INTRODUCTION

THE MALARIA PARASITE

Malaria is an ancient disease that still one of the major tropical diseases. In Thailand, the annual parasite incidence (API) of malaria is 5.20 per 1,000 in 1991. Four species of malaria parasites are infectious to man : Plasmodium falciparum, the most lethal species since it can causes complication such as cerebral malaria, Plasmodium vivax , Plasmodium malariae and Plasmodium ovale.

In the erythrocytic stage of their life cycle,malaria parasites primarily derive energy from glycolysis. They do not carry out oxidative phosphorylation and do not possess an active tricarboxylic acid cycle. The intraerythrocytic parasite does utilize oxygen, however, it is not related to energy production (Sherman, 1979; Scheibel, 1988). In vitro culture of P.falciparum requires low O_2tension as low as 0.5% for its growth, its optimal O_2 tension is 3% (Scheibel et al., 1979). The requirement of $O_2$for the parasite growth is believed to be linked to dihydroorotate (DHO) oxidation in pyrimidine biosynthetic pathway (Fig. 1.) and a simplified electron transport system (ETS) of mitochondria which is proposed to have containing flavoprotein, ubiquinone, cytochrome b and c and cytochrome oxidase (Gutteridge et al., 1979; Scheibel, 1988). Recently, evidence has been provided that mitochondria might play a role in adenosine triphosphate (ATP) synthesis through the existence of enzyme adenylate kinase (ADP +Pi <--> ATP) (Kanaai and Ginsburg, 1989).

The growth of malarial parasite in erythrocytes requires a supply of purine and pyrimidine nucleotides for production of nucleic acid. Whereas rodent and primate parasites obtain their purine requirement by salvage of preformed bases and nucleosides. They appear unable to take up pyrimidines because they lack of the relavant salvage enzymes, notably, thymidine kinase (Hill et al., 1981 ; Hammond and Gutteridge, 1982; Krungkrai et al., 1989). So they obtain their pyrimidine requirements by de novo synthesis (Gutteridge and Trigg, 1970 ; Van Dyke et al., 1970, Walsh and Sherman, 1968).

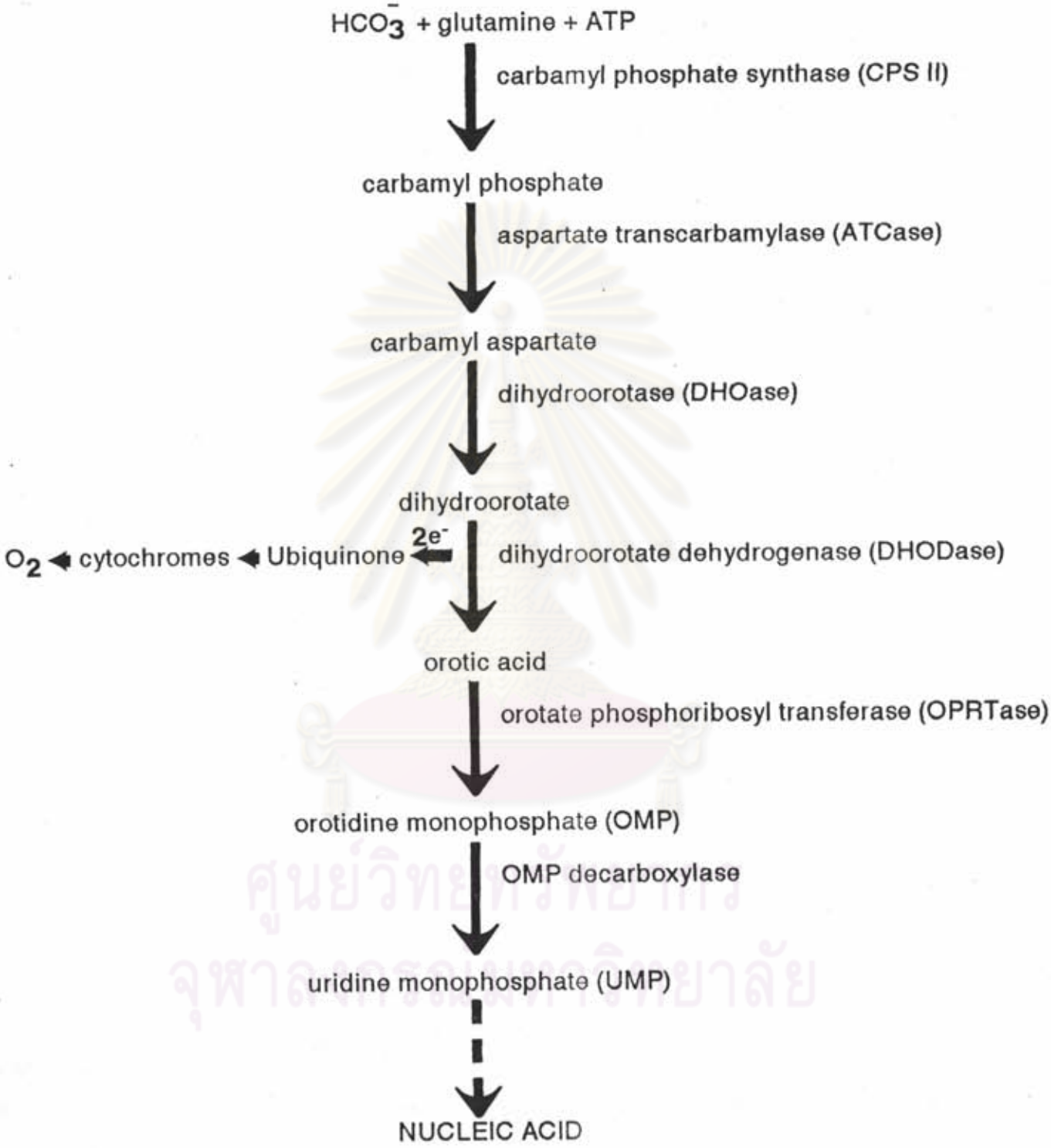

FIGURE 1 : Pyrimidine Biosynthesis Pathway.

BIOLOGY AND BIOCHEMISTRY OF MALARIA

1. Life Cycle and Intraerythrocytic Stages of Malaria

Malaria ranks as one of the commonest and most important parasitic diseases. The distribution of malaria is determined by the vector arthropods, species of anopheline mosquitoes, and major foci in Africa, India, Southeast Asia, Central and South America. Despite intensive programs of vector control, which has been successful in some countries, there has been no significant global reduction in the extent of malaria infection. Thus, it is necessary to control malaria eradication by means of chemotherapy.

Man is the intermediate host of Plasmodium, the sexual stages of the life cycle taking place in the body of mosquitoes(sporogony). There are 4 species of Plasmodium which cause malaria in man: P. falciparum,P. vivax, P. malariae and P. ovale. Other species of Plasmodium cause malaria in simians (P.knowlesi, P.cynomolgi), in rodents (P.berghei, P.yoelii, P. chabaudi, P. vinckei), and in birds (P.lophurae, P. gallinaceum).

Malaria in man is initiated by the bite of an infected female mosquito with the introduction of sporozoites into the host circulation. The sporozoites invade hepatic parenchymal cells shortly after the blood meal and undergo growth and asexual reproduction to form a large preerythrocytic shizonts (exoerythrocytic schizogony). This process results in the rupture of the infected cells and release of thousands of merozoites which penetrate the red blood cells to initiate the erythrocytic schizogony. Within the red cells, the morozoite undergoes a developmental cycle of

sequential cellular differentiation to the schizont, membrane biogenesis and nuclear divisions occur, along with generation of new spical organelles and cytoplasmic segmentation to yield new merozoites. The mature schizont ruptures the red cell and the merozoites are released to infect new red cells.

Gametocytogenesis occurs in conjuction with the erythrocytic schizogony. Some merozoites invade new red cells and produce male or female gametocytes rather than a further generation of schizonts. The host blood containing gametocytes are taken up by anopheline mosquitoes. Production of male gametes and fertilization of the female gamete occurs in the mosquito. The motile zygote then penetrates the stomach wall and forms an oocyst on the outer lining. Within the oocyst there is repeated division to produce large numbers of sporozoites and these eventually move anteriorly in the mosquito to enter the salivary gland (Bannister and Sinden, 1982; Sinden, 1984). The life cycle of Plasmodium in the mosquito and man is shown in Fig. 2.

In falciparum malaria only red cells containing rings circulate in peripheral blood, cells containing trophozoites and schizonts are sequestered by adhering to endothelium of postcapillary venules of several tissues, predominantly heartly and skeletal muscle. Sequestration of the more mature forms of the parasite prevents their circulation through spleen, a major site of parasite destruction. The sequestered parasites may also obstruct blood flow in the brain, as seen in cerebral malaria. The mechanism of sequestration may be related to the knob formation on surface membranes of falciparum-infected red cells (Suarez et al., 1985). The knobs consist of histidine-rich proteins binding to the red skeleton

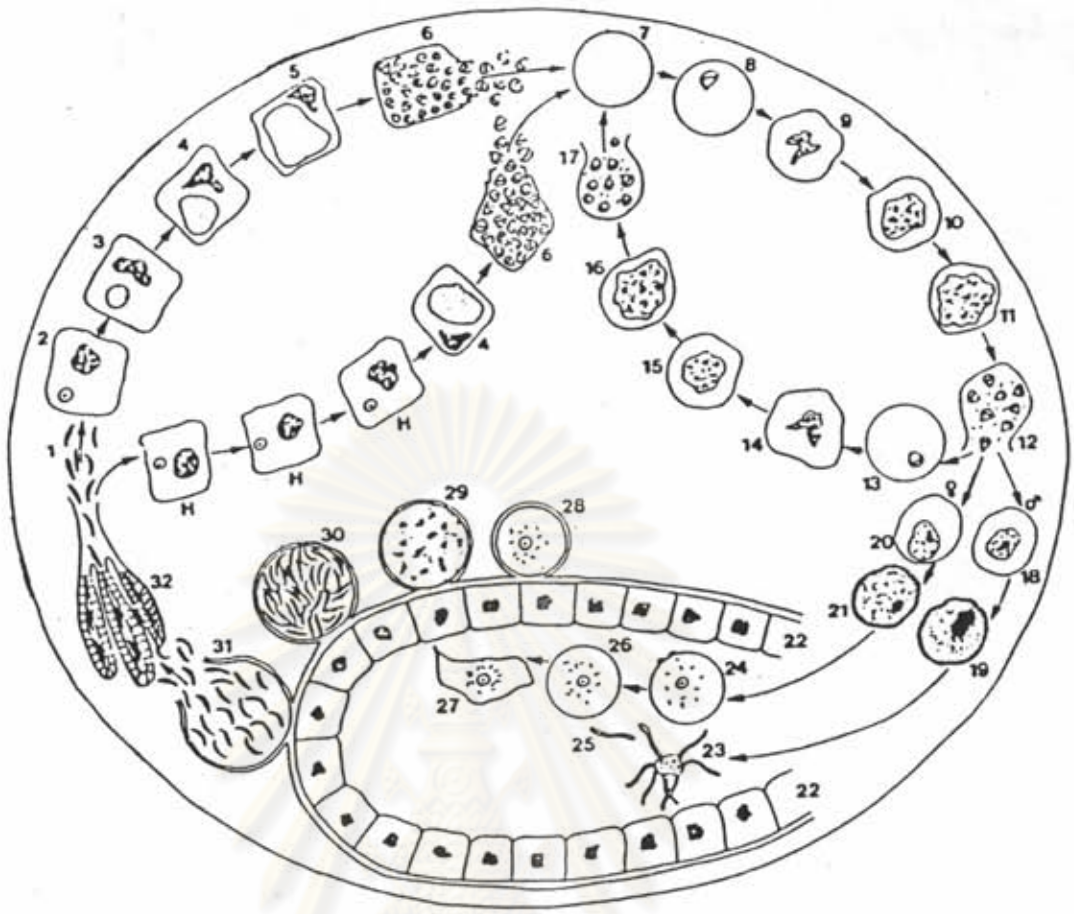

Figure 2 : The Life Cycle of The Malaria Parasite.
(from Bray and Garnham,1982)

1: sporozoites injected into skin by mosquito; 2: 2–day–old exoerythrocytic form in hepatocyte; 3, 4, 5: growing erythrocytic schizonts; H: hypnozoites in hepatocytes ; 6: mature exoerythrocytic schizonts bursting, releasing merozoites in to the blood; 7: erythrocyte; 8, 9: growing trophozoites; 10, 11: growing schizonts; 12: mature schizont releasing merozoites; 13, 14, 15, 16, 17: erythrocytic cycle repeated; 18, 19: growth of the microgametocyte; 20, 21; growth of the macrogametocyte; 22: mosquito has taken gametocytes up into its mid–gut; 23: exflagellation of microgametocyte; 24: macrogametocyte escapes from erythrocyte to become a macrogamete; 25: microgamete; 26: macrogamete about to be fertilized; 27: zygote or ookinete; 28, 29, 30: oocyst growth on the mid–gut serface; 31: oocyst bursting, releasing sporozoites; 32: sporozoites in the mosquito salivary glands.

to form electron-dense cups. The cups produce knobs by forming focal protrussions of the red cell membrane that are the specific points of binding between infected red cells and the endothelium (Leech et al.,1984).

2. Biochemistry of Malaria

The red cell receptor for P. falciparum is probably located on the glycophorin (Pasvol, 1984). After invasion the 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, glucose metabolism and the pentose phosphate pathway and amino acid and lipid metabolisms. 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. Glycolysis

Glucose is the major source of energy obtained from host plasma and lactate is the end product of the pathway. The production of lactate by P.falciparum is stage-dependent, schizonts show the higher production (Pfaller et al., 1982). Enzymes involved in the glucose breakdown have been found in every species of the parasites, but only lactate dehydrogenase from P.falciparum has been purified and kinetically studied (Vander Jagt et al., 1981). Additionally, heterogeneity of the enzymes in the pathway is found in a wide variety of malaria, e.g., lactate dehydrogenase, glucose phosphate isomerase, and pyruvate kinase (Sherman, 1979).

2.2. Pentose Phosphate Pathway

This pathway is not increased on parasitization of the red 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.

2.3. Tricarboxylic Acid Cycle (Krebs cycle)

The parasite has no complete cycle of tricarboxylic acid, and only two enzymes in the pathway have been demonstrated : succinate dehydrogenase and malate dehydrogenase (Sherman, 1984). Additionally, the intact mitochondrion has been recently observed which undergoes a complex pattern of growth, development, and replication during the erythrocytic phase of P. falciparum (Divo et al., 1985a), suggesting that the parasite lacks the functional Krebs cycle. However, it needs for growth and development (Blum and Ginsburg, 1984). The parasite also shows a detectable electron transport system which has not been well characterized (Sherman, 1979; Homewood and Neame, 1980). P. falciparum is found to be the obligate microaerophile (Schiebel et al.,1979) that the oxygen is believed to be utilized for oxidative reaction in pyrimidine de novo synthesis (Gero et al., 1984).

2.4. Amino Acid Metabolism and Protein Synthesis

There are four potential sources of amino acids for the intra-erythrocytic Plasmodium. (1) De novo synthesis from folate-mediated reactions e.g., glycine, methionine,(2)CO_2fixation, which can only supply a limited amount of amino acids e.g., glutamic acid. Only one of the enzymes in the CO_2fixing pathway, namely phosphoenolpyruvate carboxylase, has been identified in P.berghei (Sui, 1967), however, the presence of CO_2fixation glutamate dehydrogenase has been recently reported in P. faciparum by Blum and Ginsberg (1984). (3) The free amino acid pools of the blood plasma and erythrocyte. It is found that isoleucine and methionine supplied exogeneously 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. The Hb 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 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).

2.5. Phospholipid and Cholesterol Metabolism

Malaria-infected red cells show an increase in total lipids, and changes 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 phospholipids 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).

2.6. Genome Organization and Nucleic Acid Synthesis

The genome size of plasmodia is estimated to be 3.8×10^8 base pairs (Hough Evans and Howard, 1982). It is unusual in 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 2 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 malarial DNA at a very high copy number and appears to be distributed widely throughout the genome (Goman et al.,1982;Guntaka et al.,1985) as found in all of the eukaryotic genome. P.falciparum has been recently found to contain at least 7 chromosomal DNA molecules (Kemp et al., 1985). Although the DNA base composition of malaria falls into distinct categories (McCutchan et al.,1984 the base composition of the RNA has been shown to be the same of all malarias being typically protozoan in its (G + C) content of 35% (Sherman, 1983).

The rate of nucleic acid synthesis by the malarial parasite is dependent on the stages of intraerythrocytic development. DNA synthesis, as measured by the incorporation of labeled compounds, is initiated at the trophozoite stage and remains constant throughout schizogony in P.falciparum (Inselberg and Banyal,1984) and P.chabaudi (Newbold et al., 1982). For RNA synthesis, it occurs throughout the cycle of intraerythrocytic development e.g., P.knowlesi (Conklin et al., 1973), P.chabaudi (Newbold et al., 1982).It was also found that pyrimidine de novo synthetic enzymes is dependent on the stage of the intraerythrocytic cycle of P.falciparum,

the highest activities for all enzymes being found at the trophozoite stages (Gero et al., 1984).

There are two possible sources of purine and pyrimidine bases and nucleosides for nucleic acid synthesis by the intraerythrocytic parasite: synthesis from simple precursors, and material 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 enzymes : adenosine deaminase and purine nucleoside phosphorelase), appears to be the major purine base salvaged by P.falciparum (Webster and Whaun,1981a; 1981b). The purine metabolic pathway in the malarial parasite is well characterized, from hypoxanthine to both guanosine and adenosine nucleotides (Webster and Whaum, 1981a; 1981b; 1982). At least 6 enzymes are involved 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 phosphorelase 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., 1969). 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 offer a possible basis of the design of novel antimalarial agents.

The pyrimidine de novo pathway in such parasites represents a series of enzymatic steps (Fig.1.) that are unique in the parasitized cell and are the obvious target for selective chemotherapy as de novo synthesis is absent in the mature erythrocyte (Kelly and Smith, 1978).

In mammals and other higher eukaryotes, the first three enzymes of pyrimidine biosynthesis, carbamyl phosphate synthase II (CPS II),asparate transcarbamylase (ATCase) and dihydroorotase (DHOase) are carried by a molecular weight 240,000 daltons multifunctional protein (Kelly et al., 1986; Jones, 1980). In 1990 Krungkrai et al have purified the third enzyme of this pathway, dihydroorotase (DHOase),from P.berghei (rodent malaria parasite) and Crithidia fasciculata to apparent homogeneity. The DHOase from the two parasitic protozoans has been shown to be a monofunctional protein which differs from the mammalian enzyme which is a part of trifuntional protein with the first two enzymes of the pathway. The fourth enzyme of the pathway, dihydroorotate dehydrogenase (DHODase), has been characterized in C. fasciculata and Trypanosoma brucei (Pascal et al., 1983). This enzyme has been proposed as the site of action of a class of experimental antimalarial

drugs, but there is little information on this protein in Plasmodium species. Orotate phosphoribosyltransferase (OPRTase) and orotidine –5'– phosphate decarboxylase (ODCase) catalyze the final steps of de novo synthesis of uridine monophosphate (UMP) in mammalian cells and exist as a bifunctional protein (Jones, 1980), but in P.falciparum, the most important cause of human malaria, these proteins has been described as discrete entities (Rathod and Reyes, 1983).

Dihydroorotate dehydrogenase (DHODase), the fourth sequential enzyme in the de novo biosynthesis of pyrimidine, has been shown to be a particulate enzyme that catalyzes the oxidation of dihydroorotate (DHO) to orotate(OA) . In many bacterial systems , the DHODase is membrane–bound and its catalytic action is intimately linked to the cell's respiratory systems (Karibian and Couchoud, 1974 ; Larsen and Jensen, 1985). Similarly in a variety of eukaryotic cells, the enzyme is physically associated with the membrane of the mitochondrion, and again, its action appears to be linked to respiratory electron transport (Jones, 1980;Chen and Jones 1976;Forman and Keneny, 1978; Hines et al.,1986; Gero and O'Sullivan, 1985). By analogy to other systems we expect this enzyme to be associated with outer surface of inner membrane of the mitochondria in the malaria parasite. This enzyme has also been purified from P.berghei to apparent homogeneity from a Triton X–100 extract of the mitochondrial pellet in combination with anion–exchange, affinity and gel filtration chromatographic techniques. The preliminary data shows that the purified DHODase from P.berghei is physically different from the host enzyme and requires ubiquinone –30, –45, –50 for its maximal activity (Krungkrai et al., 1991).

With the relatively limited information suggesting key differences between the malaria parasite and host in pyrimidine biosynthesis, and some evidence suggesting that parasitic protozoa might be acutely sensitive to existing or novel drugs which act at this site. This thesis is focused on this aspect of parasite metabolism, pyrimidine biosynthesis through purification and characterization of the enzyme DHODase in P.falciparum in expectation that these studies will provide a basis for the development of novel anti-malarial compound.

OBJECTIVES

1. To identify the enzyme dihydroorotate dehydrogenase (DHODase) in P.falciparum, human malaria parasite.

2. To purify the enzyme DHODase from P.falciparum .

3. To characterize the enzyme DHODase from P.falciparum .