciparum* food vacuole contains three proteases, the cysteine protease falcipain and the aspartic proteases plasmepsin I and plasmepsin II. Each of these enzymes probably participates in globin hydrolysis. All three enzymes cleave denatured hemoglobin *in vitro* and the hydrolysis of hemoglobin by parasite and food vacuole extracts is inhibited in an additive manner by cysteine and aspartic protease inhibitors. Plasmepsin I may act uniquely on hemoglobin degradation in early erythrocyte parasites, as it is synthesized, processed and present active in young ring-stage parasites.

Hemozoin formation

The hydrolysis of globin by erythrocytic parasites liberates large amounts of heme. Free heme is toxic to parasites as malaria parasites have not been shown to degrade heme enzymatically, they apparently incorporate heme molecules into particulate hemozoin as a detoxification mechanism. Hemozoin appears to consist of polymerized heme in the form of β-hematin, a noncovalent coordination complex with the ferric iron of each heme moiety bound to a carboxyl side chain of the adjacent heme molecule. A recent study found evidence that the iron in hemozoin is more likely to be bound to a hydroxyl than a carboxyl group, suggesting that hemozoin consists of monomeric hemin bound to protein or other molecules. It has been proposed that hemozoin synthesis is catalyzed by an enzyme, named heme polymerase. However, this enzyme has not been definitively isolated from the malarial parasites. Heme polymerizing activity of malarial extracts is not abolished by boiling and protease treatment suggesting that heme formation occurs spontaneously as heme is liberated in the food vacuole. Thus, it appears that hemozoin formation does not require enzymatic activity, but rather apply the presence of hemozoin polymers active nucleation centers for polymer elongation. Antimalarial drugs appear to act by preventing hemozoin formation, producing free radicals in the food vacuole or, in the case of experimental compounds, preventing globin hydrolysis. The 4-aminoquinolines (chloroquine) and aryl alcohols and artemesinin or its analogues appear to act by blocking the formation of hemozoin from heme molecules once they are liberated from hemoglobin and the antiparasitic effects are presumably engendered by the toxicity of free heme, possibly by disruption of membranes

Malarial lipids

Eukaryotic cells are composed of a complex set of membrane-enclosed intracellular organelles that are distinguished by the unique lipid compositions of their corresponding membranes. The major function of lipid is the structural integrity of the cell membrane and

provide a permeability barrier between exterior and interior compartments within and between cells.

During the erythrocytic developmental cycle of *Plasmodium* a considerable increase in total lipid content in the malarial-infected erythrocyte, irrespective of the *Plasmodium* species has been observed. In normal erythrocytes, cholesterol and PL constitute major lipids with a molar ratio of around 1. The main phospholipids are phosphocholine (PC) (35 to 40%), phosphatidylethanolamine (PE) (30 to 35%), sphingomyelin (SM) (15%), and phosphatidylserine (PS) (10%), whereas phosphatidylinositol (PI) and other PL, such as phosphatidic acid (PA), cardiolipids, and lyso-PL account for less than 3% of total PL. Fatty acid (FA) and natural lipids are barely detectable. The malaria infection causes a marked increase by five folds in PL and neutral lipids (fatty acid, diacyl glycerol, triacyl glycerol) as well as redistribution in lipid composition in erythrocytes irrespective of species. Malarial parasite is incapable of de novo synthesis of sterols in all the developmental stages and cholestrol in all the erythrocytes is in dynamic equilibrium with the cholestrol plasma lipoproteins. The parasite invasion or maturation could be responsible for the reported increases or loss of cholestrol.

The most abundant lipid in parasite membranes, PC, can be synthesized in *Plasmodium* via three main pathway (i) from choline via de novo synthesis, (ii) from ethanolamine via de novo synthesis of PE and methylation (this also includes synthesis from PE formed from ethanolamine originating from serine decarboxylation) and (iii) from incorporation of serine into PS, followed by decarboxylation into PE (and possibly also from ethanolamine obtained via serine decarboxylation), and then sequential methylation to form PC. The first two pathways involve a DAG-dependent reaction.

Phosphatidyl ethanolamine biosynthesis requires serine and ethanolamine in eukaryotes. In *Plasmodium*, serine is the most incorporated polar head, and serine incorporation is probably sufficient to supply most of the PE requirements of the parasite. *Plasmodium* is unique in possessing two pathways to provide PE from serine viz, PS decarboxylation and direct serine decarboxylation (Fig. 10).

Phosphatidyl serine decarboxylase activity, converting PS into PE, is increased in *P. knowlesi* and *P. falciparum* infected erythrocytes and PE is possibly then methylated to PC. PS is present at a very low level in *Plasmodium*, but it is a key intermediate in PE synthesis (through decarboxylation), which also serves as a precursor for PC through subsequent methylation. Phosphatidyl serine is abundantly biosynthesized by infected erythrocytes and which is converted to phosphatidyl ethanolamine and phosphatidyl choline. Phosphatidyl inositol has an important role in anchoring proteins to the membrane and as a precursor to signaling molecules such as polyphosphoinositides. PI synthase (CDP-1, 2-diacyl-*sn*-glycerol: 3-phosphatidyltransferase, EC 2.7.8.11) catalyzes the reaction of CDP-DAG and *myo*-inositol to form PI and CMP. In *Plasmodium* PI shows the highest relative increase after infection. The different metabolic pathways present in *Plasmodium* make it difficult to determine the related amounts of PC and PE synthesized by each pathway and also raise the question as to whether each pathway corresponds to distinct compartments and further *Plasmodium* possesses the regulatory mechanism to maintain an equilibrium. In *Plasmodium*, no direct reciprocal interactions were noted between de novo and methylation pathways for PC biosynthesis. The only interactions that were observed occurred at very high and physiological precursor concentrations (i.e., mM range) and could be related to direct alteration of transport or enzymatic steps, but not to modifications of gene expression.

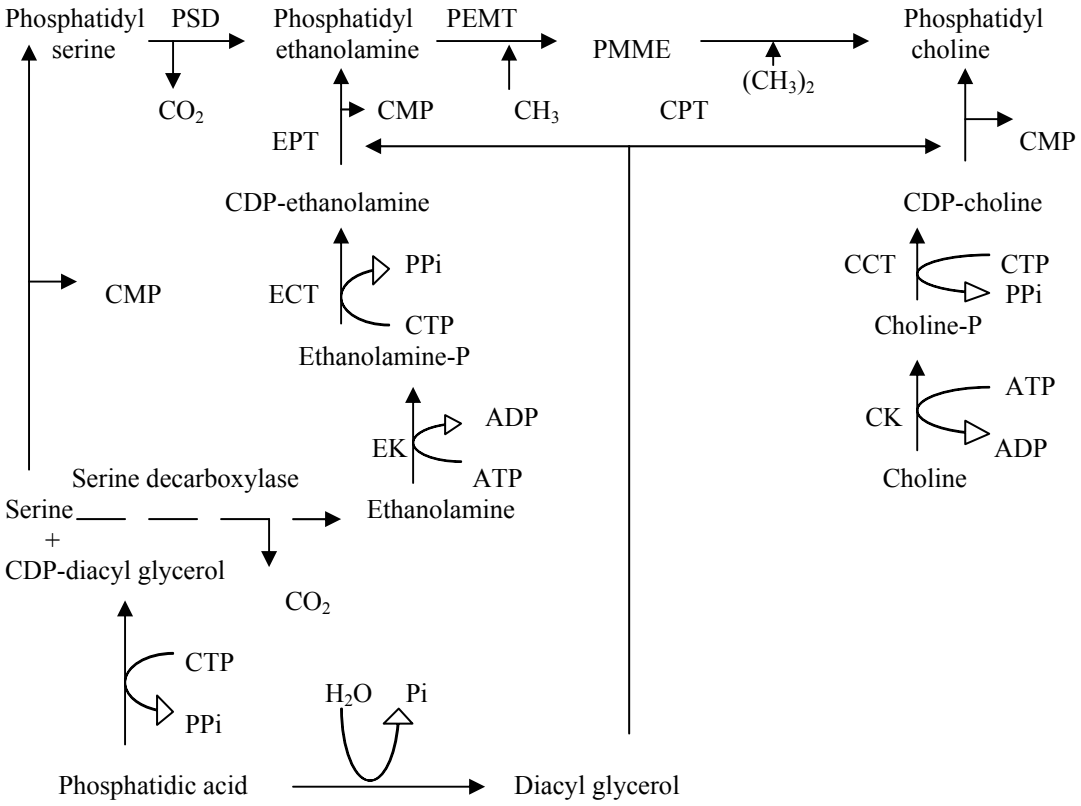

Fig.10: Biosynthesis of phospholipids in *Plasmodium*. The serine decarboxylation reaction is parasite specific

CPT = Choline phosphotransferase, CCT = Choline phosphatecytidylyl transferase, CK = Choline kinase, PSD = Phosphotidyl serine decarboxylase, PEMT = Phosphotidyl ethanolamine methyl transferase, EK = Ethanolamine kinase, ECT = Ethanolamine phosphatecytidylyl transferase, EPT = Ethanolamine phosphotransferase

Phosphospholipase activity causes degradation of imported intact lipid molecules and fine adjustment of the composition of the lipid fatty acyl group through deacylation/reacylation reactions, thus modulating membrane function. Presence of various phospholipase activities in *Plasmodium* spp.has been reported and calcium dependent phospholipase C hydrolyzes polyphosphoinosirides in *P. falciparum*. This activity is activated only at the late intraerythrocytic stages and plays a role at the exflagellation step in gamete formation. Phospholipase A2 and A1 have also been observed in malarial parasites. Presently there is little direct evidence of modifications of the membrane FA composition by deacylation and reacylation of existing membrane phosphoglycerides by malarial parasites. However the parasitic PC is closely correlated with the substrate specificity of choline phosphotransferase, which catalyzes the final step in the Kennedy pathway and could be the key enzyme in regulation of phospholipid in *Plasmodium* spp.

PL metabolism is highly compartmentalized in the infected erythrocyte, as noted in biochemical studies and with pharmacological tools, and distinct pools of PE probably exist in infected erythrocytes regardless of whether they are synthesized from serine, ethanolamine, or serine-derived ethanolamine. Alternatively, the parasite is probably capable of intense lipid trafficking between its different membranes, as highlighted by complete

remodeling in the PL molecular species of the host erythrocyte plasma membrane, which probably does not involve any significant phospholipase activities.

Lipids have also been involved in various other phenomena related to malarial infections. The digestive vacuole of *P. falciparum* is the site of hemoglobin degradation, heme polymerization into crystalline hemozoin, and antimalarial drug accumulation. Heme polymerization is important since failure of heme detoxification is a likely mechanism of action of quinoline antimalarial drugs. This activity was attributed to many potential mechanisms involving a specific enzyme, catalysis by histidine-rich protein and also by a plasmodium PL containing fraction. It was also recently shown that hemozoin, the by-product of hemoglobin digestion, may enhance lipid peroxidation and could catalyze oxidation of unesterified FA hydroxyeicosatetraenoic acid (HETE), whose path physiological impact on organism remains to be determined.

Pyrimidine biosynthesis pathway

Pyrimidine is *de novo* synthesised in all the Plasmodia. Dihydroorotate dehydrogenase (DHODH), localized in the mitochondria catalyzes the rate-limiting step of uridine monophosphate (UMP) during the pyrimidine biosynthesis in malarial parasite. The enzyme catalyzes the oxidation of dihydro-orotate to orotate using the flavin cofactor (FMN) in the very first step while in the second step it catalyzes the reoxidation of FMNH via respiratory chain type quinines (coenzyme Q). All the cytoplasmic forms of dehydrogenases oxidize $FMNH_2$ through NAD^+ or fumarate, while membrane or mitochondrial forms require respiratory quinines. The parasite's DHODH gene belonging to the mitochondrial type membrane enzyme has been sequenced. Since the erythrocytic stages of the parasites lack a number of enzymes of the tricarboxylic acid (TCA) cycle, the electrons from reduced quinines generated from DHOD reaction, are funneled to the electron transport chain of the parasite respiratory pathway. Therefore, the inhibitors of the enzymes (different from human host) involved in dihydrofolate synthesis would prove to be good antimalarial agents (Fig. 11).

Apicoplast Metabolism

The apicoplast in malaria parasites is a plastic like organelle having a 35-kilo base circular genome including elements of a prokaryotic translation and transcription system. Apicoplast metabolism has significant implications in the parasite life cycle and it is the target of many clinically used antimalarial and antibiotics. Malaria parasites are susceptible to prokaryotic transcriptional inhibitors such as rifampicin and DNA gyrase inhibitors such as quinolones. Two very important targets Type-II fatty acid biosynthesis (FAS-II) and non mevalonate pathway leading to synthesis of isopentenyl diphosphate subunits are located in the apicoplast and these are very good targets to develop new drugs. Thiolactomycin inhibits this enzyme and is known to inhibit *P. falciparum* growth also. Another inhibitor Triclosan is active against parasite growth in cell culture and in rodents model through inhibition of enoyl-acyl carrier protein reductase. Fosmidomycin having antimalarial activity is known to inhibit 1-deoxy-D-oxylo-5-phosphate synthase (DOX-5) and offers a lead molecule for antimalarial drug development.

Chemotherapy

The chemotherapy of malaria basically involves killing of the asexual parasites and providing supportive therapy to the host to boost its immune system. The sexual forms in the blood circulation are non-pathogenic but are important in drug resistance development. The efficacy and specificity of anti-infective drugs depend on their ability to interfere with parasite metabolism that differs significantly from the human host. During its life cycle in human erythrocytes the plasmodium parasite requires several metabolic adaptations and innovations, which render it susceptible to chemotherapeutic attack. The parasite degrades hemoglobin in its acidic food vacuole producing free heme, which react with molecular oxygen and generate reactive oxygen species as toxic byproducts. A major pathway of detoxification of heme moieties is polymerization of heme to heamazoin. Majority of antimalarial drugs act by disturbing the polymerization (and / or the detoxification by any other way) of heme; thus killing the parasite with its own metabolic waste. A series of reports exist for different drugs and their efficacy against malaria by this mechanism.

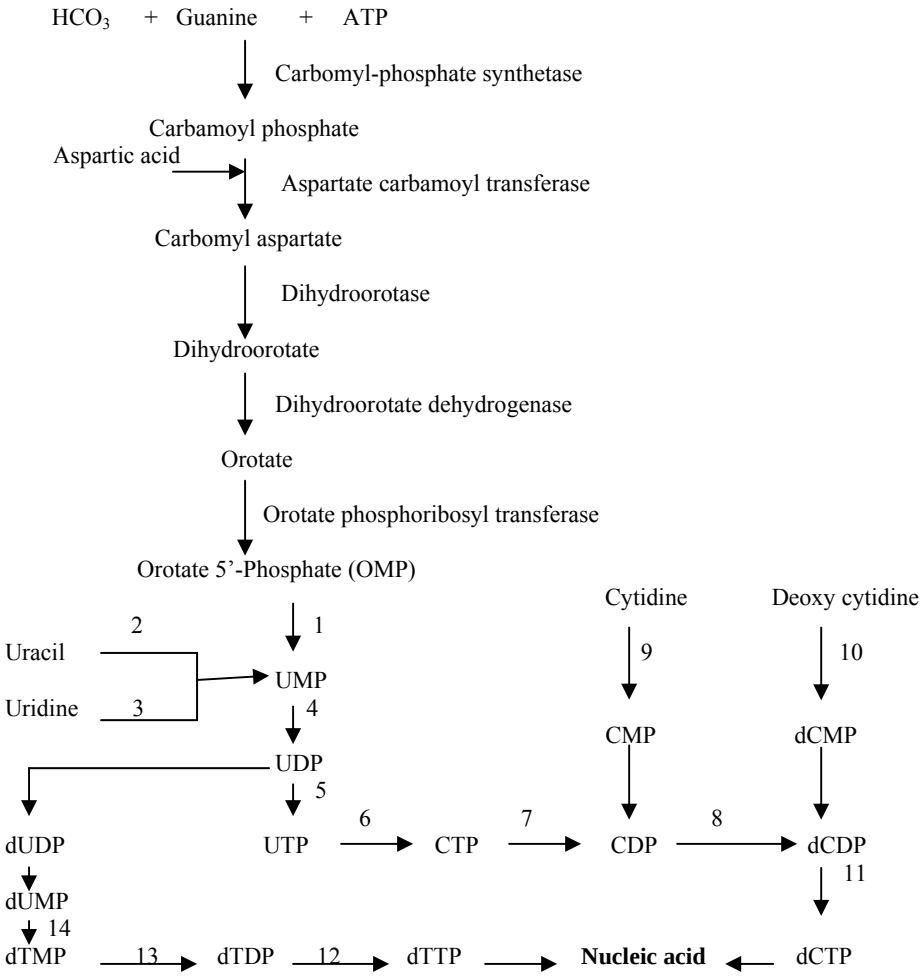

Fig.11: De novo biosynthetic pathway for pyrimidines in parasite

(1) orotidine 5'- phosphate decarboxylase, (2) uracil phosphoribosyltransferase, (3) uridine kinase, (4) nucleotide monophosphate kinase, (5) nucleotide diphosphokinase, (6) CTP synthetase, (7) non specific phosphatase, (8) ribonucleotide reductase, (9) cytidine kinase, (10) deoxycytidine kinase, (11) nucleotide diphosphokinase, (12) nucleotide diphosphokinase, (13) deoxy TMP kinase, (14) thymidylate synthetase.

Chloroquine is the effective and cheap drug both for prophylactic and chemotherapeutic viewpoint. Most of the *P. falciparum* strains are resistant to it. Mefloquine is effective against many resistant strains of *Plasmodium falciparum* but undesirable side effects have been observed for it.

Halofantrin is an effective antimalarial due to short half life and the drug is not used as prophylactic agent due to observed side effects.

Artemisnin and its derivatives appear to be best drugs for treatment of severe malaria and no drug resistance has been reported for it. The peroxide bond is essential for the activity. This peroxide bond undergoes reductive activation by heme librated through hemoglobin digestion resulting in carbon free radicals, which exert the parasiticidal effects. Artemisinin derivatives being gametocidal reduce the transmission of the disease also. Artemisinin is derived from Chinese herb *Artemisia annua*. Artesunate a water-soluble humisuccinate derivative and oil soluble ethers artemether and arteether are most widely used drugs for drug resistant parasites. Many synthetic peroxides, acyclic peroxide 1,2-dioxane ketals, amine peroxides, acyclic peroxides have been found to contain antimalarial activities (Table 2).

Table 2: Antimalarials and their possible mode of action

S. No.	Antimalarials	Possible mode of action
1.	Choroquine	Heme polymerase activity
2.	Amodiaquine	Heme polymerase activity
3.	Quinine	Heme polymerase activity
4.	Mefloquine	Heme polymerase activity DNA intercalating agent
5.	Halofantrine	Heme polymerase activity, DNA intercalating agent and arithmogenic
6.	Sulfonamides	Incorporation of p-aminobenzoic acid into dihydropteroic acid
7.	Primaquine (8-aminoquinoline)	Gametocidal
8.	Quinidine (Cinchona alkaloids)	Schizonticidal and Gametocidal
9.	Artemisinin / Artemether	Forms free-radicals mediated adducts with hemozoin and may exhaust oxidative defense of parasite
10.	Desferoxamine	Iron chelator, reduces free Fe required for ribonucleotide reductase
11.	Pyrimethanine	Dihydrofolate reductase inhibitor
12.	Hydroxy napthoquinone	Inhibit dihydroorotate dehydrogenase and decreases synthesis of pyrimidines and inhi bits electron transport at bc complex of parasite mitochondria.
13.	Atovaquinone	Schizontocidal, inhibit cytochrome-c reductase
14.	Tetracyclines	Inhibit protein synthesis by binding 30S ribosome and blocking access of aminoacyl t-RNA to acceptor site on m-RNA ribosome complex

Drug resistance in malarial parasite has become one of the most important problems in disease control in recent years because of single drug therapy. Drug resistance has been reported *in vivo* for almost all the antimalarial drugs except artemisinin and its derivatives. Since the efficacy and easy availability of antimalarial drugs is declining, cost effective strategies are needed to extend the useful life spans of antimalarial drugs. The combination of two or more drugs has been observed to slow down the development of resistance and offers a synergestic effect too. Malarone, a combination of proguanil and atovaquone, have a synergistic effect and this combination is a very effective for malarial infection. The other drugs in combination used for antimalarial treatment are, sulphadoxine combined with mefloquine, and sulphadoxine combined with chloroquine. The other drugs combination includes chlorproguanil/dapsone, fansidar and co-arteether (luefantrine+artemether). The use of artemisinin with other antimalarials is very effective due to rapid parasite clearance by artemisinin, fewer parasites are exposed to other drug and minimize the probability that a resistant mutant will survive therapy and also reduce overall malaria transmission rates.

The development of resistance and side effects of currently used antimalarials has led to the development of new molecules viz., clindamycin, thiolactomycin, hydroxamate, diaminopyrimidines, pentamidines, S-carbolines, chaleones etc. Due to the delayed developments of new antimalarial drugs there is need for a drug that allows short course treatment and not rapidly eliminated to delay emergence of resistance. Hence the combination therapy may provide solution to the problem of development of drug resistance.

Leishmania

The disease is always a zoonosis (that is normally resident in animals). Gerbils are a major reservoir of *L. tropica* in Central Asia. Dogs and cats and squirrels are also infected in many regions. Rodents are the reservoir of *Leishmania species* in Latin America.

Recently, there has been an increase in visceral leishmaniasis due to the AIDS pandemic *Leishmania*/HIV co-infection is considered to be a real "emerging disease", especially in southern Europe, here 25-70% of adult VL cases are related to HIV infection and 1.5-9.5% of AIDS cases suffer from newly acquired or reactivated VL. Epidemics of visceral leishmaniasis still occur in southern and eastern Sudan and at the junction of Eritrea, Ethiopia and Sudan. Recently there has been a resurgence of visceral leishmaniasis in Brazil.

L. donovani promastigotes incorporate [^{14}C]acetate into lauric, myristic, palmitic, stearic oleic, linoleic, linolenic and C_{20} and C_{22} fatty acids, indicating that they contain not only the apparatus for the *de novo* synthesis of fatty acids but also the desaturases and elongating systems required for the conversion of stearic acid into unsaturated and very long chain fatty acids.

The *Leishmania* spp. are unicellular protozoa that exist in two distinct morphologic forms. In the alimentary tract of their insect vectors, the parasite exists extracellularly as the flagellated, motile promastigote. In the phagolysosomal system of host mononuclear phagocytes, the parasite occurs intracellularly in the nonmotile amastigote form. The environment of the phagosome becomes acidified after parasites or bacteria are ingested by polymorphonuclear leukocytes. It seems the amastigote has adapted to survive and multiply in an acid environment.

Surface enzymes

Phosphomonoesterases (i) phosphatases: membrane bound acid phosphatases - purification and properties. Promastigotes of one particular clone of *L. donovani* contain at least three distinct surface membrane-bound acid phosphatases as observed using histochemical-electron microscopic and subcellular fractionation techniques. The majority of the cell-associated acid phosphatase activity in *L. donovani* promastigotes is located on the external surface of the parasite. The membrane-bound acid phosphatase activity can be solubilized and resolved into one major and two minor phosphatase isoenzymes.

The physiologic role of these acid phosphatases is now understood. The acid phosphatases by hydrolyzing phosphomonoesters may provide the parasite with a source of inorganic phosphate for growth. It has also been suggested because of their surface orientation that the leishmanial phosphatases may be involved in pathophysiologically significant surface interactions between the host cell and the parasite. In fact there is some recent evidence in support of this hypothesis.

Few studies have been carried out on the lysosomal hydrolases of *Leishmania* amastigotes. L. mexicana subsp. *Amazonensis* amastigotes contain large amounts of acid phosphatase activity, some of which is contributed by an ectoenzyme associated with the plasma membrane or the flagellar pocket. It would be useful to isolate and characterize the acid phosphatase of the amastigote and to compare it with promastigote phosphatases.

In a comprehensive comparative study of various phosphomonoesterases in various *Leishmania* species, high levels of acid phosphatase activity in extracts of *L. donovani, L. mexicana* subsp. *Mexicana* and *L. mexicana* sub sp. *Amazonesis* was observed. It was also demonstrated that *L. mexicana* subsp. mexicana amastigotes and promastigotes contain acid phosphatase on their external surfaces. However, inhibitor and substrate specificity studies revealed qualitative differences in the particulate acid phosphatase activities of promastigotes and amastigotes. Furthermore, by using the electron microscope in a semiquantitative histochemical technique, it was observed that surface staining of acid phosphatase was less intense for amastigotes than for promastigotes.

Nucleotidases

Attached to the outer surface of the plasma membrane of *L. donovani* along with the acid phosphatase are two distinct nucleotidases, 5'-nucleotidase and 3'-nucleotidase. Nucleotidases are phosphomonoesterases which remove the phosphate group from phosphorylated sugars (ribose of deoxyribose) linked by an N-glycosidic bond to a purine or a pyrimidine. In the case of 3'-nucleotides, phosphate is attached to the hydroxyl group on C-3 of the pentose; in the 5'-nucleotide series, the phosphate moiety is attached to the hydroxyl group on C-5 of the sugar. The 5'- and 3'-nucleotidases are phosphomonoesterases that catalyze the reactions indicated below, both of which generate orthophosphate (P_i) and nucleoside (base pentose).

$$\text{Base-pentose-3'phosphate} + H_2O \xrightarrow{\text{3'nucleotidase}} \text{base pentose} + P_i$$

$$\text{Base-pentose-5'phosphate} + H_20 \xrightarrow{\text{5'nucleotidase}} \text{base pentose} + P_i$$

On a specific activity basis, plasma membranes from *L. donovani* promastigotes contain 40 times more 3'-nucleotidase than 5'-nucleotidase. However, crude extracts of *L. mexicana* subsp. *Mexicana* promastigotes or amastigotes contain only two- to threefold more 3'-nucleotidase than 5'-nucleotidase. In terms of function, it is thought that these nucleotidases play a nutritional role. *Leishmania* spp., as well as other trypanosomatid protozoa (e.g. Trypanosoma and Crithidia spp.), can not synthesize purines de *novo* and therefore dependent upon an exogenous supply of preformed purines which are utilized by means of a purine "salvage" pathway. Nucleotides are not taken up by these organisms, but nucleosides are, therefore, the leishmanial 5-nucleotidase, which can hydrolyze both ribonucleotides and deoxyribonucleotides and the 3'-nucleotidase, which is specific for ribonucleotides appear to provide the parasite with the purine nucleosides required for growth. The specific activities of 3'-nucleotidease and 5'-nucleotidase are nearly the same in promastigotes and amastigotes.

Proteases

There are few reports of studies of the proteases of *Leishmania* species. Extracts of *L. mexicana* subsp. *Mexicana* promastigotes and amastigotes possess proteases that are capable of hydrolyzing protein substrates that include azocasein hide powder azure and α-casein. The two leishmanial stages can be distinguished on two grounds first, the specific activities of the amastigotes proteases are much higher than those of promastigotes second, the pH optimum on azocasein is at pH 5.5 for the amastigotes and near neutrality for the promastigotes. Synthetic protease substrates such as N-benzoyl-Arg-4-nitroanilide were also cleaved by crude extracts of both forms of the parasite for N-benzoyl-Arg-4-nitroanilide, the pH optimum for each was 8.0 and the specific activities of the two were similar.

Protein kinases

Leishmania spp. contains relatively high levels of protein kinase activity. Protein kinases are a class of enzymes which catalyze the transfer of the γ-phosphate group from nucleoside triphosphates (usually ATP) to the hydroxyl group of serine, threonine or tyrosine residues in proteins. It can be shown by using radioactive phosphorous and metabolically labeled cultures of promastigotes that the membrane proteins of *L. donovani* undergo rapid phosphorylation dephosphorylation *in vivo*. Furthermore, motile and live *L. donovani* promastigotes, when incubated at pH 7.0 in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer catalyze the transfer of ^{32}P from [γ ^{32}P ATP] to exogenous histone acceptors present in the extracellular medium. Comparing these results with those obtained with broken cells, we estimated that more than half of the total protein kinase of *L. donovani* promastigotes is localized to the outer surface of the parasite.

The crude lipid fraction obtained by exhaustively extracting promastigotes with chloroform methanol (2:1, vol: vol) accounts for 2 to 15% of the dry weight of a number of species of *Leishmania*, including *L. donovani, L. branziliensis, L. mexicana, L. tropica, L. enricii, L. hertigi, L. adleri* and *L. tarentolace* neutral lipids and polar lipids accounted for 14 to 55% and 45 to 86% of the total lipid respectively. For most strains, the total lipid content and the neutral/polar lipid ratio was the same for cultures grown in minimum essential medium plus 10% fetal calf serum versus defined medium RE III plus 0.06% defatted peptone.

The neutral lipid fraction of *L. tarentolae* (grown in brain heart infusion), when analyzed by silica gel thin layer chromatography, exhibited the following composition (by weight): sterols

(ergosterol), 43.3%; triacylglycerols (triglycerides), 43.0%; sterol esters, 9.1%; alkoxydiacyl glycerols, 1.7%; diacylglycerols, 1.5%; monoacylglycerols, 1.0% and a trace of free fatty acids. Neutral lipids accounted for about 25% of the total lipids of *L. donovani* promastigotes, 60% of the neutral lipid fraction was accounted for by sterols and 30% was accounted for by diglycerides (diacylglycerols). Alkoxy lipids were first detected in *Leishmania* spp. The temperature at which the promastigotes are cultured influences their lipid content and fatty acid composition. *L. donovani* promastigotes incorporate [^{14}C] acetate into lauric myristic palmitic, stearic oleic, linoleic, linolenic and C_{20} and C_{22} fatty acids, indicating that they contain not only the apparatus for the *de novo* synthesis of fatty acids but also the desaturases and elongating systems required for the conversion of stearic acid into unsaturated and very long-chain fatty acids.

Glucose metabolism

Carbohydrates are metabolized primarily by aerobic fermentation in the promastigotes, they oxidize sugars incompletely to a mixture of organic acids e.g. succinate, acetate, pyruvate etc. They do not store polysaccharides. Nevertheless the fact remains that glucose is taken up and utilized by the *Leishmania* spp. However, different species oxidize glucose at different rates. *L. donovani* for example consumes glucose more rapidly than *L. braziliensis*. Summarizing decades of investigation involving tracer studies and enzymologic studies with extracts of various *Leishmania* spp. glucose is most likely converted to triose phosphates by a combination of the pathway of glycolysis and pentose phosphate pathway. Triose phosphates are metabolized to pyruvate via the intermediary of phosphoenolpyruvate. Pyruvate is converted to oxaloacetate most likely by means of the pyruvate carboxylase reaction and oxaloacetate is converted sequentially to malate, fumarate and succinate. It is not clear whether mitochondrial or cytoplasmic enzymes are involved in the conversion of oxaloacetate to succinate.

The pentose phosphate pathway or hexose monophosphate shunt is present in *Leishmania* promastigotes. An appreciable fraction of the glucose is used via the pentose phosphate pathway. With increasing culture age of *L. donovani*, there is a decrease in the ratio of $^{14}CO_2$ produced from [1-^{14}C] glucose to that from [6-^{14}C] glucose. The glycolytic enzymes in the trypanosomatids, including promastigotes and amastigotes of *Leishmania* species are contained in a unique organelle called the glycosome. Enzymes of the glycolytic pathway that catalyze the reactions between and including hexokinase and glyceraldehyde-3-phosphate dehydrogenase are confined largely to the glycosome. In contrast, most of the phosphoglycerate kinase and pyruvate kinase activities were found to be cytosolic in both stages of the parasite. The first enzyme in the pentose phosphate pathway, glucose-6-P-dehydrogenase is largely cytosolic.

Leishmania promastigotes are capable of performing both glycolysis and gluconeogenesis pyruvate kinase of *L. major* promastigotes is an important regulatory site in glycolysis, specifically the enzyme is activated by its substrate, phosphenolpyruvate in a positively cooperative fashion and heterotropically by fructose 1-6, biphosphate. During the transformation from the promastigote to the amastigote form, the levels of PEP carboxykinase and malate dehydrogenase activities increase greatly while that of pyruvate kinase decreases markedly in *L. mexicana*.

All of the tricarboxylic acid cycle enzymes can be found in all of the *Leishmania* species that have been examined in this regard. However, although a complete tricarboxylic acid cycle

apparatus exists the levels of certain key enzymes of the pathway (e.g., citrate synthase and α-ketoglutarate dehydrogenase) are so low that it appears that the role of the tricarboxylic acid cycle is to trap reducing equivalents through reduction of oxaloacetate to succinate, excretion of the later, and conversion of isocitrate to glutamic acid, which would detoxify or organify ammonia and make glutamate available for further transamination reactions. Promastigotes do not oxidize free fatty acids very rapidly. In contrast, however, amastigotes have a well-developed capacity for oxidizing long-chain fatty acids to CO_2 and water.

High capacity of amastigotes for fatty acid oxidation may reflect an adaptation to the kind of substrate they encounter in intracellular habitat.

The inhibition of fatty acid oxidation in *L. mexicana* by compounds that are known to inhibit cytochromes or β-oxidation enzymes suggests that classic mitochondrial β-oxidation is the pathway responsible for the degradation of long-chain fatty acids in mitochondria. Nevertheless studies in recent years have documented that a variety of eukaryotic cells oxidize very long chain fatty acids by a peroxisomal pathway distinct from the mitochondrial one.

Amino acids are significant source of energy, especially in promastigotes. Rates of amino acid utilization in general are higher in promastigotes than in amastigotes. Following amino acids were rapidly extracted from the growth medium and degraded viz., asparagine, glutamine, leucine, lysine, methionine and threonine. It appears that L-arginine is assimilated by *L. donovani* promastigotes by means of the α-oxoglutarate pathway that involves the intermediary of γ-guanidinobutyramide and the enzyme L-arginine decarboxyoxidase.
The Leishmania spp. like other hemoflagellates, appear to be incapable of synthesizing the purine nucleus *de novo* and therefore require exogenous purines for growth. It seems that like mammalian cells, the *Leishmania* spp. possess two distinct phosphoribosyltransferases, one specific for adenine and another specific for guanine.

The *L. donovani* adenosine kinase has physical and kinetic properties, which distinguish it from adenosine kinases of other eukaryotic cells. Since nucleotides do not enter into cells readily it is thought that adenine nucleoside permeates the plasma membrane first and then is converted to its nucleotide (AMP).

The purine salvage pathways in the *Leishmania* spp. are different from those used by mammalaian cells. Promastigotes and amastigotes of *L. mexicana* subsp. *mexicana* utilize the salvage pathway. The key enzymes in this process are adenine deaminase and guanine deaminase, which convert adenine and guanine to hypoxanthine and xanthine respectively. The phosphoribosyltransferases of the parasite then convert hypoxanthine and xanthine to inosine monophosphate (IMP) and xanthine monophosphate respectively. These nucleotides can be converted to AMP and GMP by the following enzymes, adenylosuccinate synthetase, adenylosuccinate lyase, IMP dehydrogenase and GMP synthetase

The main difference in purine metabolism between the *Leishmania* spp. and humans is that the hypoxanthine-guanine phosphoribosyltransferase of *Leishmania* spp. but not of humans, utilize allopurinol as a substrate and convert the drug to the corresponding ribonucleotide. The amastigote is the main target for this type of leishmanial drug. The most extensively studied of these drugs are the pyrazolopyrimidines. In contrast, when allopurinol is metabolized to allopurinol riboside, it gives the appearance of being relatively stable metabolically because its formation is favoured in the presence of purine nucleoside

phosphorylase. One of these enzymes namely aspartate carbamoyltransferase (aspartate transcarbamylase), is very sensitive to inhibition by N- (phosphonoacetyl)-1-aspartic acid, a transition state analog. The *Leishmania* spp. appear to possess a unique thymidylate synthase, an enzyme which catalyzes the reduction methylation of deoxyuridine monophosphate.

Table 3: Possible of action of antileishmanial compounds

S. No.	Antileishmanial compounds	Possible site of action
1.	Melar soprol (arsenic compound)	Pyruvate kinase
2.	Pentamidine	Inhibit kinetoplast topoisomerase II (Inhibit nucleic acid synthesis), Inhibit polyamine synthesis (by inhibiting S-adenosyl methioni ne decarboxylase), Inhibit oxidative phos phorylation
3.	Eflornithine	Inhibit synthesis of polyamine by inhibiting ornithine decarboxylase
4.	Metronidazole	Its NO_2 group acts as e^- acceptor for microbi al e^- transport protein, so interfere with ener gy metabolism and disrupt helical structure of DNA causing strand breakage
5.	Sodium stibogluconate	Topoisomerase I, Energy metabolism, (glycolysis and β- oxidation), Alter thiol-redox potential, DNA fragmentation, Apoptosis like death * Exact cellular target is unknown
6.	Meglumine antimoniate (Pentostam) (SbV)	Energy metabolism, (glycolysis and β-oxidation), Alter thiolredox potential, DNA fragmentation, Apoptosis like death * Exact target is unknown
7.	Amphotericin B	Ergosterol biosynthesis pathway
8.	Miltefosin	Changes in alkyl-lipid metabolism and Phospholipid biosynthesis * Exact target is unknown
9.	Paromomycin	Mitochondrial ribosomes, alteration in membrane fluidity, lipid metabolism and key mitochondrial activity
10.	Sitamaquine	Induction of respiratory dysfunction and mitochondrial membrane depolarization
11.	Allopurinol	Inhibits enzymes of purine salvage pathway, conversion to ribonucleoside triphosphate analogues and incorporation into RNA thereby inhibiting macromolecular biosynthesis.

The polysaccharide character of the material is indicated by its ability to react with certain lectins and the fact that it shows positive reaction with the periodic acid. The trichloroacetic acid released polysaccharide factor reacts with peanut lectin, indicating the presence of nonreducing galactose residues. The highly acidic anionic product obtained was resistant to digestion by a variety of proteases, hyaluronidase chondroitinase ABC and endoglycosidase and when ran on sodium dodecyl sulfate polyacrylamide to find that its molecular weight was in the 15,000 to 30,000 range. The lipophosphoglycan (LPG) of *L. donovani* promastigotes is localized to the parasite's cell surface and it could be released from the cell surface into the culture medium. The observation that the presence of albumin in the medium enhanced the rate of release of LPG from cells prompted to postulate that the lipid moiety of LPG interacts with some hydrophobic binding site on albumin. A number of functions have been suggested for excreted factor. First, it has been shown that excreted factor inhibits the activity of the lysosomal-β-galactosidase or a competitive inhibitor of the enzyme. The *Leishmania* Spp. may play a role in the survival of the parasite during encounters with hydrolytic substances in the alimentary tract of its sand fly vector. The purified LPG from *L. donovani* is capable of inhibiting the activity of protein kinase C derived from rat brain; the glycoconjugate was a competitive inhibitor with respect to the lipid activator, diolein and a noncompetitive type inhibitor with respect to the other lipid activator of protein kinase C namely phosphatidylserine.

Suggested Readings

1. Von Brand, T. (1979). Biochemistry and Physiology of Endoparasites. Elsevier, North Holland, Biomedical Press, Amsterdam, New York.
2. A.L. Lehninger, David L. Nelson, Michael M. Cox. (2003) Principals of Biochemistry. Worth Publishers Inc. USA, CBS Publishers & Distributors, Delhi.
3. David E. Metzler. (2003). Biochemistry The Chemical reactions of Living Cells. Vol 1, 2. Academic Press, San Diago, California
4. Irwin W. Sherman. (1998). Malaria : Parasite biology, pathogenesis and protection. ASM Press, Washington, D.C.