INTRODUCTION

The Malarial Parasite and Enzyme Succinate Dehydrogenase

Malaria, a major infectious disease in tropical countries, is caused by species of Plasmodium, a genus that belongs to protozoa and transmitted by the bite of infected mosquitoes. Malaria still remains severe health problem and major impediment to economic development due to the spread of *Plasmodium falciparum* parasites which is resistant to currently available antimalarial drugs. The intraerythrocytic parasites derive energy from anaerobic glycolysis, they do not carry out tricarboxylic acid cycle and oxidative phosphorylation. In vitro culture of *P. falciparum* requires low O_2 tension as low as 0.5% of its growth, its optimal O_2 tension is 3% (Scheibel et al.,1979). In the intraerythrocytic stage of their life cycle, it utilizes oxygen, however, it is not related to energy production (Sherman, 1979 ; Scheibel, 1988). The utilization of O_2 for the parasite growth does not derive energy, but O_2 may be used as electron acceptor in simplified electron transport system of mitochondria, which is divided into four functional units or complexes, each composed of several proteins. All components are embedded in the inner mitochondrial membrane. Complex I (NADH - coenzyme Q reductase) carries hydrogens from NADH to coenzyme Q. It contains 26 polypeptides and the prosthetic group is composed of flavin mononucleotide and six or seven nonheme iron sulfur clusters. Complex II

(succinate - coenzyme Q reductase) contains 4 polypeptides, including the flavin adenine dinucleotide - dependent enzyme succinate dehydrogenase and a few nonheme iron sulfur clusters, and passes electrons from succinate to coenzyme Q. Complex III (Coenzyme Q - cytochrome c reductase) carries electrons from coenzyme Q to cytochrome c. It contains at least 6 polypeptides and two types of cytochromes. Complex IV (Cytochrome c oxidase) is composed of two cytochromes (a and a_3), two copper atoms, and at least seven protein subunits (Fig 1,2). Coenzyme Q and cytochrome c act as "shuttles" between complexes and are also known as " mobile carriers ", cytochrome c is a low molecular weight hemoprotein, coenzyme Q is a lipoidal quinone. The major function of the aerobic respiratory chain of mammalian host mitochondria is the electrogenic translocation of protons out of the mitochondrial membrane to generate the proton motive force that derives ATP synthesis. Respiratory systems of parasites tend to show greater diversity in electron transfer pathways than those of host mammalian and many have exploited unique respiratory chains as adaptation to their natural habitats within their hosts.

Succinate dehydrogenase (SDH) catalyzes the oxidation of succinate to fumarate and transfers the reducing equivalents directly to the electron transport system. It has several particularly interesting properties; (1) it is a membrane-bound dehydrogenase , a member of both the tricarboxylic acid cycle and the electron transport system, (2) its activity is modulated by several activators and inhibitors, (3) it is a complex enzyme containing nonheme iron, acid labile sulfur and covalently bound flavin adenine

dinucleotide (FAD).

SDH is located in the cytoplasmic membrane in bacteria or in the chromatophore membrane of photosynthetic bacteria (Reddy and Weber, 1986; Kita et al., 1989; Moll et al., 1991) and on the matrix side of mitochondrial inner membranes of eukaryotic cells (Hatefi and Stiggall, 1978 ; Tushurashvili et al., 1985). It has been purified from many species of organisms (Davis et al., 1977 ; Hederstedt, 1986 ; Kita et al., 1989 ; Pennoyer et al., 1988 ; Saruta et al., 1995), by releasing from the membrane with nonionic detergent, such as triton X-100. The purified enzyme is water soluble and has a molecular weight (M_r) about 100,000 Da. It contains covalently bound FAD, nonheme iron, acid labile sulfur and cytochrome b. Its composition is similar to the enzyme isolated from beef heart mitochondria (Davis and Hatefi, 1971 ; Tushurashvili et al., 1985). Both contain equimolar amounts of two unequal subunits noncovalently bound to each other. The large subunit, M_r about 60-70 KDa, contains covalently bound FAD, namely flavoprotein (Fp). The small subunit has M_r about 25-30 KDa, namely iron protein (Ip). The Fp and Ip subunits are hydrophilic and form the catalytic portion of the complex that transfer the reducing equivalent from succinate to a water soluble dye, such as dichlorophenolindophenol, via SDH or from reduced methyl viologen to fumarate via fumarate reductase. Two small hydrophobic membrane anchoring polypeptides, cytochrome b subunits, M_r about 13 - 20 KDa, seem to be essential for the interaction between the enzyme complex and quinone species. Amino acid compositions of the SDH subunits of the both bacterial

and mammalian enzymes are very similar, although the later is slightly larger. The bacterial Ip is less polar than the mammalian Ip. Structural and functional similarities between the two enzymes are also expressed in reconstitution experiments of the mammalian succinate-Q reductase and succinoxidase. The bacterial SDH can substitute for the mammalian enzyme to form a hybrid reductase and oxidase, respectively. Both Fp and Ip contain nonheme iron and acid labile sulfur that, together with cysteinyl residues, are the building blocks of several iron sulfur centers. These centers render soluble SDH sensitive to inactivation by O_2. The enzyme should, therefore, be kept under anaerobic or reducing conditions in the presence of succinate (Table 1).

The exact stoichiometry, localization, and function of each iron sulfur center in the mammalian enzyme are presently not known.The photosynthetic bacteria also have similar sets of iron sulfur centers. The general view is that the Fp subunit contains two Fe_2S_2 clusters, designated S-1 and S-2 center. Center S-1 is reduced by succinate. Center S-2 is very low redox potential, and it can be reduced by dithionite. The Ip subunit probably contains a Fe_4S_4 cluster designated S-3. Center S-3 is very susceptible to destruction by O_2 in the soluble enzyme. It is essential for expression of the low Km site for ferricyanide, and it is involved in the electron transport from succinate to quinone in the succinate-Q reductase. The substrate binding site of SDH is located in the Fp subunit. Reducing equivalents from the oxidation of succinate are transfered via the FAD to iron sulfur center S-1, S-3, and ultimately to quinone. Electron transport to quinone can be inhibited by 2-

thenoyltrifluoroacetone or carboxanilides, such as carboxin. Both inhibitors block electron transfer between center S-3 and quinone, but they do not affect the reduction of center S-3 by succinate. The cytochrome b of SDH, with an amount nearly equimolar to flavin, is a new species of cytochrome b discovered by Davis et al. (1973). It is not reducible by succinate, but when reduced chemically (dithionite) it is rapidly oxidized by fumarate or coenzyme Q.The reduced cytochrome b does not react with carbon monoxide.

It is important to study basic mechanisms of electron transport system and oxygen requiring system in malarial parasite that is neccesary for parasite survival. Recently the electron transport complex III & IV have been demonstrated (Fry and Beesley, 1991 ; Krungkrai et al., 1993), but studies on complex II have not yet carried out in these parasites. In malaria, SDH might be in mitochondria or in cytosol. In this study enzyme SDH from *P. falciparum* (T_9 isolate) is purified and characterized. The basic information obtained is expected to be useful for the development of new antimalarial drug affecting this enzyme in the future.

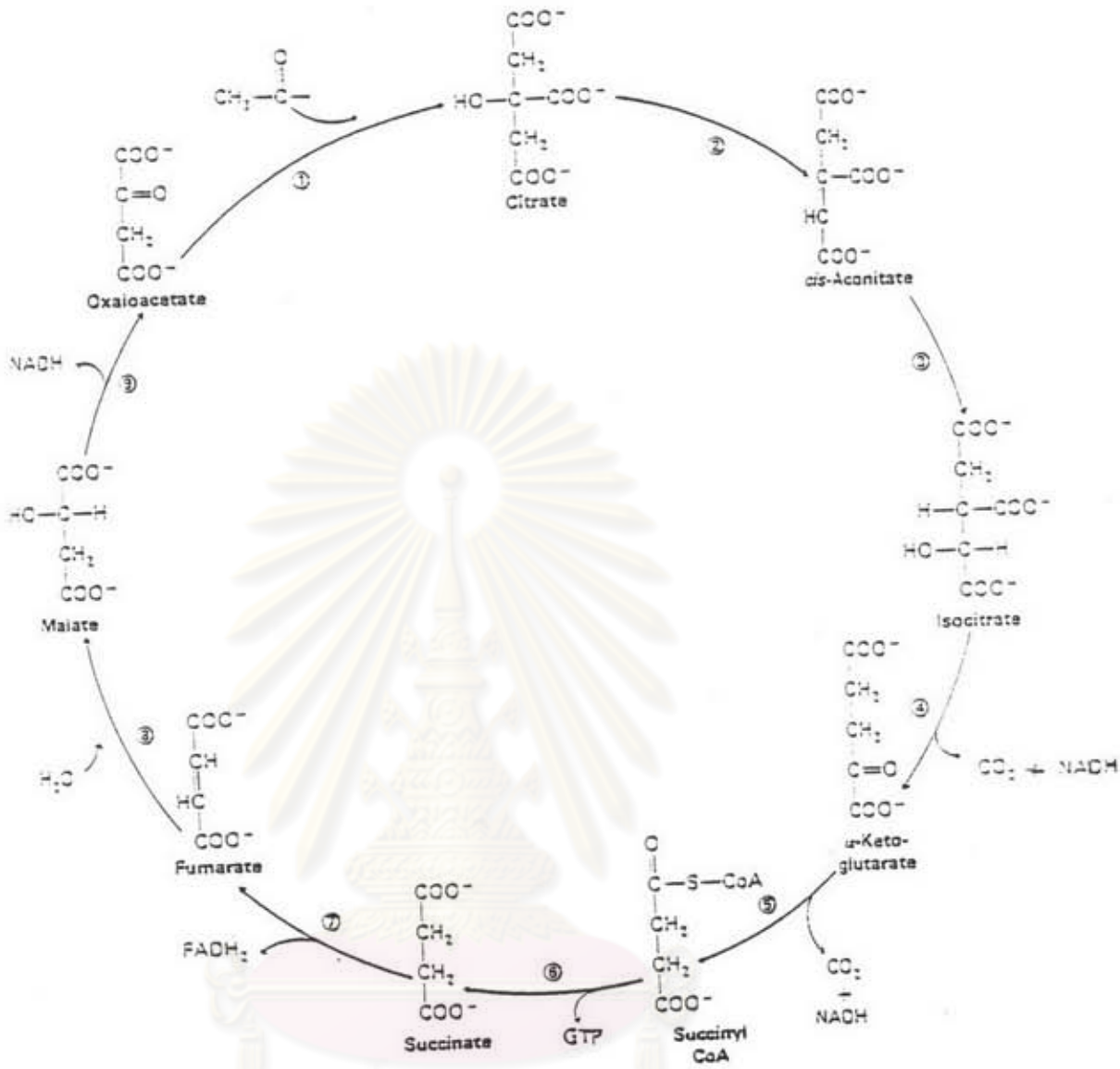

Figure 1. The tricarboxylic acid cycle. Number 1, Citrate synthase ; 2 and 3, Aconitase ; 4, Isocitrate dehydrogenase ; 5, α-Ketoglutarate dehydrogenase ; 6, Succinyl Co A synthetase ; 7, Succinate dehydrogenase ; 8, Fumarase ; 9, Malate dehydrogenase.

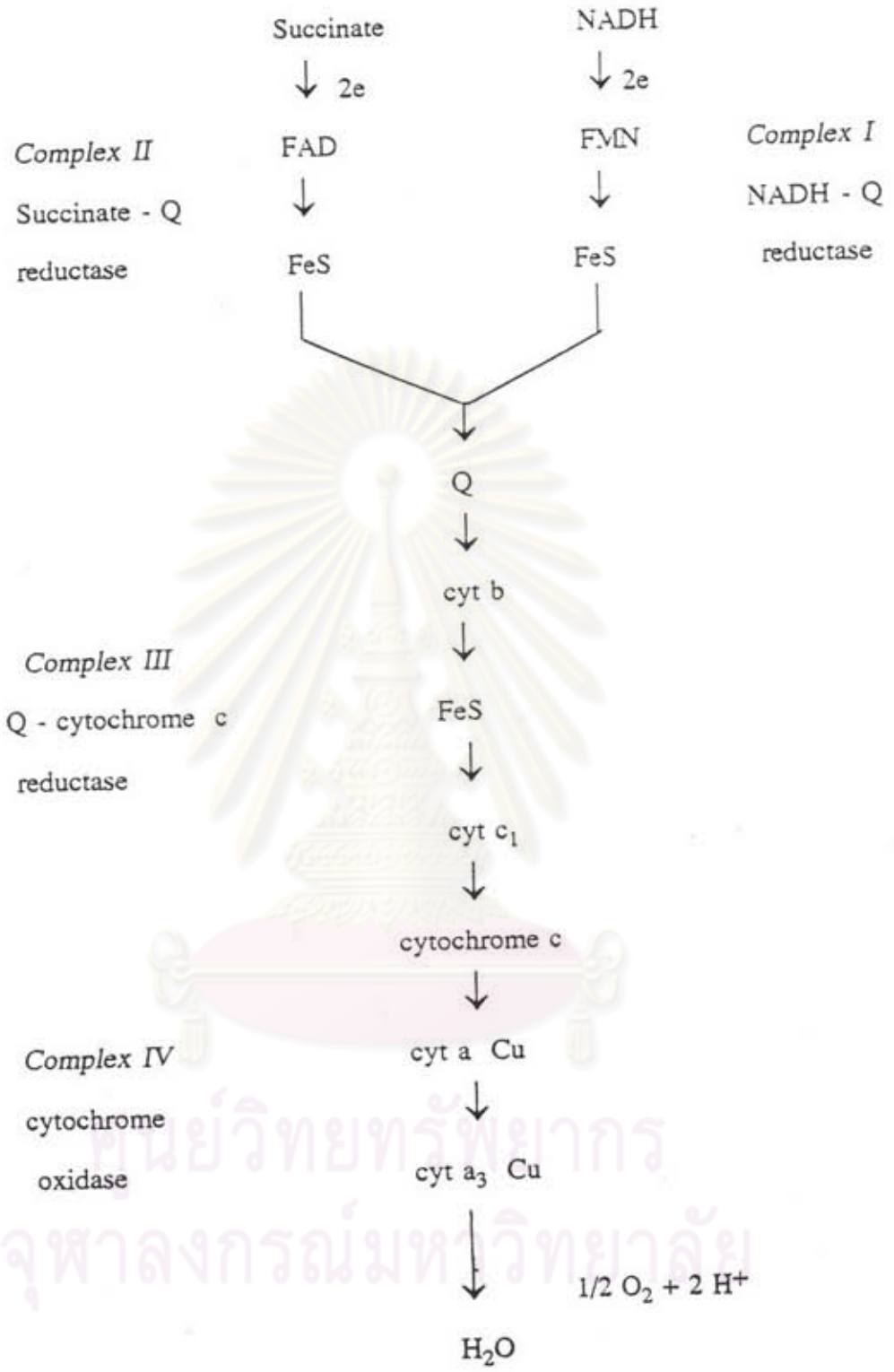

Figure 2. Electron transport chain in mammalian mitochondria.

component	*E. coli* Kita et al. (1989)	*P. denitrificans* Pennnoyer et al. (1988)	Beef heart Tushurashvili et al. (1985)
FAD	9.85[1]	5.6	6.0
non heme iron	67.4	NR[2]	53.0
acid labile sulfur	61.8	NR	50.0
cytochrome b	8.88	3.7	1.2

1 values are expressed as nmol / mg protein.

2 NR , no report.

Table 1. Composition of complex II among three organisms.

Biology and Biochemistry of Malaria

1. Life cycle

The human malaria parasite actually consisting of four species of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) undergoes over a dozen distinguishable stages of development as it moves from the arthropod vectors (species of anopheline mosquitoes) to the human host and back again. One way to conceptualize this complex life cycle is to consider it in three distinct parts; the liver phase, the blood phase and the mosquito phase.

Depending on the developmental stages and species, malaria parasites can be spherical, ring shape, elongated, or crescent shaped, and can be ranged in size from 1 to 20 microns in diameter. By comparison, a normal red blood cell has a diameter of about 7 microns.

Although the four species of human malaria parasites are closely related, there are major differences among them. *P. falciparum*, the most pathogenic of the four species, has been found to be more closely related to avian (*P. lophurae*, *P. gallinaceum*) and rodent species (*P. berghei*, *P. yoelii*, *P. chabaudi*) of Plasmodium than to the other primate and human species (McCutchan et al., 1984).

1.1 Liver Phase

The liver phase of malaria begins when the female anopheline mosquito injects the sporozoite stage of the parasite into the human host during a blood meal. After just a few minutes, the sporozoites arrive at the liver and invade the liver cells (hepatocytes). Over the course of 5 to 15 days, depending on the species, the sporozoites undergo a process of asexual reproduction (known as schizogony, the " spliting process ") that results in the production of as many as 30,000 "daughter" parasites, namely merozoites. It is the merozoites that, once released from the liver, carry the malaria infection into the red blood cell (erythrocytes).

1.2 Blood Phase

When merozoites are released from the liver into the blood stream, asexual blood-stage reproduction, or erythrocytic schizogony, has begun. Parasite invasion of red blood cells unfolds in four steps: attachment of the merozoite to the erythrocyte, rapid deformation of the red blood cell, invagination of the erythrocyte membrane where the parasite is attached and subsequent envelopment of the merozoite, and the resealing of the erythrocyte membrane around the parasite (Aikawa et al., 1978 ; Perkins, 1989; Bannister and Dluzewski, 1990 ; Wilson, 1990).

After invasion, the parasite lies within a membranous parasitophorus vacuole, where it synthesizes nucleic acids, proteins, lipids, mitochondria, and ribosomes and assembles these components into new merozoites (Ginsburg, 1990b). The entire erythrocytic asexual cycle takes between two and three days to run its course, depending on the species. Once merozoite assembly is completed, the erythrocyte ruptures and merozoites are released into the plasma, where they attach to other erythrocytes and begin the process anew. Some merozoites, for reasons not well understood, differentiate into the sexual forms of the parasite, the gametocytes. The factors that determine the sex of the gametocyte are unknown. Gametocyte development takes between 2 days (for *P.vivax*) and 10 days (for *P.falciparum*). The release of merozoites precipitates malaria's classical paroxysms of fever, chills, headache, myalgia, and malaise.

1.3 Mosquito Phase

When gametocytes are taken up during a mosquito's blood meal, a number of factors, including temperature, concentrations of oxygen and carbondioxide, pH, and a mosquito exflagellation factor, are thought to contribute to the maturation of gametocytes. Male microgametes are released during a process called exflagellation. Fusion of the female macrogamete with a single microgamete results in fertilization and the formation of the ookinete. The ookinete migrates to the wall of the mosquito midgut, where it penetrates the peritrophic membrane and epithelium and comes to rest on the external surface of the stomach. Over a period of days, this stage of the parasite matures into an oocyst containing up to 10,000 motile sporozoites. When the oocyst

ruptures, the sporozoites enter the mosquito circulation and travel to the salivary glands, where they are injected into the human host when the mosquito feeds. The number of sporozoites that enter the human host during a single blood meal is thought to be highly variable.

The life cycle of Plasmodium in the mosquito and man is shown in Fig.3

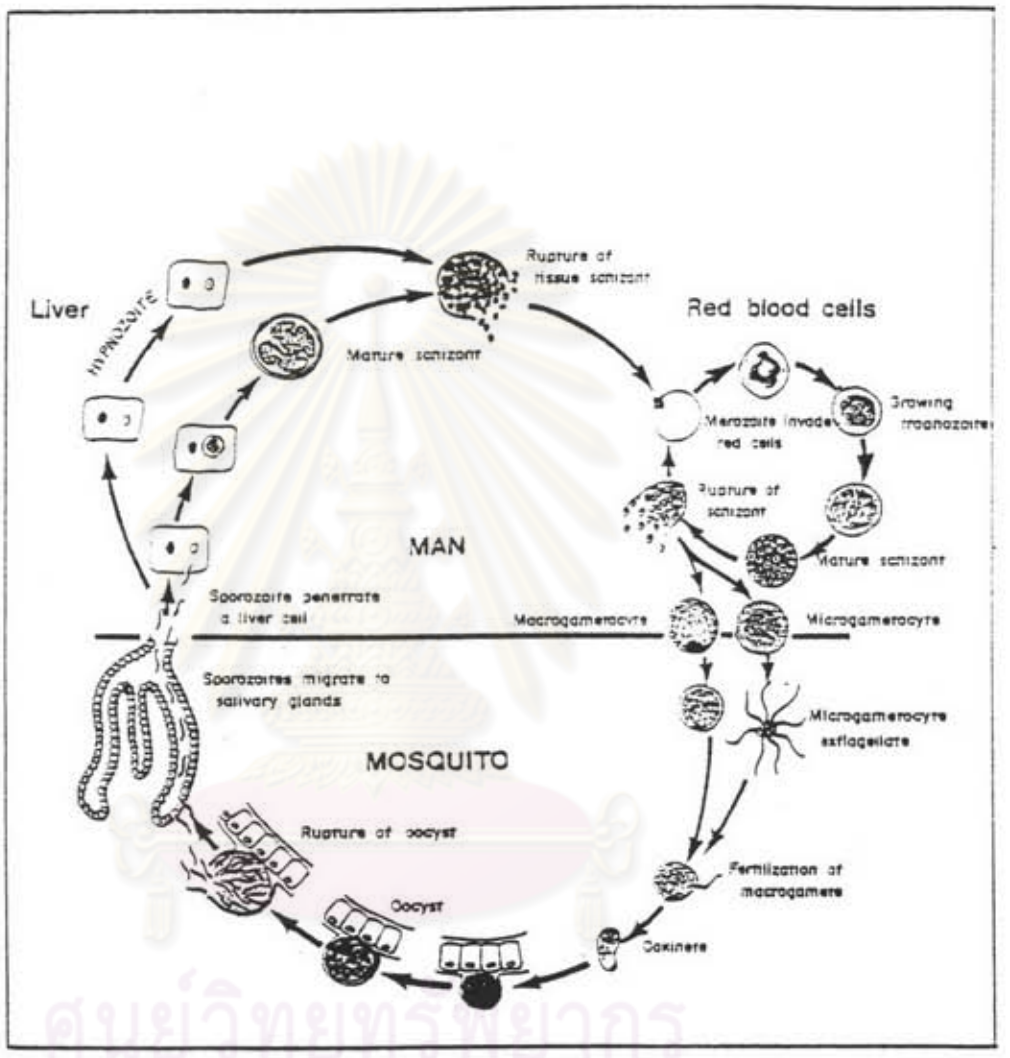

Figure 3. The life cycle of the malarial parasite.

2. Biochemistry of Malaria

The red cell receptor for *P. falciparum* is probably located on the glycophorin (Pasvol, 1984). After invasion infected cell undergoes considerable morphological and metabolic changes. These changes accompanying maturation are related to parasite biochemical activities involving the biosynthesis of protein, DNA and RNA, carbohydrate metabolism, amino acid and lipid metabolisms. The relatively recently acquired ability to cultivate *P. falciparum* in vitro has greatly expanded biochemistry to study parasite nutrition and metabolism .

The biochemistry of malaria parasites in the erythrocytic phase has been extensively reviewed by Sherman (1979; 1983; 1984) and Homewood and Neame (1980).

2.1 Carbohydrate Metabolism

The erythrocytic stages of malaria do not store glycogen or other reserve polysaccharides, therefore they rely on host supplied glucose. In *P. falciparum*, glucose can be replaced by fructose, but the parasite will not develop in vitro when another sugar, such as galactose, mannose, maltose or ribose are substituted. (Geary et al., 1985a). The end products of glucose catabolised vary with the species of Plasmodium: simian and rodent malaria infected red blood cells convert 70-85% of glucose to lactate, whereas in red blood cells infected with avian malarial parasites, CO_2, organic, and

amino acid as well as lactate are produced (Sherman, 1979 ; Homewood, 1980).

All species of malaria appear to posses the glycolytic enzymes of the Embden-Meyerhoft pathway. All of the glycolytic enzymes have not been identified in a single species (Roth et al., 1988) and therefore it is an assumption that the entire pathway exists in all species of Plasmodium.

The pentose pathway is not increased on parasitisation of the red blood cells. The existence of glucose - 6 - phosphate dehydrogenase (G6PD), the first enzyme in the pathway, remains to be further elucidated (Hempelmann and Wilson, 1981). Recently, Usanga and Luzzatto (1985) reported that *P. falciparum* contains its own enzyme which can be expressed in the G6PD deficient red cells. However, the second enzyme in the pathway, 6-phosphogluconate dehydrogenase, is consistently identified in malarial parasites and is different from that of host red cells (Sherman, 1979; 1984; Homewood and Neame, 1980). The remaining enzymes in the pathway have not been studied in the parasites.

The parasite has no complete cycle of tricarboxylic acid. The enzyme of the tricarboxylic acid cycle identified with some degree of certainly in avian, human and rodent malaria is malate dehydrogenase (Sherman, 1984; Unnasch, 1992) . But, it has been reported that some strains of *P. berghei* lack of enzyme malate dehydrogenase (Homewood and Neame, 1980).

Isocitrate dehydrogenase has been identified in *P. lophurae* (avian malaria) *P. falciparum* (human malaria) (Vander Jagt et al., 1989) and *P. knowlesi* (simian malaria) (Sahni et al. , 1992), but can not be detected in *P. berghei* (rodent malaria). Succinate dehydrogenase activity has been found in *P. gallinaceum* and *P. lophurae* (avian malaria), but not in *P. berghei* (Sherman, 1979).

2.2 Amino Acid Metabolism and Protein Synthesis

There are four potential sources of amino acids for the intraerythrocytic Plasmodium. (1) De novo synthesis from folate mediated reaction e. g. glycine, methionine. (2) CO_2 fixation , which can only supply a limited amount of amino acids e.g. glutamic acid. Only one of the enzymes in the CO_2 fixing pathway, namely phosphoenolpyruvate carboxylase, has been identified in *P. berghei* (Siu, 1967), however, the presence of CO_2 fixation has been recently reported in *P. falciparum* by Blum and Ginsburg (1984). (3) The free amino acid pools of the blood plasma and erythrocyte. It is found that isoleucine and methionine supplied exogenously are necessary for parasite growth, probably because hemoglobin (Hb) is deficient in these amino acids. The increased uptake of various amino acids by malaria infected red cells has been reviewed by Sherman (1977). However the detailed mechanism of uptake should be further studied. (4) The host cell Hb. Hb is likely to be the major source of amino acids for parasite protein synthesis. It is ingested via the cytostome and then the food vacuoles at the base of the cytostome vacuole containing the

protein are pinched off. The degradation of Hb in the food vacuole take place by at least 2 catalytic enzymes : cathepsin D (Sherman and Tanigoshi, 1981) and aminopeptidase (Vander Jagt et al.,1984). The food vacuoles contain the degradative products of hemoglobin with electron particle, namely malarial pigment (or hemozoin). It has been suggested that the food vacuole is lysosomal particle (Sherman, 1983).

The protein synthesis of malaria appears to be typically eukaryotic: the parasite has its own ribosomes which have a sedimentation constant of 80S and can be dissociated into 60S and 40S, the synthesis is inhibited by cycloheximide and puromycin, but not choramphenicol or streptomycin (Sherman, 1979). Most proteins of *P. falciparum* are reported to be synthesized by every stage of growth, and unchanged the cycle through to the ring stage following merozoite invasion of the red cells. Some proteins synthesized are found to be dependent on the stages of the parasite (Myler et al., 1983).

2.3 **Phospholipid and Cholesterol Metabolism**

Malria infected red cells show an increase in total lipids, and change in the phospholipid to cholesterol ratio which is associated with parasite membrane (Holz, 1977). It is found that malaria does not synthesize cholesterol and fatty acids de novo (Vial et al., 1984: Holz, 1977). In falciparum malaria cholesterol is probably obtained preformed from the host (Vial et al., 1984). The parasite obtains free fatty acids from the host

plasma (Holz, 1977; Sherman, 1979; Homewood and Neame, 1980). The parasite shows its ability to synthesize phospholipid de novo from their precursors such as palmitate, serine, choline, inositol and glycero - 3 - phosphate in *P.falciparum* (Vial et al., 1982a) and in *P. knowlesi* (Vial et al., 1982b). The metabolism of phospholipid in *P. falciparum* is a unique pathway and may constitute a potentially fruitful chemotherapeutic approach to malaria (Vial et al., 1984). Plasmodium also appear to lack the capacity to synthesize fatty acids de novo from acetate, but they can incorporate exogenous fatty acids into their phospholipids, thereby maintaining a lipid fatty acid composition distinct from that of the host cell (Vial et al., 1990).

2.4 **Genome Organization**

Malaria parasites are lower eukaryotes which are haploid for their life cycle, with a brief diploid phase prior to meiosis in the mosquito vector. The haploid genome comprises 2 - 2.5 x 10^7 base pairs of DNA. It is unusual that it has extremely low (G+C) content : *P. falciparum* , *P. berghei* and *P. lophurae* are 18 %, *P. knowlesi* and *P. fragile* 30 %. *P. cynomolgi* and *P. vivax* with multiple bands in addition to the two major bands at 18 % and 30 % (McCutchan et al., 1984), however, Williamson et al. ,(1985) reported that *P.knowlesi* has (G+C) content of 38 %. It was also found that the repeat element is present in malaria DNA at a very high copy number and appear to be distributed widely throughout the genome (Goman et al., 1982; Guntaka et al., 1985) as found in all of the eukaryotic genome.

There are three genomes in *Plasmodium* (Wilson et al., 1991). (1)The nuclear genome contains 14 chromosomes. The technique of pulse field gel electrophoresis has allowed researchers to visualize the chromosomes, which range in length from 600 to 3,500 kilobases. Genes for various parasites proteins has been located on individual chromosomes (Kemp et al., 1985, 1987b). (2) Mitochondrial genome, known as the 6 kilobase (Kb) tandemly repeated element encodes three genes of subunit I , III of cytochrome oxidase as well as cytochrome b , and fragmented rRNA genes. (3) 35 Kb circular genome contains large and small ribosomal RNA genes, transfer RNA genes and genes encoding rpo B and rpo C subunits of RNA polymerase.

2.5 **Pyrimidine Biosynthesis and Purine Salvage**

There are two possible sources of purine and pyrimidine bases, nucleosides and nucleotides, for nucleic acid synthesis by the intraerythrocytic parasite : synthesis from simple precursors, and using materials from outside the cell. Malaria parasites cannot synthesize purines de novo , and must obtain them from the host (Sherman, 1979). Hypoxanthine, obtained from both host plasma and from adenosine metabolism (involving 2 enzyme : adenosine deaminase and purine nucleoside phosphorylase), appears to be the major purine base salvaged by *P. falciparum* (Webster and Whaun, 1981a; 1981b). The purine metabolism pathway in the malaria parasite is well characterized, from hypoxanthine to both guanosine and adenosine nucleotides

(Webster and Whaum, 1981a; 1981b; 1982). At least 6 enzymes involving in purine metabolism have been identified in *P. falciparum* (Reyes et al., 1982). Some of the enzymes are well characterized e.g. adenosine deaminase of *P. falciparum* (Daddona et al., 1984) and of *P. lophurae* (Schimandle et al., 1983), purine nucleoside phosphorylase of *P. lophurae* (Schimandle et al., 1985).

Malaria parasites are unable to utilize exogenous pyrimidines, and must synthesize them de novo (Sherman, 1979). Thymidylate (TMP) is the precursor of nucleic acid synthesis, and its precursor is in turn deoxyuridylate (dUMP). All of the enzymes necessary for the de novo synthesis of dUMP have been identified in *P. berghei* extract (Hill et al., 1981), *P. falciparum* extract (Gero et al., 1984; Reyes et al., 1982; Scott et al., 1986). The flux of $H^{14}CO_3$ through the de novo pyrimidine biosynthetic pathway is well defined to complete the metabolic pathway in *P. falciparum* (Hammond et al., 1984). Only two enzymes,orotate phosphoribosyltransferase and oroditylate decarboxylase, are well characterized in *P. falciparum* (Rathod and Reyes, 1983). The two pathways of nucleic acid synthesis, purine salvage and pyrimidine de novo, render possible targets for the design of novel antimalarial agents.

2.6 **Energy Transformation and Mitochondria**

There has been considerable debate about whether the erythrocytic stages of mammalian malaria parasite posses mitochondria, the energy

producing organelles essential for all life forms. The falciparum parasite uses glucose as its primary energy source. In fact, glucose utilization is significantly greater in the infected erythrocyte than in the uninfected cell. Progress has been made in characterization of all enzymes involved in glycolysis in *P. falciparum* (Roth et al., 1988; Roth, 1990). However, there is no evidence supporting the presence of a tricarboxylic acid cycle, a key energy producing process of the mitochondria.

The presence of mitochondria in the erythrocytic asexual stages of *P. falciparum* has recently been shown, but the actual function is not well understood (Divo et al., 1985b). Recent advances in the molecular biology of the mitochondria DNA of malaria parasites may help to untravel the role of the mitochondria (Gardner et al., 1988). Antibiotics used to treat falciparum infection, such as the tetracyclines, clindamycin, and erythromycin, appear to work by blocking the development of parasite mitochondria (Prapunwattana et al., 1988). Of great interest in this regard is the recent finding that mitochondria DNA of *P. falciparum* encodes an RNA polymerase which is closely related to prokaryotic polymerase and is sensitive to rifampicin, potentially explaining the antimalarial activity of this drug (Gardner et al., 1991).

The erythrocytic stages of many mammalian malaria parasites appear not to derive their metabolic energy through classical electron transport. The mitochondria may participate in ion transport, but the role of the organelle in metabolism is unclear. It is not known whether components analogous to

those present in the mammalian terminal electron transport system function in the malaria parasite, and for what purpose, since the organism, like many other parasitic protozoans and all parasitic worms so far studied has rather limited terminal respiration (Scheibel, 1988). Mammalian malaria parasites are aerobic fermenters, capable of partially decomposing metabolic substrates to fermentation products, but are unable to oxidise them completely to carbon dioxide and water. Since the substrates are not completely metabolised, it would appear that terminal respiration is either absent or rate limiting in the parasite. Available evidence supports the view that malaria parasites are microaerophilic, homolactate fermenters (Scheibel et al., 1979).

AIM OF THE THESIS

In this thesis, the enzyme succinate dehydrogenase in *P. falciparum* (T_9 isolate) was studied as follow :

1. demonstration of its existence of the enzyme in the parasite
2. purification and characterization of the enzyme
3. determination of the kinetics of the enzyme and the inhibitory effects of some inhibitors.