CHAPTER I

INTRODUCTION

Malaria is one of the most important infectious diseases in Thailand, affecting millions of people. The Annual Parasite Incidence per thousand population (API) throughout the country has been greatly reduced during the past 35 years. In 1947 the API was 286. This rate was reduced until the period of 1966-1972 when the incidence appeared to stabilize, varying from 2.2 to 3.6 per 1000 population. Since that time it has risen annually,to 7.1 in 1979, 8.9 in 1980, and 10.6 in 1981. The incidence decreased to 9.1 in 1982 and 5.2 in 1983 but it is now on the rise again. In 1984 the API was 6.12 (Harinasuta, et al., 1982; Prasittisuk, 1985). The malaria infection is still on the high rank of one of the top ten diseases in Thailand (Amaranuntakit, 1984).

1. Taxonomy and life cycle of malaria

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium* that can be transmitted to man and other vertebrate hosts by the bite of the infected female *Anopheles* mosquito.

1.1 Taxonomy of the malarial parasite

Plasmodium can be classified into 3 groups according to the specificity of host —— mammalian, avain and reptile malaria parasites (Wernsdorfer, 1980). The commom four species of *Plasmodium* that cause human malaria are *P. falciparum, P. vivax, P. malariae* and *P. ovale*. Three of the four species of human *Plasmodium* found in Thailand are *falciparum, vivax* and *malariae*. The parasite incident record in 1982 showed 65.3% *P. falciparum*, 34.4% *P. vivax* and 0.02% *P. malariae* (Prasittisuk, 1985). However in some areas, malaria infection of *P. falciparum* can be found in the range of 98.38-99.19% (Amaranuntakit, 1984). The most deadly of human malaria is caused by *P. falciparum;* if treated unappropriately, the infection may cause cerebral malaria and may result in mortality.

1.2 The life cycle of the malarial parasite

Man and other vetebrate hosts, eg. rodents, birds and reptiles are infected with malaria on being bitten by an infected female *Anopheles* mosquito. Viable sporozoites are inoculated and invade liver cells, where they multiply asexually to produce merozoites. These merozoites are liberated and invaded red cells, where they form characteristic "rings" which grow to become mature trophozoites and undergo another asexual multiplication to produce many new merozoites. The red cells erupt, releasing the merozoites which soon invade other red cells and begin a new cycle (Figure 1 shows the life cycle of a

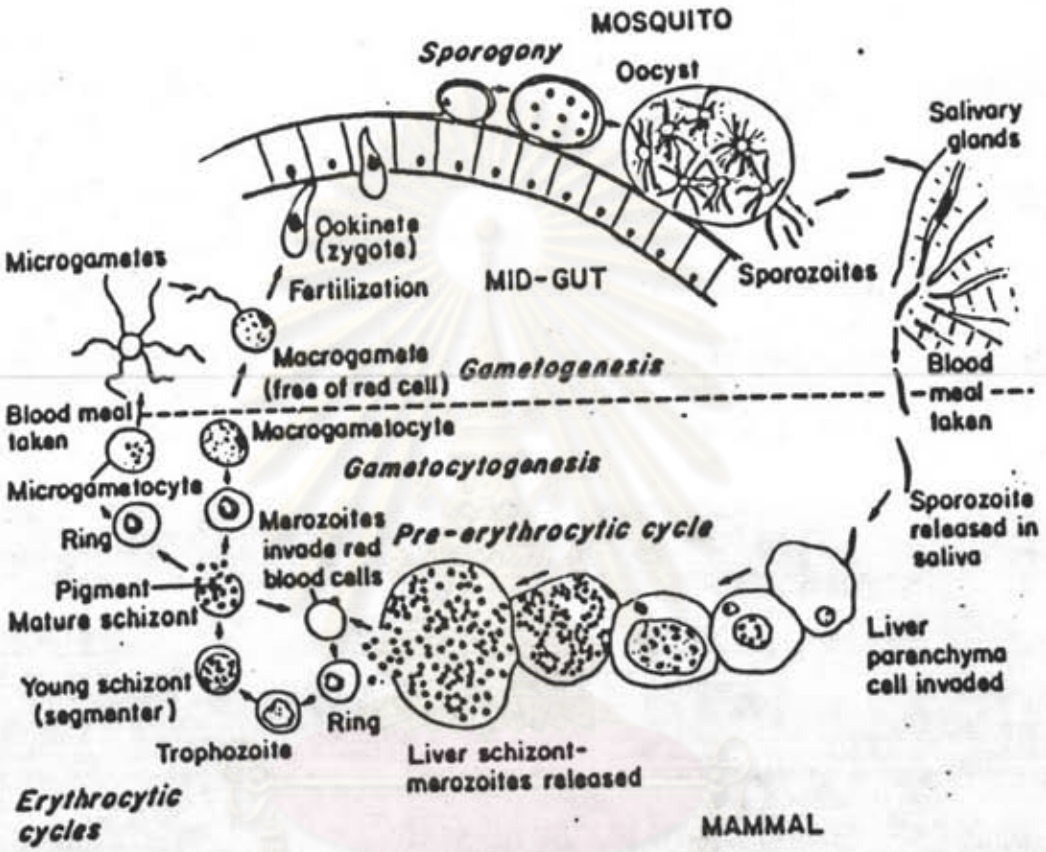

Figure 1 The life cycle of a mammalian *Plasmodium*.
(Phillips, 1983)

mammalian *Plasmodium*). The cycle of growth of human malaria parasites withih the red cells is 48 to 72 hours depending on the malaria species, and tends to be synchronous, producing periodic fevers with successive releases of the merozoites. Some parasites develop into the alternative sexual mean in the red cells to become male and female gametocytes, which are the forms infective to anopheline mosquitoes. The gametocytes are taken up with the blood meal, develop into gametes and combine sexually to form the zygote in the mosquito midgut. The zygote matures into a motile ookinete, which forces its way to the external wall of the midgut, where it forms an oocyst. The oocyst bursts after maturation, releasing thousands of spindleshaped sporozoites into the haemacoel of the mosquito. These migrate to the salivary glands, from which they are readily to inoculate into the new host.

2. <u>The immune response of the vertebrate host to malaria parasites</u>

Plasmodium infections can be broadly assigned to one of the three catagories according to the cause they take. The parasite may either (1) multiply and rapidly kill the host, (2) be quickly controlled and totally eliminated from the blood of the host, or (3) be reduced to low and usually sub-clinical levels after the acute phase of the infection in the blood but persist for long periods. Animals, including man which survive from malarial infections and show resistance to re-infection do so because they have

mounted an effective protective immune response. Where the acquired resistance is incomplete and the parasites persist, the animal is referred to as being in a stage of "premunition" and this, with respect to transmission, is better for the parasite than one in which it either rapidly kills the host or is itself rapidly eliminated. Immunity to malaria in man is acquired only after repeated exposure over several years and is mainly directed against the erythrocytic stage (Phillips, 1983).

3. Malarial chemotherapy

3.1 Type and mechanism of malarial drugs

Many drugs are available against specific stages of malaria. They can be classified into 3 groups on the basis of their modes of action (Peters and Howells, 1978). The first group comprises antimetabolites acting on the folate pathway (Ferone, 1977). As the parasite synthesizes its own folate cofactors *de novo*, it is susceptible to the action of sulpha drugs, which inhibit dihydropeteroate synthase, the enzyme catalysing the coupling of pteridine pyrophosphate, with p-aminobenzoate (Figure 2). Mammalian hosts are not susceptible to these drugs because they obtain folate from dietary sources. Both the host and parasite, however, have dihydrofolate reductase for conversion of dihydrofolate to tetrahydrofolate. Pyrimethamine and other antifolates, bind much more tightly to the plasmodial enzyme than to the mammalian enzyme, are potent antimalarials by virtue of their selective inhibition of

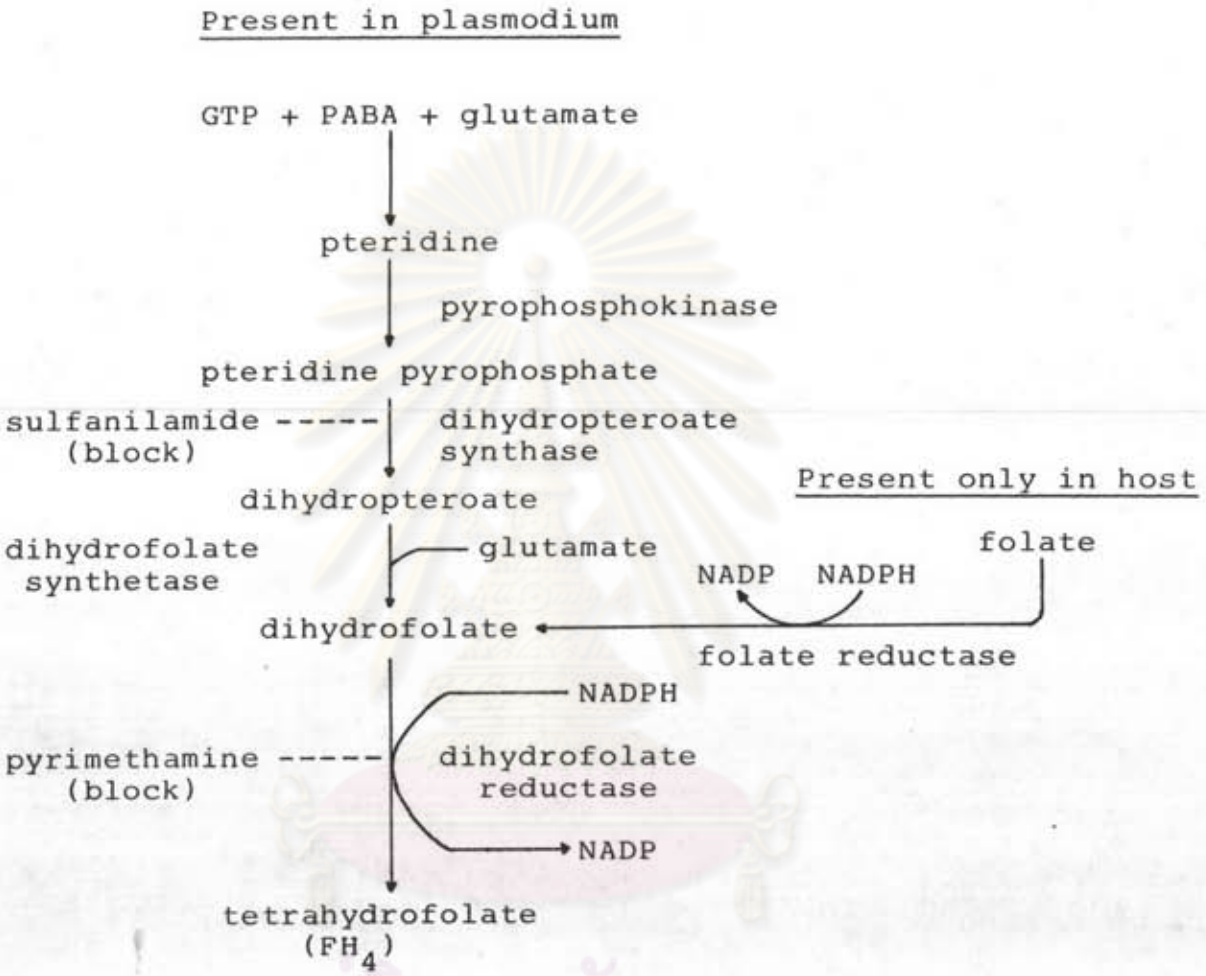

Figure 2 Metabolism of folate coenzyme in plasmodium and host (Sherman, 1979).

the parasite enzyme. Combination of sulphonamides and dihydrofolate reductase inhibitors leads to a marked potentiation effect, possibly because of their action on the two enzymes in the same metabolic pathway. The potentiating effect of pyrimethamine and sulfa drugs to dihydrofolate reductase of *P. chabaudi* was reported (Siriwaraporn and Yuthavong, 1985).

The second groups of antimalarials are 8-aminoquinolines, typified by premaquine, which are active against all stages of the malaria parasite. The third group of antimalarials are blood schizontocides comprising 4-aminoquinolines and quinoline-4-methanols, such as chloroquine, quinine and mefloquine. Besides all of the above mentioned, some antibiotics such as tetracycline can be used in theraputic of malaria by using it in combination with one of the three groups above.

4. Antimalarial drug resistance

4.1 Development of drug resistance

Although many agents that can inhibit the growth of plasmodium have been found, it still has problem in the chemotherapy of malaria because the parasites have developed resistance to certain antimalarial drug. The resistance of *P. falciparum* to chloroquine was first observed in Columbia in 1961, not long after in Brazil, and subsequently in many parts of South-East Asia (Young and Moore, 1961; Degowin and Powell, 1965; Harinasuta, et al.,

1965). In Thailand chloroquine had been used since World War II and *P. falciparum* started to show poor response to chloroquine in 1962 (Harinasuta, et al., 1962). Harinasuta (1976) and Colwell (1976) reported that chloroquine resistance in *P. falciparum* had spread throughout the country. Chloroquine was then replaced by sulfa-drug which could be used alone or in combination with pyrimethamine. The most widespreade use is Fansidar. Each tablet of Fansidar contains 500 mg sulphadoxine and 25 mg pyrimethamine. Sulphadoxine and pyrimethamine act synergistically to block enzymes in plasmodium pyrimidine synthesis (Rollo, 1955).

4.2 Pyrimethamine resistance

In 1972, Fansidar was first used in Thailand, the cure rate was 85% which was very effective (Hall, 1974). However, the cure rate of this drug to *P. falciparum* was decreased. At Malaria Clinic in Phra Phuttabat during the five past years revealed a marked drop in the cure rate 80%, 83%, 56%, 50% and 10% respectively (Harinasuta, et al., 1980). In 1979-1980, Armed Forces Research Institute of Medical Sciences (AFRIMS) studied military and civilian patients who acquired their malaria near Thai-Kampuchean border and found that only 9.1% were cured by 2 tablets of Fansidar and 19.4% by 3 tablets. It was assumed that *P. falciparum* had developed resistance to pyrimethamine. In 1980, Thaithong and Beale studied *in vitro* drug resistance tests of *P. falciparum* in Thailand and found that the majority of *P. falciparum* was resisted to pyrimethamine and was widespread throughout almost every provinces.

5. Biochemistry of pyrimethamine resistance in plasmodium

It would be interesting to study the mechanization of the action and the resistance of drug pyrimethamine including the dosage affecting the treatments. But in the study of *P. falciparum*, which is the prime cause of infection in human malaria, there are many obstacles such as the preparation of blood samples having large quantity of plasmodium by *in vitro* cultivation, also the materials, chemicals and growth ingredients used are expensive. Therefore, plasmodium which is useful in the research and study leading to the understanding of the mechanisms of action and drug resistance of human malaria, comprises of *P. berghei*, *P. yoelli*, *P. vinckei* and *P. chabaudi* existing in small rodents like mice and rats as hosts which are the cheapest and most easily maintained laboratory animals. Also the preparation of blood samples could be done in large quantity with high parasitemia as required. Besides the physiology and life cycle are close to that of human malaria. Another important reason is that this is the only group of plasmodium that could be induced to develop resistance to various type of drugs; for example, resistant to chloroquine and pyrimethamine. Walliker, et al., (1975) obtained a line of *P. chabaudi* which could resist to 19 mg of pyrimethamine/kg of body wt when treated the mice with pyrimethamine intraperitoneally for 4 days. Resistance is inherited in a stable manner through serial blood passages (Diggens, et al., 1970; Morgan, 1974; Rosario, et al., 1978).

Pyrimethamine resistance may be due to the spontaneous mutation of parasite or it is induced under the drug pressure as happened in the rodents (Rollo, 1952; Yoeli, et al., 1969; Diggens, et al., 1970).

Ferone (1969) and Diggens, et al., (1970) demonstrated that the content of dihydrofolate reductase enzyme from pyrimethamine-resistant strains *P. berghei* was higher than that in pyrimethamine-sensitive strains and the affinity of pyrimethamine to enzyme was more loosely.

Ploydanai (1982) studied pyrimethamine resistance in *P. falciparum* by *in vitro* method and found that the specific activity of dihydrofolate reductase in isolate which was resistant to pyrimethamine was higher than in isolate which was sensitive to pyrimethamine. Besides this, studies of the uptake of pyrimethamine, done by detecting the amount of ^{14}C-pyrimethamine in red blood cells, revealed that when the parasitized cells were allowed to contact with ^{14}C-pyrimethamine *in vitro* for a short period of time, the uptake of pyrimethamine in isolate which resistant to pyrimethamine was lower than that in the sensitive one significantly.

Ratanaphan and Ruenwongsa (1983) reported that the specific activity of thymidylate syntase enzyme in *P. chabaudi* which was resistant to pyrimethamine was reduced. The ability of enzyme in binding with substrate deoxyuridylate (dUMP) did not markedly change but it could bind with 5,10-methylene-tetrahydrofolate better by about two times.

Nuchadomrong (1985) studied the effects of pyrimethamine resistance on the uptake of pyrimethamine and some folate metabolizing enzymes in *P. chabaudi* and found that the uptake of pyrimethamine into red blood cells infected with pyrimethamine-resistant *P. chabaudi* was higher than that in red blood cells infected with pyrimethamine-sensitive *P. chabaudi* and in uninfected red blood cells respectively. Dihydrofolate reductase from pyrimethamine-resistant *P. chabaudi* bound to pyrimethamine less effective than that from the sensitive clone; and the affinity of enzyme from resistant clone seemed to be better than from sensitive clone. For serine hydroxymethyltransferase enzyme, there were no significant differences in the binding between enzyme and substrate, serine and THF, from both clones; and the amount of enzyme in pyrimethamine-resistant parasite was slightly greater than that in pyrimethamine-sensitive one. Another enzyme that was studied was dihydropteroate synthase. It was found that the enzyme from both sources were not different in their affinity to their substrate, PABA and DHPP; and the activity of the enzyme from pyrimethamine-resistant parasite was slightly higher than that from the sensitive one.

From the research documents complied it is evident that the mechanism in pyrimethamine resistance in plasmodium had received very little studies, particularly the process of dihydrofolate synthesis which is the specific process of the plasmodium, the process of metabolism of tetrahydrofolate, the regulation of deoxythymidylate synthesis and

in particular other mechanisms relating to metabolism of pyrimethamine both *in vitro* and *in vivo*.

6. Methods for the determination of pyrimethamine

Various analytical methods for the determination of pyrimethamine have been reported in the literatures.

Hitchings (1952) estimated the pyrimethamine content in serum and urine by using *Streptococcus fecalis* (A.T.C.C. No. 4083) as test organism and determined the content from the growth-inhibiting activity. Weidekamm, et al., (1982) developed a microbiological method for quantifying pyrimethamine which required the separation of the compounds before analysis.

The spectrophotpmetric method for determination of pyrimethamine in urine and plasma, used by Schmidt, et al., (1953) was based on extraction into an organic solvent and direct spectrophotometric measurement of the extract at 270 nm. This method lacks specificity as well as sensitivity.

In 1968, Jones and King attempted to measure pyrimethamine level in urine utilizing the weak natural fluorescence of the compound. These investigators chromatographed an extract of urine on a TLC plate and scanned the product in a specially modified spectrophotofluorometer. They reported a lower limit of measurement of 100 ng/ml for supporting clinical trials. DeAngelis, et al., (1975) reported a method for quantitation of pyrimethamine and

related diaminopyrimidines in body fluid and tissues. The ultraviolet absorption of the pyrimidine ring at 275 nm was utilized to quantitate these compounds on TLC plates with scanning instruments. The lower limit of sensitivity of 10 ng/ml plasma was achieved.

Jones, et al., (1981) developed a method for measuring pyrimethamine by using gas-liquid chromatography (GLC). The minimum detectable quantity of pure compound injected on column was 50 pg; and this allowed plasma concentrations ranging from 4-200 ng/ml to be comfortably assayed. Previous determination for pyrimethamine using GLC had lower limit of sensitivity of 100 ng/g tissue (Cala, et al., 1972). Bonine, et al., (1981) reported a GC method for the determination in blood and urine which required two extraction steps and the collection of fractions.

Jones and Ovenell (1979) and Timm and Weidekamm (1982) developed a sensitive, rapid and selective high-performance liquid chromatographic (HPLC) method to measure levels of pyrimethamine in plasma. The drug was extracted from plasma and quantified on HPLC column. The sensitivity limit was about 10 ng/ml of plasma. Recently, Edstein (1984) reported a HPLC method for the quantification of sulfadoxine and pyrimethamine which employed a single-extraction step and the limit of quantification was 5 ng/ml of plasma for pyrimethamine.

From the above mentioned, the spectrophotometric method had no specificity and low sensitivity whereas the

analysis in the TLC, GC, microbiological methods and HPLC had to employ complicated procedures and time consuming; and some methods need expensive instruments.

7. Objective

This research is aimed at the develpoment of a high sensitivity, specificity and also a simple method which could be done in short period of time in order to determine the levels of pyrimethamine in body fluids and tissues. The competitive binding assay developed was based on the binding properties of dihydrofolate reductase with pyrimethamine. The developed method was then used in the studies to monitor the turnover of the levels of pyrimethamine in plasma, red blood cells and liver of mice infected with sensitive and resistant *P. chabaudi* after receiving single dose of pyrimethamine. It is believed that the results gained would help to better understand the mechanism and the process in the infection, pyrimethamine resistance and the malaria therapy in the living creatures including human beings.

In this research, *P. chabaudi* was chosen to be a model for studies. The reason being that it has advantage in biological features which ia very close to that of the human parasite *P. falciparum*, also the parasite growth follows the pattern of synchronous schizogony which enables the planned growth according the requirements (Newbold, et al., 1982) and it could be induced high resistance to pyrimethamine (Walliker, et al., 1975).