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Early steps in folate synthesis: Targets for antimalarial chemotherapy

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City University of New York, 1992

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Early Steps in Folate Synthesis: Targets for Antimalarial Chemotherapy.

by

Yun Zhang

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1992

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

12/6/91
Date

Chair of Examining Committee

December 18, 1991
Date

Executive Officer

Supervisory Committee

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Abstract

EARLY STEPS IN FOLATE SYNTHESIS:
TARGETS FOR ANTIMALARIAL CHEMOTHERAPY

By

Yun Zhang

Adviser: Steven R. Meshnick, M.D., Ph.D.

De novo folate biosynthesis is required by malarial parasites and is the target of several antimalarial agents. Two early steps in this pathway, the synthesis of dihydropteroate and the transport of *p*-aminobenzoic acid (PAB), are studied here.

The concentrations required for 50 % inhibition (IC_{50}s) and the Michaelis-Menton inhibitory constants (K_is) of the *Plasmodium falciparum* dihydropteroate synthetase (DHPS) were determined for six sulfa drugs. These drugs inhibited the *in vitro* growth of *P. falciparum* (50 % lethal concentrations, LC_{50}s) at concentrations of 30 to 500 nM; these concentrations were 100 - 1000 times lower than their IC_{50}s and K_is (6 to 500 μM). The uptake of *p*-aminobenzoic acid was not inhibited by the sulfa drugs. However, sulfa drugs impaired total folate synthesis in malaria parasites. Furthermore, infected red cells took up more sulfamethoxazole than did uninfected red cells; the intraparasitic sulfamethoxazole concentration was estimated to be 20 times higher than that in the media. It is therefore suggested that sulfa drugs can exert their antimalarial effects at concentration 100 to 1000 times lower than they inhibit DHPS because the drugs are concentrated in parasites.

Since malaria parasites utilize exogenous PAB to synthesize folate, the transport of PAB into parasites is essential. We show that normal and parasite-infected red cells transport PAB in a saturable and energy dependent manner, with a dissociation constant of 83 nM and 111 nM, respectively. In parasite-infected red cells, PAB transport has a similar time course, affinity for PAB, energy dependence, and inhibitor sensitivity as in

normal red cells. Therefore, the same transport mechanism(s) operative in normal red cells must be rate-limiting in infected red cells. This PAB transport depends on cellular ATP (or a proton gradient) but not a sodium gradient, and is partially sensitive to both a substrate and three inhibitors of the red cell anion exchanger. We conclude that PAB transport is mediated by two independent transporters, the anion exchanger and a hitherto unknown specific transporter.

p-Aminosalicylic acid (PAS) inhibits PAB transport competitively, with an inhibition constant of 378 nM. It may be useful as an antimalarial, particularly in conjunction with other agents that inhibit *de novo* PAB synthesis.

ACKNOWLEDGEMENTS

The work described in this thesis was done in the laboratory of Dr. Steven R. Meshnick at The City University of New York Medical School. It is a pleasure to acknowledge the many people who have helped to make the accomplishment of this work and my graduate education a fruitful and gratifying experience.

First and foremost, I wish to express my deepest gratitude to my mentor and sponsor, Dr. Steve Meshnick. Through these years he has provided me with constant guidance, insight, and emotional surpport. Not only the experience of carrying out this research, but also the creative and rigorous approach to science that I have learned from him will be my resource in the years to come.

I thank Drs. John Eaton and Gary Rudnick for the many insightful and informative discussions; Drs. Thomas Haines, David Calhoun, Maria Tomasz, and Cy Bacchi for being members of my doctoral committee and for their interests, valuable criticisms, and supports which go far beyond their duties; Mark Quillan, Salim Merali, Yingzi Yang, Tor Smeland, Allen Ranz, Viera Lima, Dr. Dani McBeth, Raj Maturi, and Yvonne Francis for their friendships, much help, and the creation of an enjoyable working environment; Dr. Horst Schulz and Ms. Sophie Weintraub for their help through my graduate education.

Lastly and most specially, I thank my mother Sheng Shu-qiu, and my father Zhang Feng who sadly is no longer here to share this with me, for their love, sacrifice, and encouragement. It is their high standards and work ethic which have influenced me greatly in every thing I have done.

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LIST OF ABBREVIATIONS

CSP: circumsporozoite protein
CCCP: *m*-chlorophenylhydrazone
DDT: dichloro-diphenyl-trichloroethane
DHFS: dihydrofolate synthetase
DHFR: dihydrofolate reductase
DHPS: dihydropteroate synthetase
DITS: 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid
FPLC: fast performance liquid chromatography
IAA: iodoacetamide
IC50: concentration required to inhibit dihydropteroate synthetase by a 50%
Ki: Michaelis-Menton inhibitory constant
Kd: disassociation constant
LC50: concentration requiredto inhibited the *in vitro* growth of *P. falciparum* by a 50%
PAB: *p*-aminobenzoic acid
PABG: *p*-aminobenzoyl glutamate
PAS: *p*-aminosalicylic acid
PFF: PAB- and folate- free
PPPK: hydroxymethyldihydropterin pyrophosphokinase
PVM: parasitophorous vacuole membrane
RS: RPMI 1640 medium with 10 % serum
TCA: tricholoroacetic acid

1. INTRODUCTION

1.1 Malaria:

Malaria is still a major health threat in the world. It afflicts hundred millions of people each year, it is debilitating, and it often is life threatening.

After World War II, a worldwide campaign was launched to control malaria. Chloroquine as an antimalarial agent was proven safe and effective. DDT (dichloro-diphenyl-trichloroethane) was also developed around the same time, and was successfully used to control the invertebrate vectors of malaria parasites, anopheline mosquitoes. Early success in malaria control using chloroquine and DDT drastically reduced the number of malaria cases and led to the hope of malaria eradication (WHO Manual, 1972). However, the rapid spread of chloroquine-resistant strains of malaria parasites has shattered that hope. Since the 1960's the number of malaria cases has been increasing steadily (Bruce-Chwatt et al., 1981). Currently, it is estimated that there are 500 million malaria cases each year, with 2 million deaths, occurring mostly in Africa, South America, and Southeast Asia (Warhurst 1987). Furthermore, incidence of malaria cases in traditionally non-malarial countries has also been rising steadily, due to the introduction of infected anopheline mosquitoes via air transport and blood transfusion (Cook, 1988).

Four protozoan parasites, of the genus *Plasmodium*, are responsible for the human malaria disease. These are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Malaria caused by these different parasites have different symptoms and severities. *P. falciparum* causes an acute and potentially lethal illness. While on the other end of the spectrum, *P. malariae* produces a very chronic infection that may continue for 50 years or more without serious morbidity. Infections by *P. ovale* and *P. malariae* result in subacute morbidity.

Clinically, uncomplicated disease is characterised by fever, splenomegaly, and later anemia. Life threatening complications of *falciparum* malaria result from extensive hemolysis and from diminished microcirculation and tissue hypoxia caused by cytoadherence of parasites (Wyler and Pasvol, 1986). Women and children are particularly vulnerable to malaria infection.

1.2 *Plasmodium* parasites:

1.2.1 Life cycle

Plasmodium parasites have a sexual phase in mosquitoes (except lizard parasites which are transmitted by sandflies) and asexual cycles in red blood cells and other tissues of the vertebrate hosts. These parasites develop in a highly regulated manner through different extracellular and intracellular forms (Garnham, 1984).

The merozoite is the form of parasite which is periodically released to the blood stream of an infected host and its release is concomitant with periodic fevers and chills. Merozoites invade erythrocytes soon after they are released, and subsequently develope into intraerythrocytic asexual stages, namely, rings, trophozoites, and schizonts. Multinucleated schizonts mature and give rise to more merozoites, which then rupture infected red cells, become released into the bloodstream again, and infect more red blood cells (Figure 1.1). This asexual erythrocytic cycle can continue many times until the host succumbs or until parasites are destroyed by the immune system, alone or with aide of chemotherapy. In the case of *P. falciparum*, this erythrocytic cycle can also be indefinitely sustained *in vitro*, which provides a great convenience for research.

In addition to breaking into the bloodstream and invading more red cells, newly formed merozoites can also develop intracellularly into male or female gametocytes, which

if ingested by mosquitoes, change into male and female gametes. During the sexual phase, which occurs in mosquitoes, gametes unite to form zygotes, which then develop into oocysts. In a process called sporogony, each oocyst gives rise to 10,000- 20,000 sporozoites. Sporozoites are injected into the host during a blood meal, invade liver cells, and develop into tissue schizonts within hepatocytes. Each tissue schizont then devides to form at least 10,000 merozoites, which start the erythrocytic cycle upon being released to bloodstream from the liver.

1.2.2 Culture systems

Attempts have been made, with varying degrees of success, to culture all stages of *P. falciparum* and some stages of other species. As mentioned above, *in vitro* cultivation of the asexual erythrocytic stages of *P. falciparum* has been most successful and is widely used (Trager and Jensen, 1976). Production of gametocytes and ookinetes during cultivation of the erythrocytic stages of *P. falciparum* has been possible (Ponnudurai et al., 1982). The complete hepatic cycles of *P. vivax* and *P. falciparum* were also accomplished, where sporozoites introduced into cultures of human hepatocytes invaded the liver cells, developed to maturity, and released merozoites infective for red cells (Mazier et al., 1984 and 1985). Most recently, extracellular development from merozoites and rings of *P. falciparum* has been reported (Trager et al., 1990), so these parasites might be able to develop in the absence of host cells if proper conditions are provided.

In addition to the culture systems for different stages in the life cycle of the *plasmodium* parasites, the whole life cycle of *P. falciparum* can be maintained in animal models. Classical genetic crosses have been accomplished (Wellems et al., 1991).

1.2.3 Biology of *plasmodium* parasites: current interests

Their unique parasitic life style makes *plasmodium* parasites attractive organisms for addressing many fundamental questions, such as how parasites invade and develop in host cells and how the development through different stages in their life cycle is regulated. Due to the tremendous public health threat of malaria, research efforts have been mostly oriented toward the control of malaria. However, the link and mutual benefit between such efforts and the understanding of the molecular mechanisms of the organism can not be underestimated.

Merozoite and sporozoite are the two extracellular forms which invade host erythrocytes and hepatic cells, respectively. Knowledge has been gained about the ultrastructures, stage-specific antigens, host proteins involved in their attachment and invasion, as well as the effects of antibodies against both parasite antigens and host proteins on invasion (Breuer, 1985; Howard, 1986; Pasvol, 1984). The incentive underlying this research is clearly its potential application in both chemotherapy and vaccine development. Interuption of any of the invasion steps would block the development of parasites; the extracellular forms of parasite are good targets for vaccine development, because they are more exposed to immunological attack as compared to intracellular stages, which are sheltered by host cells.

Molecular biological approaches have been used mostly to isolate and characterize malaria antigenic determinants which are potentially useful for vaccine developement (Kemp et al., 1987). Best studied *plasmodium* genes include that for circumsporozoite protein (CSP), a major sporozoite antigen; S-antigen, a soluble heat-stable protein released by parasite infected erythrocytes; merozoite surface antigens; and histidine-rich proteins which are a family of proteins involved in the knoblike protrusions as well as variety of other structures and functions (Sharma and Godson, 1985; Wellems et al., 1986). In the

process of these studies, light has been shed on the plasticity of *plasmodium* genome and the evolution of these parasites (Pologe and Ravetch, 1986; McCutchan et al., 1984).

The development of anti-malarial vaccines has been attempted (Miller et al., 1986; Nussenzweig et al., 1984; Charoenvit et al., 1991). Malaria parasites, however, are particularly shrewd in eluding host defenses. Immunity against malaria can be produced and maintained only by frequent immunological boosting with live parasites (Playfair, 1982). Furthermore, malaria antigens are species, stage, and even strain specific (Howard, 1984). All these pose serious difficulties for vaccine developement. It is controversial as to when and if malaria vaccine would be possible.

While the search for malaria vaccines continues, chemotherapy is still the method of choice for malaria control. Some of the current interest in this area will be discussed in the next section.

1.3 Malaria Chemotherapy:

Antimalarial drugs are traditionally classified either according to their biological targets, namely, the specific stage in the malarial developmental cycle inhibited by the drugs (Bruce-Chwatt et al., 1981), or according to their chemical structure (Warhurst, 1987). Since the focus of this thesis is to understand mechanism of action of sulfa drugs and to explore new therapeutic options targeted at the folate synthesis pathway, a brief review of mechanisms of action of a few most important anti-malarial drugs is relevant.

1.3.1 Quinine, quinidine, and mefloquine.

Quinine is the active ingredient of cinchona bark, which has been used by Indians for centuries and offered them protection from malaria. Quinidine is the D-epimer of quinine

while mefloquine is a synthetic analog (Fig.1.2a). These compounds are all blood schizontocides, that is, they inhibit the growth of intracellular parasites in erythrocytic cycle.

There is evidence that both quinine and mefloquine form complexes with hemin in parasites (Fitch, 1986; Warhurst, 1981). Because malaria parasites have unusually large amount of hemozoin, a polymer of hemin, this binding between drugs and hemin can explain the concentration of the drugs in parasites and their selective toxicities. The hemin-quinine complex, however, is not toxic to parasites. Quinine, but not mefloquine, intercalates into DNA (Estensen et al., 1969; Davidson et al., 1977), which may or may not be the mechanism of action of the drug.

Both quinine and mefloquine resistance have been reported (Malin et al, 1990; Boudreau et al.,1982). Mefloquine resistance has been shown to correlate with the amplification of pf-mdr gene, a member of multi drug resistance gene family which was first identified in multi drug resistant cancer cells (Wilson et al., 1991).

1.3.2 Primaquine

Primaquine, or 8-aminoquinoline (Fig.1.2 a), has acitivity against all stages of the parasites in their life cycle. It is particularly active against gametocytes and liver stages.

Primaquine is demethylated and hydroxylated to several highly redox-active compounds in liver (Strother et al., 1984). One such metabolite, 5-hydroxyl-6-demethyl primaquine, is 20 times more active than primaquine. Since the antimalarial activities of these metabolites correlate with their abilities of generating superoxide, the mechanism of action of these compounds may be that they cause oxidative damage (Bates et al., 1990).

1.3.3 Chloroquine

Chloroquine, or 4-aminoquinoline, as mentioned earlier, was once the most effective antimalarial agent. It is a chemical modification of quinine (Fig.1.2 a). Choloroquine is also a blood schizontocide. The action of chloroquine is characterized by rapid onset, long serum half-life, and minimum side effects.

Chloroquine, as a anti-malarial agent, has been the subject of extensive research effort. There are still controversies, however, regarding the mechanism of action of chloroquine. DNA, lysosome, and membrane have all been suggested as the targets of chloroquine action (reviewed by Meshnick, 1990).

The theory that chloroquine acts by interfering with DNA metabolism originated from early observations that it binds to DNA. It has recently been shown that chloroquine intercalates into DNA, and the binding affinity is highly salt-dependent and sequence-dependent (Kwakye-Berko and Meshnick, 1989 and 1990). Under physiological conditions, the K_d is in the millimolar range. Despite the apparently low affinity, however, the binding of chloroquine to DNA could be significant and damaging because of the following two reasons: 1) it is sequence dependent and 2) at therapeutic concentrations of the drug, 0.03-1.0 % of intercalating sites would be occupied (Meshnick, 1990), which is a significant number when we consider the total number of intercalating sites in a malarial genome.

The lysosome accumulation hypothesis suggests that chloroquine impaires lysosomal functions of parasites. It is postulated that the unprotonated form of the drug can penetrate membranes by diffusion. Once inside lysosomes of parasites, however, chloroquine gets protonated because of the low pH, losses the ability to diffuse out, and becomes trapped inside lysosomes. The presence of large amount of chloroquine subsequently raises the pH

of lysosomes and impairs their functions (Meshnick, 1990). The selective concentration of chloroquine in parasite lysosomes has been demostrated (Aikawa, 1972).

Finally, it has also been proposed that chloroquine can form a complex with hemin and this is the cause of its toxic effect. Evidence supporting this hypothesis include that chloroquine treatment causes clumping of hemozoin (McChesney and Fitch, 1984); chloroquine-hemin complex is lytic to red cells (Fitch, 1986); and certain malaria strain lacking hemozoin is chloroquine-resistant (McChesney and Fitch, 1984).

Chloroquine resistant malaria strains are now common in most of the malaria endemic areas (Peters, 1987). Chloroquine resistance correlates with an increased efflux of the drug. It was proposed that this efflux is mediated by a pump homologous to the mammalian p-glycoprotein coded by the mdr (multidrug resistance) gene because: 1) the chloroquine efflux in malaria parasite infected red cells, like the drug efflux in multi-drug resistant mammalian cells, is sensitive to verapamil and other Ca^{2+} channel blockers (Krogstad et al., 1987), and 2) increased expression of the pfmdr 1 gene correlates with chloroquine resistance (Foote et al., 1989). However, by genetic crosses, the chloquine resistance gene was mapped to a locus on chomosome 7, which is not a mdr locus (Wellems et al., 1991). A current view on chloroquine resistance integrating both of the above data is that at least two genes are involved in chloroquine resistance, one is the mdr gene while the other is at the chloroquine resistance locus and is yet to be identified (Foote et al., 1991).

1.3.4 Qinghaosu

Qinghaosu (artemisinin) is a novel antimalarial drug developed from a traditional Chinese herbal remedy qinghao. *In vitro* sensitivity studies and clinical trials have shown

its effectiveness and quick action against both choloroquine-sensitive and -resistant strains of *P. falciparum* (Gu et al. 1983; li et al. 1982; Jiang et al. 1982).

Generation of free radicals and interuption of membrane structure have been correlated to the antimalarial action of qinghaosu. Three lines of evidence suggest the involvement of free radicals in the qinghaosu action: qinghaosu causes lipid peroxidation (Meshnick et. al., 1989); antioxidants antagonize the effect of qinghaosu (Meshnick et. al., 1989; Krungkrai et al., 1987); and endoperoxide linkage is required for the antimalaria activity of qinghaosu (Gu et al., 1980). Most recently, it has been shown that qinghaosu binds to hemin, and this may mediate the generation of free radicals (Meshnick et al., 1991).

Qinghaosu also causes structural changes in membranes of the ER and mitochondria and localizes in membranes (Ellis et al., 1985). This lead to the hypothesis that qinghaosu may act primarily by altering membrane integrity. But up to 40% of qinghaosu was found not to effect temperature or entropy of the phase transition in reconstituted membranes, suggesting qinghaosu does not interupt the lipid structure of membranes (Browning et al., 1989).

Qinghaosu-resistance, while it has never been seen clinically, is potentially a problem. Strains resistant to qinghaosu have been developed in the laboratory by growing malaria under sub-lethal levels of the drug (Hubbert et al. 1989) and have also been isolated from mutagen-treated populations (Inselberg, 1985).

1.3.5 Antifolates

Antifolates are inhibitors of enzymes in the malaria folate synthesis pathway. They are important antimalarials, especially for chloroquine resistant strains. The mechanism of

action involves tetrahydrofolate deprivation in malaria, but much experimental data remains unexplained. These will be discussed in detail in the folate synthesis section.

1.3.6 Other antimalarial agents

There are other antimalarial drugs the mechanism of action of which are less known. Some metal chelators, such as desferrioxamine which may deprive parasites of iron, and diethyldithiocarbamate, which may form a toxic complex with copper, have antimalarial activity. Naphthoquinones, which inhibit mammalian electron transport, may exert their antimalarial activity by inhibiting an electron transport-dependent enzyme, dihydrooratate oxidase (reviewed by Meshnick and Marr, 1990).

1.3.7 Perspectives of anti-malarial chemotherapy

Because of the spread of chloroquine-resistant malaria strains and problems associated with most other antimalarials, new antimalarial drugs are urgently needed (Panisko and Keystone, 1990). In developing new antimalarials, two types of research are most helpful. One is to understand mechanisms of actions of available drugs. This can aid in the rational devolopment of new and more effective drugs by chemical modification. In addition, a true understanding of the mode of action of an effective drug can often reveal some biological characteristics of parasites, which in long term, would also certainly help with the search for new drugs. The second type of research is to study metabolic pathways or processes unique to parasites. In order to be selectively toxic to parasites, a drug has to disrupt this type of pathway. Inhibitors of such biological processes are, therefore, good candidates for drug design.

As will be dicussed in Section 1.4, folate synthesis is a pathway unique to malaria parasites. Antifolates, inhibitors of this pathway, are important antimalarials. Despite the

extensive research about the pathway and about the mechanisms of action of antifolates, there are still many unknowns in this area. A better understanding of this pathway is important for the development of new antimalarials.

Finally, there is a general lack of chemotherapeutic options for infections caused by prozozoan parasites. Parasitic protozoa such as *Toxoplasma gondii*, and *Cryptosporidium* are emerging as major opportunistic infections in those infected with HIV and in those with other immunosuppressive conditions (Piot et al., 1988). Progress in malaria chemotherapy would provide useful information for the control of parasitic diseases in general.

1.4 Folate synthesis in malarial parasites:

De novo synthesis of folate is a metabolic pathway unique to microorganisms. Inhibitors of this pathway have been successfully used in malarial chemotherapy. It is therefore important to understand the folate synthesis in malaria.

1.4.1 Tetrahydrofolate and its synthesis in mammalian cells

Tetrahydrofolate and its derivatives are active forms of folate cofactors required by both microorganisms and mammalian cells. For the sake of comparison, it is useful to firstly look at how they are utilized as well as how they are synthesized in mammalian cells.

Tetrahydrofolate is composed of three domains, a substituted pterin, *p*-aminobenzoic acid, and glutamate (Fig. 1.3). Its derivatives often have one carbon unit bound to N_5, N_{10}, or both of the two positions (Fig. 1.3), and are used as coenzymes for a variety of enzymes in one carbon metabolism. In biosynthetic reactions, the one carbon units can be transfered to acceptor molecules. An example of this is the synthesis of deoxythymidylate, where the methyl group in 5,10-methylene-tetrahydrofolate is transfered to deoxyuridylate.

Tetrahydrofolate, on the other hand, can accept one carbon unit from degradative reactions to regenerate these one carbon unit-carrying tetrahydrofolate derivatives. The one carbon units carried by tetrahydrofolate derivatives have different oxidation states. These different tetrahydrofolates can be interconverted. Reducing power is consumed in the forms of NADPH and NADH and stored in tetrahydrofolates when they are converted to more reduced forms (Fig.1.3).

Folic acid was firstly found in spinach and other "foliage", and was known to be an essential nutritional requirement for man and other mammals. Accordingly, there are folate transport systems in most mammalian cell types (Antony et al., 1989; Henderson et al., 1988). After being transported into mammalian cells, folate is first reduced to dihydrofolate and then to tetrahydrofolate by dihydrofolate reductase. Inhibitors of dihydrofolate reductase, such as methotrexate (4-amino-10-methyl-ptroylglutamic acid), have been used as anticancer agents (reviewed by Blakley, 1984). This is because neoplastic cells have higher metabolic rate and are therefore more susceptible to a shortage of tetrahydrofolate coenzymes.

1.4.2 *De novo* folate synthesis in malaria parasites

Malaria parasites require tetrahydrofolates in a similar manner as do mammalian cells. However, they can not use exogenous folates, as evidenced by facts such as that a folate transport system has not been reported, an enzymatic activity which reduces folate to dihydrofolate could not be found (Ferone, 1977), and most importantly, the inhibition of the *de novo* folate synthesis is detrimental for malaria, as will be discussed in the next section. Malaria parasites depend on their *de novo* folate synthesis pathway.

In the folate synthesis pathway, 6-hydroxymethyl pterin is first activated by pyrophosphorylation (hydroxymethyldihydropterin pyrophosphokinase, or PPPK), and

then condensed with *p*-aminobenzoic acid (dihydropteroate synthetase, or DHPS). Dyhydrofolate synthetase (DHFS) adds a glutamate to dihydropteroate to give rise to dihydrofolate. Finally, dihydrofolate reductase (DHFR) reduces the dihydrofolate to tetrahydrofolate (Fig. 1.4). In all the cell types examined, functional intracellular tetrahydrofolates are in polyglutamated forms. A single pteroylpolyglutamate synthetase can add a number of glutamates sequentially to the newly synthesized tetrahydrofolates (reviewed by McGuire and Coward, 1984). In *P. falciparum* infected red cells, 5-methyl-tetrahydropteroylpentaglutamate was shown to be synthesized *de novo* (Krungkrai, 1989), suggesting the existence of a pteroylpolyglutamate synthetase, although it has not yet been identified.

What are the sources of 6-hydroxymethylpterin and PAB used for the folate synthesis? In both microorganisms and mammalian cells, pteridine ring can be synthesized from GTP and there are enzymes to synthesize different kinds of pterins (2-amino-4-hydroxypteridines) from a pteridine ring (reviewed by Brown, 1984) (in addition to being precursor of folate synthesis in microorganisms, pterins themselves are also cofactors for a variety of enzymes). A malaria GTP cyclohydrolase, one of the enzymes needed in order to synthesize pteridine from GTP, has been identified and characterized (Krungkrai, 1985), suggesting the existence of a similar pathway in malaria.

The source of PAB for the folate synthesis in malaria is at least partially extracellular. PAB stimulates parasite growth both *in vitro* and *in vivo* (reviewed by Ferone, 1977). A PAB-deficient milk diet inhibited the growth of *P. berghei* (murine malaria), *P. cynomolgi* (simian malaria), and *P. falciparum in vivo*, an effect which was reversed by PAB supplementation (Hawking et al., 1954; Jacobs et al., 1964; Kretschmar et al., 1973). PAB is required by *P. knowlesi* (simian malaria) *in vitro* (Anfinsen et al., 1946), although *P. falciparum* appears to grow in its absence (Divo et al., 1985). Furthermore, as

will be discussed in 1.4.4, PAB antagonizes the effects of antifolates for all the malarial parasites studied, including *P. falciparum* (Anfinsen et al., 1946; Wakins et al., 1985; Brockelman et al., 1982). These observations indicate clearly that malaria parasites utilize extracellular PAB.

In addition to utilizing extracellular PAB, however, malaria parasites may also be capable of *de novo* PAB synthesis. Four enzymetic activities in such a pathway were identified in *P. falciparum* (Dieckmann et al., 1986). The relative importance of each source of PAB for malaria parasites remains to be determined.

1.4.3 Inhibitors of malarial folate synthesis

The enzymes in the first part of the folate synthesis pathway, PPPK, DHPS, and DHFS, are absent in mammalian cells. Inhibitors of these enzymes, therefore, could be selectively toxic to parasites. Sulfa drugs, which include sulfones and sulfonamides (Fig.1.2b), have been shown to inhibit DHPS from the murine malaria parasites *Plasmodium berghei* (Ferone et al., 1973; McCullough et al., 1974) and *Plasmodium chabaudi* (Walter et al., 1980). *In vitro*, sulfadoxine and and dapsone have been shown to inhibit *P. falciparum* growth (Chulay et al., 1984; Scott et al., 1987). The relative potencies of various sulfa drugs, either in terms of DHPS inhibition or their effectiveness in preventing parasite growth, however, was not known prior to studies described in chapter 3.

Inhibitors of PPPK and DHFS should have similar effects on parasites as do sulfa drugs. However, these enzymes from malaria have not been studied. In both *Streptococcus pneumoniae* and *Escherochia coli*, PPPK and the bifunctional enzyme folylpolyglutamate synthetase-dihydrofolate synthetase have been identified and

characterized (Lopez et al., 1990; Kimlova et al., 1991). No effective inhibitors of these enzymes have been found.

The next enzyme in the folate synthesis pathway, DHFR, is present in both malaria and their mammalian host cells. But the malarial DHFR differs from the mammalian enzyme in three ways. Firstly, malarial DHFR, like all the protozoan DHFR studied, is a bifunctional enzyme which also has thymidylate synthetase activity, while the mammalian thymidylate synthetase is a separate enzyme. Secondly, the malaria enzyme is not like the mammalian enzyme in that it can not use folate as a substrate. Thirdly, drugs such as pyrimethamine (2,4-diamino-10-methyl- pteroylglutamic acid) and cycloguanil (4,6-diamino-1-(p-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine, activated form of chloroguanide) (Fig.1.2 a), can inhibit the parasite DHFR selectively (Ferone et al., 1969). *In vitro* , pyrimethamine and chloroguanide (the pre-drug of cycloguanil) have been shown to inhibit parasite growth in *P. falciparum* and to act synergistically with sulfa drugs (Chulay et al., 1984; Scott et al., 1987).

Clinically used antifolate antimalarials include fansidar (sulfadoxine and pyrimethamine) and maloprim (pyromethamine/dapsone) (Warhurst, 1987). Unfortunately, fansidar is associated with toxic epidermal necrolysis (Stevens-Johnson syndrome) (Stitt et al., 1988) and maloprim with bone-marrow depression (Cook, 1986). Furthermore, fansidar-resistant strains have been isolated in Southeast Asia and Africa (Dieckmann et al., 1986).

1.4.4 Mechanisms of action of antifolates in malaria

As discussed in previous sections, malaria parasites require tetrahydrofolate and its derivatives as cofactors; there is a *de novo* folate synthesis pathway in malaria which synthesize tetrahydrofolate from 6-hydroxymethyl pterin, PAB, and glutamate; two types

of antifolates, which inhibit DHPS and DHFR respectively, inhibit parasite growth both *in vitro* and *in vivo*. Based on these and other observations, a theory for the mechanism of action of antifolates emerged that antifolates act by depriving cells of tetrahydrofolates. This general scheme is clearly supported by a large amount of experimental data, although there are contradictory observations which are currently not explained. For sulfa drugs, effort was also made to elucidate if sulfa drugs themselves or their metabolites are primary inhibitors.

First of all, it is a consistent observation that DHPS inhibitors and DHFR inhibitors are synergistic in inhibiting malaria parasite growth. In general, two drugs inhibiting independent targets in a cell are additive while two drugs inhibiting different steps in a single metabolic pathway are synergistic. Therefore, the manner by which malaria parasites are inhibited by the combination of these types of antifolates suggests that the two targets are in the same pathway and is consistent with the theory that DHPS and DHFR are the primary targets for these antifolates.

An important observation made on the inhibition of the malaria parasite by antifolates is that the inhibition can be reversed by both PAB and folate. This effect of PAB is to be expected; inhibitors of DHPS, such as sulfonamides, structurally resemble PAB (Fig.1.2b). They have been shown in *P. berghei* and *P. chabaudi* to inhibit DHPS by competing with PAB for the binding site (McCullough et al., 1974; Walter et al., 1980). A excess of PAB can therefore reverse the inhibition by having a greater access for the binding site. Inhibitors of DHFR, similarly, mimic dihydrofolate and compete with it at DHFR (Ferone et al., 1969). An excess of PAB would result in a increased production of dihydrofolate, which in turn, would weaken the inhibitions by DHPS inhibitors.

The observation that folate reverses the inhibition of parasite growth by antifolates (Brockelmann et al., 1982; Watkins et al., 1985), however, is puzzling because it is

believed that malaria parasites can not utilize exogenous folate. In order to understand the role played by exogenous folate, Rollo et al. (1955) carried out a kinetic study of the antagonism of sulfonamide inhibition by folate and discovered that folate was competive with sulfonamide. This suggested that folate did not antagonize the inhibition of sulfonamides by bypassing the folate synthesis pathway. It was subsequently proposed that the decomposition product of folate, *p*-aminobenzoyl glutamate (PABG) (Fig. 1.3), is an alternative substrate for DHPS and is responsible for the antagonizing effect of folate.

Evidence disputing the role of PABG came from a study of the effects of both folate and PABG on the apparent LC_{50} of sulfadoxine (the concentration inhibiting parasite growth by 50 %). It was found that folate increased the LC_{50} of sulfadoxine to a much greater extend than did PABG (Watkins, 1985). Therefore, the effect of folate can not be due to its decomposition product PABG. Subsequently, it was suggested that malaria parasites may possess a folate salvage pathway (Watkins et al., 1985; Krungkrai et al., 1989), although why antifolates are effective inhibiting parasite growth was not explained.

Could metabolites of sulfa drugs be more toxic than sulfa drugs themselves? Dieckmann and Jung (1986) showed in *P. falciparum* that sulfa drugs are metabolized to pteroate analogs, as had been shown previously in *E. coli* (Roland et al., 1979). However, these pteroate analogs are unlikely to be responsible for the inhibiting effects of sulfa drugs, because they do not inhibit thymidylate synthesis, N_5, N_{10} methylenetetrahydrofolate dehydrogenase, N_5, N_{10}-methylenetetrahydrofolate cyclohydrolase, or dihydrofolate reductase, enzymes which use substrates with similar structures as the pteroate analogs in *E. coli*. High concentrations of these compounds did inhibite DHPS and DHFS, but only at concentrations much higher than obtainable (Roland et al., 1979).

1.4.5 Sulfa drug targets other than the folate synthesis pathway

In *Streptococcus pneumoniae* and *Leishmania major*, targets other than the folate synthesis pathway have been suggested for sulfa drugs. In *Streptococcus pneumoniae*, a chromosomal DHPS gene was cloned from a sulfonamide-resistant strain and was found to have a six base pair deletion as compared to the wild type gene. Transformation by a plasmid carrying this gene made a sulfa drug-sensitive strain of *S. pneumoniae* resistant to sulfa drugs. However, the enzymatic properties of the mutant protein was identical with the wild type protein in terms of K_ms, K_is, and substrate specificity (Lopez et al., 1987). It was therefore proposed that the target of sulfa drugs is not dihydropteroate synthetase activity. The target would have to, however, depend on at least a subunit coded by the gene. It is currently not known if this gene is involved in any function other than dihydropteroate synthesis.

In *Leishmania major* it was shown that the inhibition by sulfa drugs can be reversed by neither PAB nor folate. Furthermore, sulfa drugs were additive instead of synergistic with methotrexate, a DHFR inhibitor (Peixoto et al., 1987). This also suggests a sulfa drug target other than the folate synthesis pathway. It needs to be pointed out, however, that this suggestion does not contradict the notion that DHPS is the sulfa drug target in malaria. While sulfa drugs inhibit *Leishmania major* growth in the micromoler concentration range, they inhibit the gowth of *Plasmodium falciparum* in the nanomoler concentration range. So even if the sulfa drug target in *Leishmania* is also a sulfa drug target in malaria, it is unlikely to be the primary target.

1.5 Erythrocytes as host cells for malarial parasites:

As discussed in 1.2, during the erythrocytic cycle, malaria parasites develop inside red blood cells. Many red cell functions are vital for the intracellular parasites and their interuption, from a chemotherapeutic point of view, can be detrimental for parasites.

It is useful to first look at normal physiological functions of red cells: Red cells are terminally differentiated cells, with their nuclei and other subcellular organelles excluded, and corresponding functions lost. The key function of red blood cells is to transport oxygen and carbon dioxide. Accordingly, red cells are rich in hemoglobin, which binds oxygen, and possess in large quantity an anion exchanger, which rapidly exchanges bicarbonate and chloride anions to facilitate CO_2 entry into the blood in the systemic capillaries and CO_2 excretion in the pulmonary capillaries. The major metabolic pathways that persist in the red cells after the loss of their other metabolic systems are the glycolytic and phosphogluconate pathways, which together meet the energy needs and reducing requirements of the cell.

Red cells must be particularly suitable to host *plasmodium* parasites, because *plasmodium* parasites, unlike some other protozoan parasites such as *Toxoplasma* or *Leishmania* , have a very strict host cell specificity - they can not grow in cells other than red cells. Red cell structures and functions important for malaria parasites may include hemoglobin, cytoskeleton structure, and ATP and NADPH generating abilities. These were suggested by observations that parasites do not develop well in variant red cells such as sickle and thalassemic red cells, which have abnormal hemoglobins, ovalocytosis, spherocytosis, and elliptocytosis red cells, which have defective cytoskeletal structures, and glucose-6-phosphate dehydrogenase-deficient red cells, which have impaired NADPH generating ability (Friedman et al., 1978, 1980; Schulman et al., 1990; Roth et al., 1978).

Malarial parasites not only adapt themselves to the host cell's physiological environment, but also modify the functions of host cells to facilitate their own development. After being invaded by parasites, red cells undergo rearrangement in their cytoskeletal structures, incorporate parasite proteins in their membrane (Coppel et al., 1986) and cytosol (Etzion and Perkins, 1989; Li et al., 1991), and acquire an altered permeability to many metabolites and ions (Cabantchik, 1989).

One important role red cells have to play as the host cells for malaria parasites is allowing the entry of nutrients into the parasites from the extracellular medium. This function of red cells is particularly relevant to the work in this thesis because of our interest in extracellular folate precursors. Their transport into parasites represents the first step in malaria folate synthesis. Some known transport systems in normal and malaria parasite-infected red cells will be discussed in the next two sections.

1.6 Transport in erythrocytes:

To meet their metabolic needs and to fulfill their systemic functions, red cells have transporters for a variety of substrates. Among these are the anion exchanger, which enables rapid chloride/bicarbonate exchange as well as the transport of other inorganic and organic anions; the glucose carrier, which supplies red cells with glucose as an energy source; a monocarboxylate transporter, which transport lactate and pyruvate; a nucleoside transporter, which allows the salvage of purine nucleosides; an inorganic phosphate transporter; and several amino acid transporters. Three best studied of these red cell transporters are discussed here with emphasis on their transport properties.

1.6.1 The anion exchanger

The rapid chloride-bicarbonate exchange in red cells is carried out by the anion exchanger, or band 3 (named after its postion in gel electrophoresis of red cell membrane proteins, Fairbank et al., 1971). Band 3 is a major membrane protein of red cells that constitutes nearly 25 % of the total membrane protein. The protein consists of two structurally distinct domains. The N-terminal is a water soluble, cytoplasmic domain that contains binding sites for ankyrin (Bennett et al., 1980) and possibly glycolytic enzymes (Harris et al., 1989; Maretzki et al., 1989). The other domain is hydrophobic membrane domain which functions as a transport pathway.

The transport properties of band 3, such as substrate specificity, kinetics, and inhibition by different reagents, have been extensively studied and reviewed (Passow, 1986). In brief, band 3 catalyzes the rapid exchange of chloride and bicarbonate anions. In addition, it also mediates the exchanges of a variety of other inorganic and organic anions, but with very different rates (Jennings, 1989). The transport event is believed to have ping-pong kinetics and is sensitive to pH, surface charge effects, "spectator anions" (non-translocated anions which interfere with binding or translocation of substrate anions), substrate inhibition, and etc. Furthermore, the dependence of the anion exchanger on the above factors vary from substrate to substrate. The transport event does not require metabolic energy. ATP is required, however, because band 3 must be phosphorylated to function (Bursaux, 1984).

Various reagents are known to inhibit anion exchange mediated by band 3. These include phloretin, flufanamic acid and 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid (DITS), which inhibit band 3 by binding to different binding sites.

There are various models proposed for the transport event mediated by band 3 (Jennings, 1989). These can be devided into two categories: "thick barrier" and "thin barrier". The "thick barrier" theory, also called the molecular zipper model, postulates that

the transport pathway consists of a set of transmembrane helices containing arrays of paired charges. A transport event starts when an anion replaces a protein-bound negative charges that had been paired with a positive charge. The anion continues to replace the next protein-bond negative charge as it is relayed from one side of the membrane to the other across the thick permeability barrier. Such a model is consistent with a protein structure consisting of hydrophobic transmembrane helices with a few paired charges in the center. It also accouts for the observed ping-pong kinetics (Jennings et al., 1982).

The "thin barrier" theory postulates that most of the transport pathway is hydrophilic except a thin permeability barrier in the center of the pathway. Anions can diffuse through the hydrophilic channel to where the barrier is. Anion translocation then takes place when the anion exchange position with the barrier. This model is consistent with the observation that the binding site of stilbenedisulfonate, a band 3 inhibitor which binds to substrate binding site, is in a cavity that extends some distance from the outer surface of the membrane (Macara et al., 1983).

1.6.2 Glucose carrier

Glucose is the major energy source for red cells. Red cells have a glucose carrier which transport glucose by a mechanism of facilitated diffusion. This red cell glucose carrier is a membrane glycoprotein with intracellularly located N- and C-termini, and 12 membrane spanning domains.

As typical for facilitated diffusion, this transport process is driven by glucose concentration gradient and is energy-independent (Wheeler, 1985). The glucose carrier is stereo-specific for D-glucose and can not transport L-glucose. The equilibrium exchange K_m for D-glucose is in the range of 10-60 mM (Sogin et al., 1978; Gould et al., 1991). In addition to glucose, the glucose carrier can also efficiently transport mannose, galactose,

and a glucose analog 3-o-methyl-D-glucose, which has been widely used because it is non-metabolizable. The transports of all these substrates are competitive, suggesting a shared binding site and transport mechanism. The glucose carrier has low affinities for most other hexoses.

The transport of glucose can be inhibited by cytochalasin B, a fungal metabolite which bind competitively to the substrate binding site (Kasahara et al., 1977) and forskolin, a diterpene activator of adenylate cyclase which also bind to the substrate binding site and whose effect on glucose transport is independent of its effect on adenylate cyclase (Lavis et al., 1987).

There are two major models proposed for the mechanism of glucose transport: the carrier model and the alternating comformation model. In the carrier model, it is envisioned that the protein moves from one side of the membrane to the other; while in the alternating conformation model, it is suggested that a substrate binding site is alternately exposed to the two sides of the membrane.

1.6.3 Monocarboxylate transporter

Monocarboxylates such as lactate and pyruvate can also cross the membrane of red cells. It was believed for years that they cross the membrane by simple diffusion of undissociated lactic and pyruvic acid. More recently, however, it is shown that L-lactate is transported by three different mechanisms. Non-ionic diffusion accounts for 5% of total lactate movement across the red cell membrane, transport by band 3 accounts for another 5 % of total lactate transport, and finally and most importantly, a specific monocarboxylate transport system is responsible for the 90 % of the lactate movement across the red cells membrane (reviewed by Deuticke, 1982). This transporter is believed to be a proton symport system (DeBruijne et al., 1983; Deuticke et al., 1981). In contrast to the anion

exchanger, this transporter is inhibited reversibly by impermeable and permeable SH-reagents of the mercurial ($HgCl_2$, p-chlormercuribenzene sulfonate) and the dithiol (4,4'-dithiodipyridine, 5,5'-dithiobis(2-nitrobenzoate) type (Deuticke et al., 1978).

1.7 Transport in parasite-infected erythrocytes:

In order to reach intracellular parasites, extracellular substances must be transported across three membranes: the membrane of red cells, the parasitophorous vacuole membrane, and the plasma membrane of parasites. Transport systems on each and every of these membranes are important for the survival of parasites.

1.7.1 Transport across red cell membranes of parasite-infected red cells

The nutritional and ionic requirements of parasite-infected red cells are tremendously different than those of normal red cells. Consistently, it has been observed that parasitized red cells have increased fluxes of Ca^{2+} (Tanabe et al., 1982) , increased Na^+ /K^+ exchange (Dunn, 1969), increased uptake of glucose and amino acids (Sherman, 1988), increased uptake of fatty acids (Moll et al., 1988), and a decrease in sensitivities to nucleoside transport inhibitors (Gero et al., 1988). These increased transport activities are made possible, at least partially, by the changes in the permeabilities of the membrane of red cells after they are parasitized.

The permeability of normal and parasite infected red cells to a variety of carbohydrates and amino acids was measured by both iso-osmotic lysis, which measures hemolysis due to the selective entry of substrates in parasitized cells, and uptake of labelled substrates. The permeability of infected red cells is remarkablly increased as compared to normal red cells. Such a permeability change occurs 6 hours after the invasion of red cells

by parasites and increases as parasites mature (Kutner et al., 1985). This permeability change is dependent on *de novo* protein synthesis (Kutner et al., 1985; Cabantchik, 1989), leading to the suggestion that parasite proteins are present on red cell membranes and are responsible for the enhanced permeability. However, it is also known that chemical modification of membrane components including oxidative damage, changes in lipid composition and organization, and alternation in membrane fluidity may induce increases in the permeability of red cell membrane analogous to those observed with parasitism (Ginsburg et al., 1987). Therefore, parasite protein(s) required for the permeability change may also function to modify the red cell membrane instead of being directly present on the membrane.

The altered permeability of the red cells is clearly beneficial for parasites. Correlations have been found between inhibition of parasite growth and blockage of permeation systems. Both the inhibition of glutamine permeability by plant alkaloids (Elford, 1986) and the inhibition of permeability to anions, carohydrates, and amino acids by phlorizin and phloritin (Cabantchik et al., 1983) are inhibitory for parasite growth.

Regarding the biochemical nature of such a permeation pathway, there are two general types of hypothesis. One is that new membrane component(s) synthesized by parasites are responsible for the transport event. This is consistent with the observation that parasite protein synthesis is required for the generation of permeation pathways. Also supportive of such a hypothesis is that a inhibitor of the pathway, phlorizin, has a defined number of binding sites on a permeabalized red cell membrane (Kutner et al., 1987). The other hypothesis is that pores of 0.7 nm equivalent radius are generated on the red cells membrane and are responsible for the observed permeability (Ginsburg et al., 1985). This was based on a survey of the substrates of the permeation pathway, where it was found the

pathways discriminate between substrates according to their molecular sizes. These two hypotheses are clearly not exclusive of each other.

1.7.2 Transport across the parasite cytoplasmic membrane and the parasitophorous vacuole membrane:

The next membrane an exogenous substrate encounters in its jouney to the intracellular parasite is parasitophorous vacuole membrane (PVM), which is a piece of reversed red cell membrane surrounding the parasite. Little is known about the transport property of this membrane. Freeze-fracture electron microscopy showed that red cell membrane proteins are lost from PVM (Sherman, 1988), suggesting the lack of active transport mechanism on these membranes.

The plasma membrane of the parasite is the final barrier to a exogenous substrate. Since the metabolic intricacy of the intracellular parasite are comparable to that of most other eucaryotic cells, it is conceivable that the parasite possesses similar transport systems. However, the transport across the parasite plasma membrane is difficult to study because of the potential damage of the parasite membrane upon being released from the parasite's host cell. Transport of glucose, amino acids, and purine nucleosides across the parasite membrane have been studied in isolated parasites (reviewed by Sherman, 1989). This type of studies generally indicated that the transport of amino acids proceeds passive diffusion, while the transport of glucose and purine nucleosides appeared to proceed by specific transporters.

Finally, it has been proposed that macromolecules such as dextrans and proteins can gain direct access to the plasma membrane of the parasite through "parasitophorous ducts", which are membrane structures connecting the parasitophorous memebrane and red cell membrane (Pouvelle, 1991). After getting into the parasitophorous space by a passive

diffusion through the "parasitophorous ducts", these substrates can be endocytosed by the plasma membrane of the parasites.

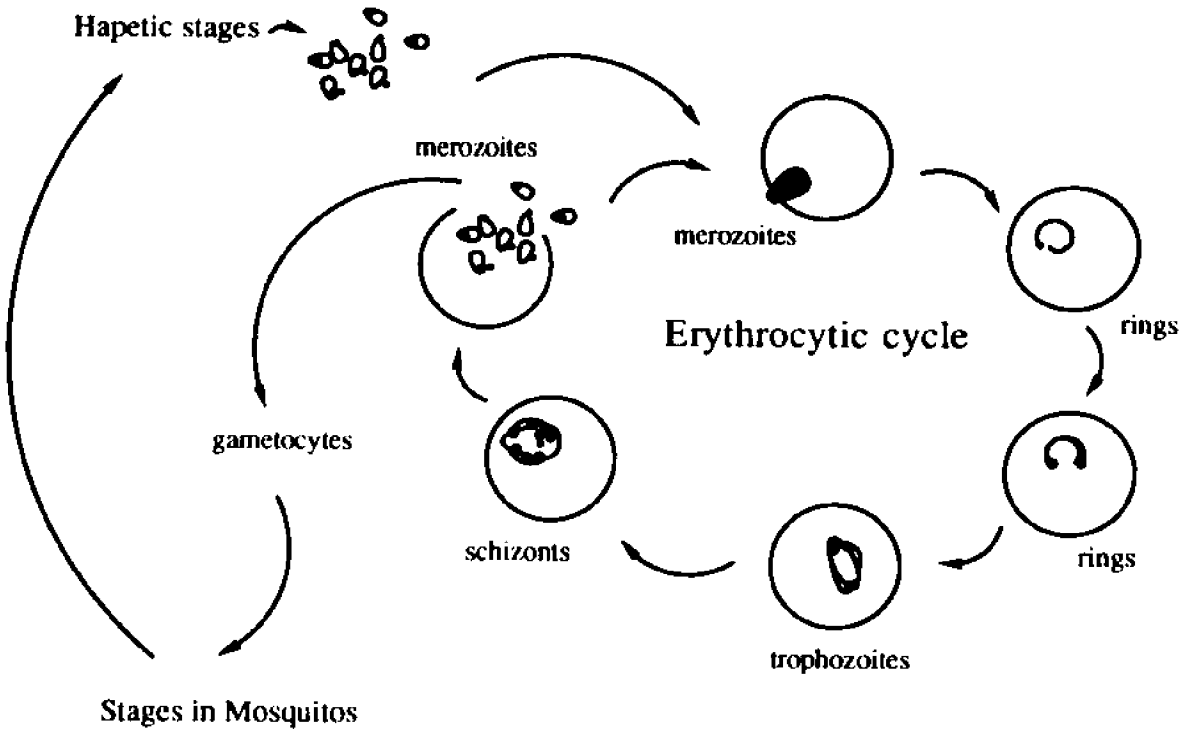
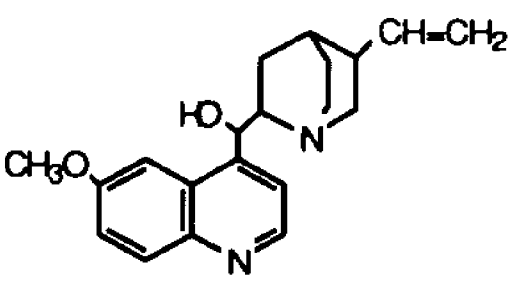
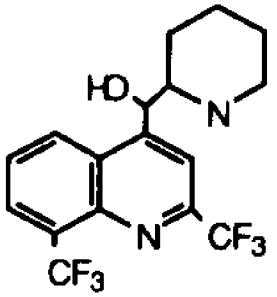


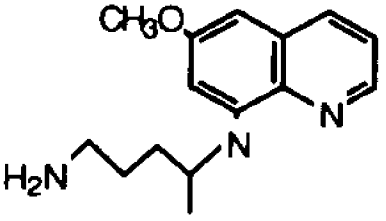
Figure 1.1 : Life Cycle of Malaria Parasites



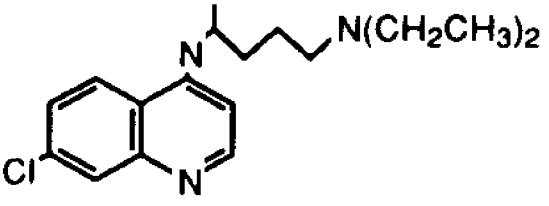
Quinine



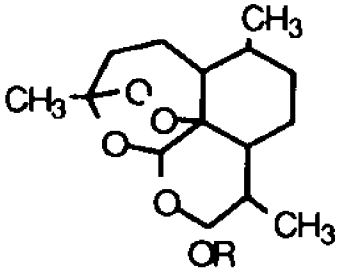
Mefloquine



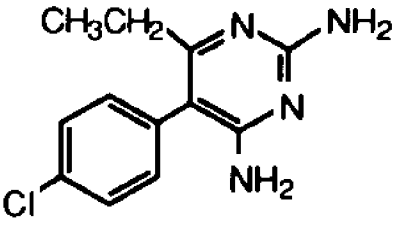
Primaquine



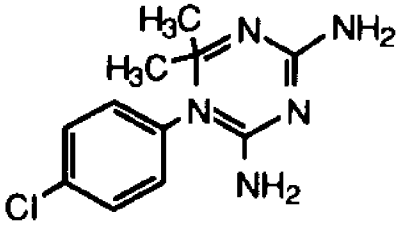
Chloroquine



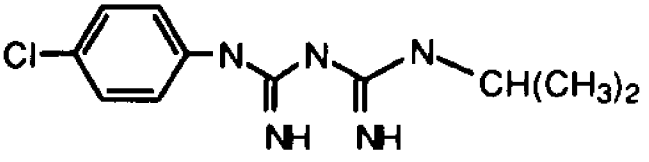
Qinghaosu



Pyrimethamine



Cycloguanil



Proguanil

Figure 1.2 a : Structures of some antimalarial drugs

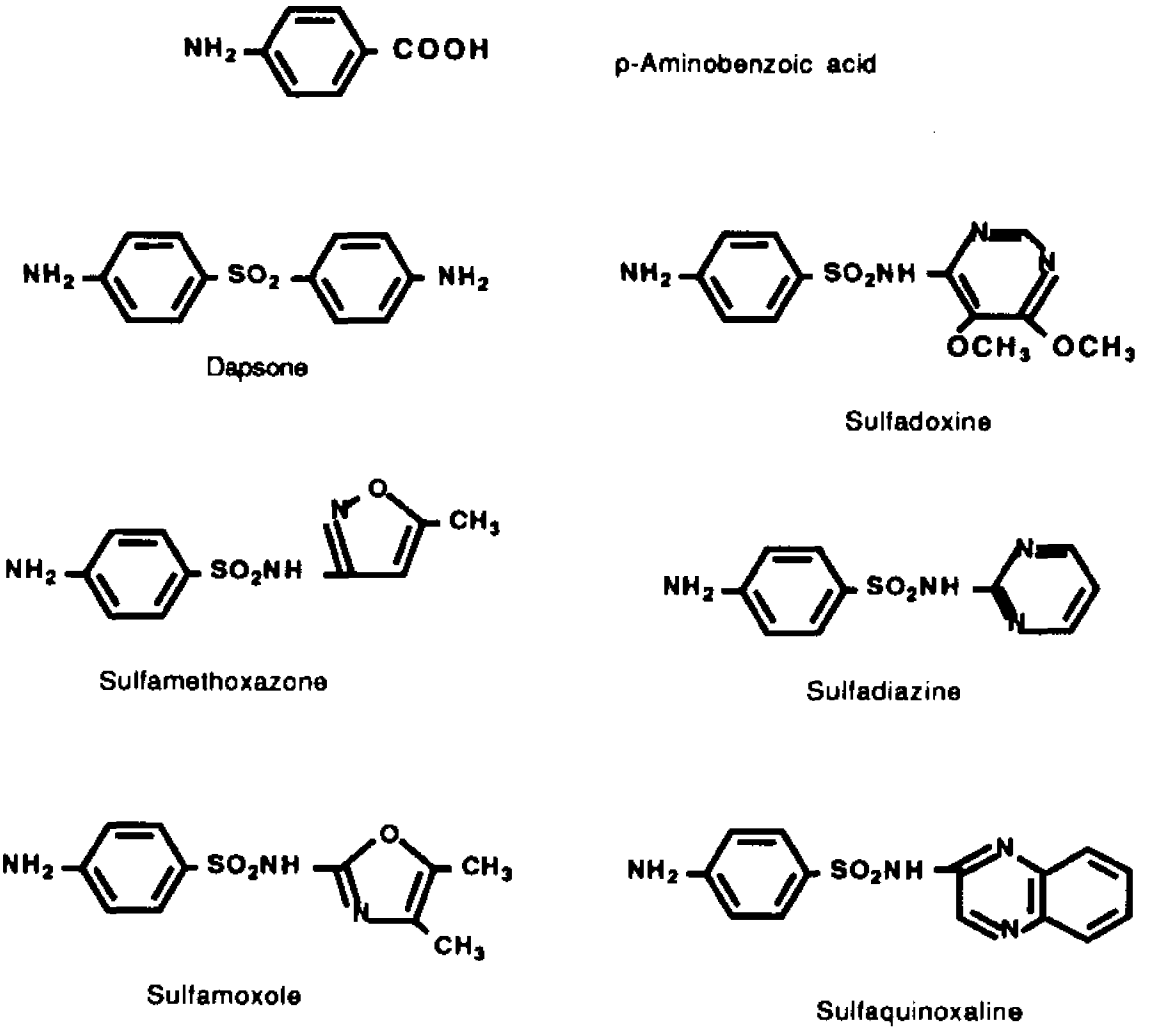


Figure 1.2 b: Structures of PAB and related sulfa drugs

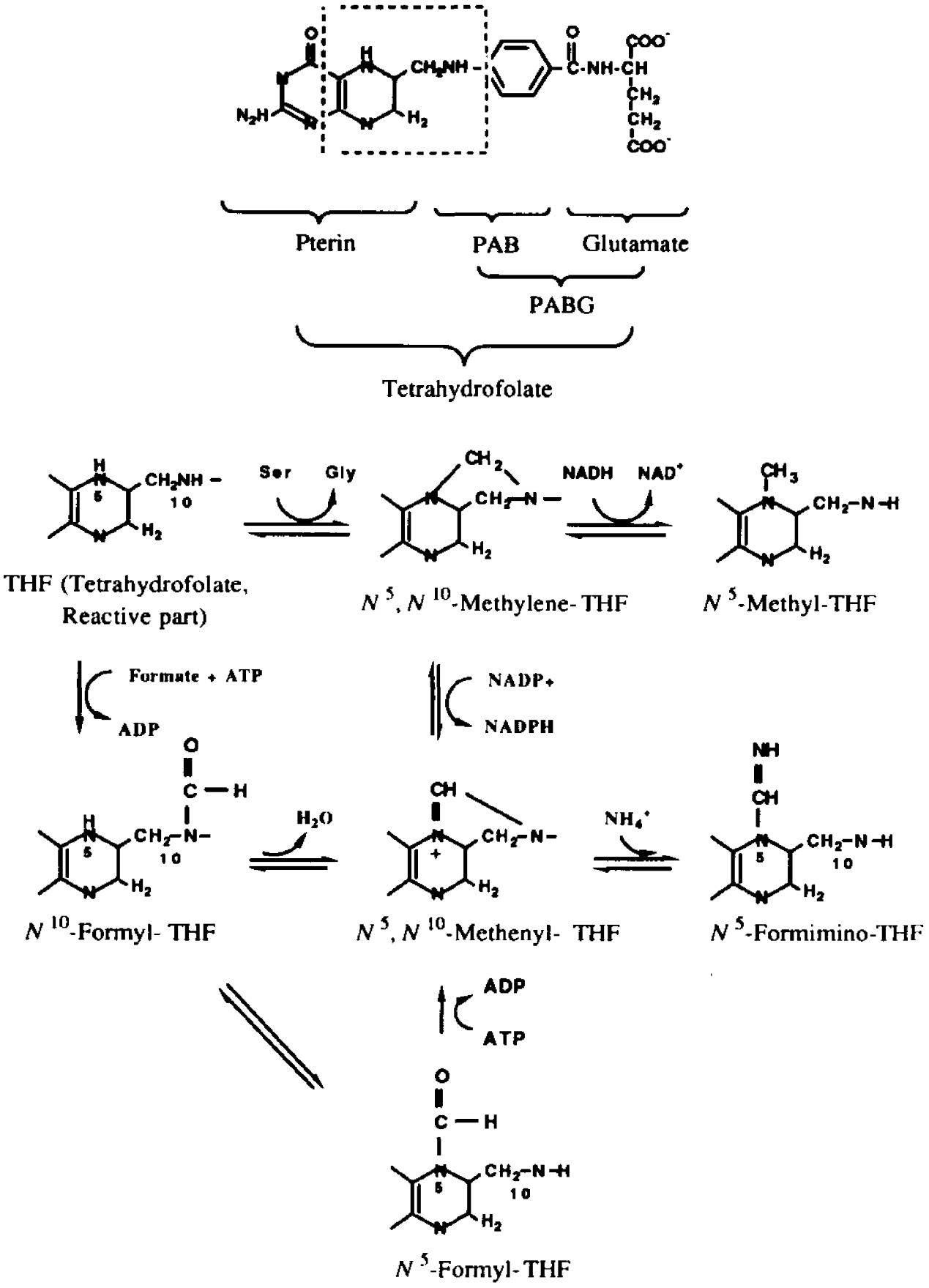


Figure 1.3 : Tetrahydrofolates and their conversions.

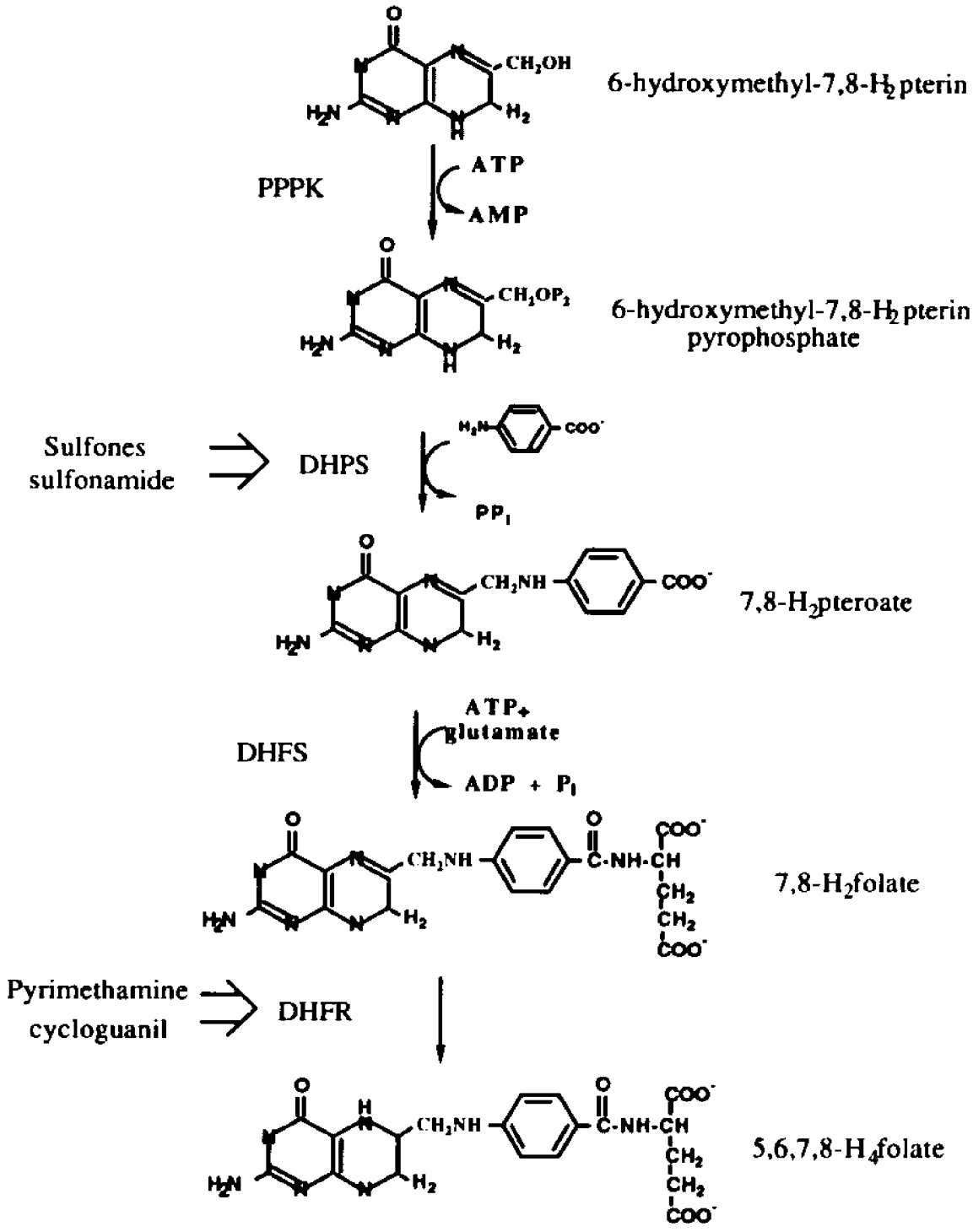


Figure 1.4 : Folate Synthesis in Malaria Parasites

2. METHODS

2.1 Malarial parasites

2.1.1 *In vitro* culture of *Plasmodium falciparum*

Parasite cultures of *Plamodium falciparum* (FCR3) were maintained in red blood cells by the method of Trager and Jensen in candle jars (1976). Culture media consisted of RPMI 1640 (GIBCO, Grand Island, NY) containing 0.2 % $NaHCO_3$, 40 mM Hepes, 35 mM dextrose, 40 mg/L gentamycin and 10 % (V/V) human type A^+ serum, and were adjusted to pH 7.1. Fresh type A^+ blood was obtained from Interstate Blood Bank (Memphis, TN) and stored at 4°C for a maximum of three weeks. Prior to being used for parasite culture, red blood cells were washed three times in RPMI 1640. Parasite culture was maintained at hemotacrit (percentage packed red cells in media) of 7 % and parasitemia (percentage parasite infected red cells) of 1 % to 10 %. Blood smears were made daily and the parasitemia were counted under microscope.

2.1.2 Concentration of red cells infected with later stages of parasites

Red cells infected with later stages of parasites were concentrated by gelatin flotation method (Fairfield et al., 1983). A 2% Gelatin solution was made by dissolving gelatin in hot RPMI, and filter sterilized using an 0.2 micron syringe filter. The gelatin solution was stablized to 36°C in an incubator before use. Parasite-infected red cells were packed in 15-ml centrifuge tubes and the supernatants were removed. To each tube of packed red cells, 5 times volume of 2% gelatin solution were added and the packed red cells were suspended by mixing. The tubes were allowed to stand completely upright at 36°C for 15-

20 minutes. The sparsely turbid upper layers now contain red cells infected with later stages of malaria parasites, namely trophozoites and schizoites, because knobs formed on the surface of these infected red cells prevented them from forming roulleaux's in gelatin and falling to the bottoms of the tubes. These upper layers were carefully removed. These parasite-infected red cells were washed in RPMI three times before they were used in experiments. Blood smears of concentrated infected red cells were made after each precedure to determined the parasitemia of these preparations.

2.1.3. Isolation of malaria parasites

Malaria parasites were isolated from infected erythrocytes by saponin lysis (Fairfield et al., 1983). Cultured parasite infected-red blood cells of 10 - 15 % parasitemia were washed three times in Trager's Buffer containing 60 mM NaCl, 60 mM KCl, 1 mM NaH_2PO_4, 7 mM K_2HPO_4, 10 mM $NaHCO_3$, 10 mM dextrose pH 7.8, and resuspended in the same buffer to four times the volumne of the packed blood. A stock solution of phenylmethylsulfolnylfluoride (PMSF, 50 mM in ethanol) was then added to a final concentration of 1 mM to inhibit proteases. One tenth volumne of a saponin-saline solution containing 0.165 % saponin and 0.85 % NaCl was added drop by drop to each tube while the blood suspension was being vortexed gentley. The solutions of partially lysed cells were then incubated in a 37°C water bath for 15 min with very gentle shaking (150 rpm). After cooling down on ice, isolated parasites were pelleted at 4°C and washed three times in Trager's buffer. The dark brown colored parasite pellets were suspended to desired densities in Tris-HCl buffer and immediately frozen at -70°C. The loss of enzyme activity was negligible in preparations stored in such a manner for up to 2 to 3 months.

2.2 *In vitro* sensitivity assay

The *in vitro* antimalarial activity of dapsone and various sulfonamides was assessed by a modification of the method of Sixsmith (Sixsmith, 1984). Parasites were washed in warm PAB- and folate- free (PFF) RPMI 1640 (GIBCO) 3 times and then cultivated for 72 hours in the same medium containing 10% human serum (PFF RS) prior to inoculation of microtiter plates. Stock solutions of the drugs were made in dimethyl sulfoxide (DMSO) and stored at -20°C for maximum of two weeks. Parasites were inoculated to a parasitemia of 0.4% and hemotocrit of 1.5% in 200 µl of PFF RS containing drugs in 96-well microtiter plates (Corning, Corning, NY). For determination of concentrations which kill 50% of parasites (LC_{50}s), 1:2 serial dilutions of drugs were made in triplicate wells. The final concentration of DMSO in each well was never greater than 1%, a concentration which did not affect parasite growth. [^{3}H-] hypoxanthine (0.5 µCi) was added to each well after 48 hours and the microtiter plates were harvested using a PHD cell harvester (Cambridge Technol. Inc., Cambridge, MA) after an additional 24 hours. The filters were counted in Aquasol 2 (New England Nuclear, Boston, MA) in a liquid scintillation counter (LKB Rackbeta, Gaithersburg, MD). LC_{50}'s were determined from plots of incorporated counts versus drug concentrations. For each drug, the LC_{50} determination was performed a minimum of 3 times.

2.3 Synthesis of 6-hydroxymethyl dihydroperin pyrophosphate

6-Hydroxymethyldihydropterin pyrophosphate, one of the substrates of dihydropteroate synthetase, was synthesized by a modification of the method of Shiota (Shiota, 1963). Fifty mg of 2-amino-4-hydroxy 6-hydroxymethyl-pteridine and 5 g of

pyrophosphoric acid was mixed in a 25 ml flask protected from light and stirred at 60°C overnight. The reaction was terminated by adding 15 ml cold distilled water. Ten milileter of 15 % Norit A charcoal (Sigma, St. Loius, MO) in water was then added and the mixture was stirred for 15 min. The mixture was filtered through a 0.45 μm Millipore filter and the charcoal pads were washed (resuspended and filtered) 3 times with distilled water. An eluting solution of 1.5 N NH_4OH and 50 % ethyl alcohol was used to elute pteridines absorbed to charcoal. The charcoal pad was first suspended in the elution solution and stired for 10 min. The mixture was filtered through a 0.45 μm Millipore filter and the eluate was kept. This procedure was then repeated four times and all the eluates were combined. The eluate was concentrated by using a Speedvac (Savant Instruments Inc., Farmingdale, NY). The product, 6-hydroxymethyldihydroperin pyrophosphate, was purified by two column chromatography precedures, using firstly an AGW50 x 8 column (Bio-Rad, Richmond, NJ) and secondly a TSK-gel DEAE 650-S (Toyopearl, Gibbstown, NJ) column on Fast Performance Liquid Chromatography (Pharmacia, Piscataway, NJ), as will be discribed in section 2.5.1 and 2.5.2. The pterindine pyrophosphate synthesized was lyophilized and stored in powder form at -70°C.

2.4. Biochemical assays

2.4.1 Dihydropteroate synthetase assay

Dihydropteroate synthetase activity was assayed by the method of Walter et al. (1980), measuring the incorporation of [^{14}C-] PAB (56 μCi per μmole, Amersham, Arlington Heights, IL) into dihydropteroate. Immediately prior to each experiment, thawed parasite preparations were lysed by adding Triton X-100 (1% stock solution) to a final concentration of 0.05 % and vortexing. The mixtures were then microfuged for 10 minutes

and the pellets of membrane debris were discarded. Low molecular weight components were removed by passing the supernatant through an NAP-5 gel filtration column (Pharmacia). This parasite lysate free of cofactors was then assayed for dihydropteroate synthetase activity. Each reaction mixture of dihydropteroate synthetase assay, in 100 µl of 100 mM Tris-HCl 10 mM $MgCl_2$ pH 8.5, contained 50 µM [^{14}C-] p-aminobenzoic acid, 2 mM B-mercaptoethanol, 5 mM sodium floride, and 100 µM reduced 6-hydroxymethyldihydropterin pyrophosphate. 6-hydroxymethyldihydropterin pyrophosphate was reduced by adding ascorbic acid to a final concentration of 3.25 mg/ml and sodium dithionite to a final concentration of 5 mg/ml at pH 8, and allowing the mixture to sit at room temperature for 15 min before being used in DHPS assays. The complete reduction of 6-hydroxymethyl dihydropterin pyrophosphate treated by this method was confirmed by the change in its absorption spectrum. Reaction mixtures were incubated for 60 minutes and reactions were terminated by heating the reaction mixtures at 100^{o}C for 1 min. The mixtures were then centrifuged, and pellets of denatured proteins were discarded. [^{14}C-] Dihydropetoate was separated from [^{14}C-] PAB by a paper chromatography procedure which will be described in 2.5.3. Controls had either malarial lysate or 6-hydroxymethyl dihydropterin pyrophosphate left out from the reaction mixture. Radioactivities corresponding to [^{14}C-] dihydropteroate in the control reactions were substracted from that of the real assays to calculate dihydropteroate activities. An unit of dihydropteroate activity is defined as one µmole per mg protein per min.

2.4.2 ATP assay

ATP was assayed by using luciferin - luciferase reaction, which generates photons (560 nm) in the presence of ATP, Mg^{2+}, and O_2 (DeLuca, 1987). Immediately before the ATP assay, frozen red cell samples were thawed and lysed by diluting them 400 fold in

distilled water. These and standard ATP samples were then diluted 20 times in Firelight Buffer (pH 7.75, Analytical Luminescence Laboratory, San Diego, CA). One hundred µl of Samples were mixed with 100 µl of Firelight Luciferin-Luciferase (Analytical Luminescence Laboratory) and the amount of light generated were measured for 10 seconds on a luminescence photometer (Analytical Luminescence Laboratory). ATP concentrations in samples were then determined from the standard curve of luminescence versus ATP concentration.

2.4.3 Hemoglobin Assay

Hemoglobin was assayed by the method of Beutler (Beutler, 1984), measuring at 540 nm the optical density of cyanmethemoglobin, which was produced from a reaction of hemoglobin with ferricyanide-cyanide reagent. The ferricyanide-cyanide reagent contained 100 mg of NaCN and 300 mg of $K_3Fe(CN)_6$ per liter, and was stored in a dark bottle at room temperature for a maximum of a few weeks. For hemoglobin assay, 10 µl of standard hemoglobin soutions or unknown samples was added to 1 mL of the ferricyanide-cyanide reagent and mixed well. Optical densities at 540 nm were measured on a spectraphotometer (Bausch & Lomb, Rochester, NY). Hemoglobin concentations in samples were determined by using an extinction coefficient derived from a standard curve measured each time.

2.4.4. Protein Assay

Protein was assayed by the method of Bradford (Bradford, 1976), which is based on the observation that the absorbence maximum for an acidic solution of Coomassie Brilliant Blue G0250 shifts from 465 nm to 595 nm when binding to protein occurs. For protein assays, 5 to 20 µl of standard (bovine plasma albumin, Bio-Rad, Richmond, CA) or

unknown protein samples were added to a mixture of 800 µl water and 200 µl dye reagent (Bio-Rad). The ingredients were mixed well and allowed to stablize for 15 minutes at room temperature. Optical densities were measured at 595 nm on a spectraphotometer and protein concentrations were determined by using an extinction coefficient derived from a standard curve measured each time.

2.4.5 Phosphate assay

The concentration of inorganic phosphate was determined by using molybdate reagent (Lowry, 1957). The molybdate reagent consisted of 0.1 % ammonium molybdate, 0.04 % ascorbic acid in acetate buffer (0.1 M acetic acid and 0.65 M sodium acetate). Solutions of KH_2PO_4 were used as standards. Fifty µl of standard or unknown phosphate samples was added to 1 ml of molybdate reagent. The ingradients were mixed and allowed to stablize for 15 minutes at room temperature. Optical density were measured at 850 nm. The phosphate concentrations of unknown samples were then determined from the standard curve of absorption versus phosphate concentration.

2.4.6 Determination of pteridine concentration

The concentration of pteridines was determined by measuring the optical density at 350 nm. A molecular extinction coefficient of 6000 at pH 7 (Shiota et al., 1963) was used for the calculation of the pterdine concentration.

2.4.7. Assays of pterin mono- pyro- and tri- phosphates

In order to distinguish 6-hydoxymethyl dihydropterin mono- pyro- and tri-phosphates, samples of pterin phosphates were first digested by alkaline phosphotase, and then assayed for both pterin and inorganic monophosphate concentrations. For the

enzymetic digestion, a solution of 1 mg per ml alkaline phosphotase (5-10 units, Sigma) in 80 mM Tris-HCl, 2 mM $MgCL_2$, and 2 mM $ZnCl_2$ pH 8 was made freshly each time. The reaction solutions were mixed with samples in a 1:1 ratio, and incubated in a 37°C water bath for 1 hour. At the end of the enzymatic digestion, an equal volume of ice cold 10 % TCA solution was added to precipitate protein and the reaction mixtures were centrifuged. The supernatants were then subject to pterin and phosphate assays. The standards of the phosphate assay, solutions of 0, 50, 100, 150, 200 μM Na_2HPO_4, were also treated in the same manner prior to the phosphate measurements.

2.4.8 Measurements of total [^{3}H-] folates in infected red cells

The total [^{3}H-] folates synthesized in infected red cells were separated from [^{3}H-] PAB (20-50 mCi per μmole, Moravek Biochemical Inc., Brea, CA) by incubating the lysate with folate binding protein and then separating the protein fraction from the fraction of small molecules on a NAP-5 gel filtration column. Parasite-infected red cells were washed with PFF RPMI and incubated with 2 μM [^{14}C-] PAB in PFF RS for 48 hours. The infected red cells were then separated from uninfected red cells by gelatin flotation. The fractions of the uninfected red cells was used as internal controls. The infected and uninfected red cells were washed and the parasitemia of both fractions were determined. These cells were lysed by adding 2 to 5 volumes of water. Samples of these lysates were taken for protein assay. An equal volume of cold 10 % TCA solution was added to each lysate to denature proteins and the lysates were clarified by centrifugation. To two equal volumes of each of the protein-free lysates, bovine milk folate binding protein (0.5 mg per ml final in TE) and water (as control) were added respectively. These mixtures were incubated for 1 hour, their volumes adjusted to 0.5 ml by water if necessary, and loaded on a NAP-5 gel filtration column (molecular weight cut off: 2,500, Pharmarcia LKB

Biotechnol., Uppsala, Sweden). The columns were eluted by water. The first 1 ml of void volume containing proteins and the subsequent 4 ml eluate were collected separately, and counted for radioactivity. The counts for the protein fractions in the control incubations (no folate binding protein) were then substracted from that in the lysates incubated with folate binding protein. Percentages of the folates synthesized were calculated by dividing these counts by the total counts for both fractions.

2.5 Chromotography

2.5.1 Column chromatography

An AG 50W x 8 (Bio-Rad) column was packed (20 x 2 cm) and equilibrated with distilled water. Concentrated 6-hydroxymethylpterin phosphates products was loaded to the column and the column was eluted with distilled water at 8 ml per min using a peristaltic pump. Eluate was collected and its OD350 (obsorption peak of pteridine) was measured periodically. The column was eluted until the eluate contained no more pteridine. The pooled eluate was then loaded to the DEAE column to separate of 6-hydroxymethyl pterin mono-, pyro-, and tri- phosphates.

2.5.2 FPLC

A column was packed using FPLC TSK-gel DEAE 650-S gel (Toyopearl, Gibbstown, NJ) and a 25 x 1.5 cm FPLC column (Pharmacia). To exclude fine gel beads, the gel was first suspended in distilled water, allowed to settle, and the liquid phase was discarded. This procedure was repeated until the liquid phase was clear. The gel was then suspended in 50 mM Tris-HCl, pH 7.2, sonicated for 5 minutes, and degassed. The

column was packed with pressure from an air vent which approximated the pressure of the column on the FPLC system.

After the column was equilibrated with 50 mM Tris-HCl, 50 mM LiCl, pH 7.2, it was loaded with degassed eluate from the AG 50W x 8 column, and eluted with a linear gradient of 50 mM LiCl to 110 mM LiCl at a flow rate of 8 ml per minute on a Pharmacia FPLC system. The eluate was collected in fractions and pooled according to absorption peaks. Each pooled fraction was assayed for their pteridine and phosphate concentrations.

2.5.3 Paper chromatography

The product of the dihydropteroate synthetase reaction, [^{14}C-] dihydropteroate, was separated from the substrate, [^{14}C-] PAB, by a paper chromatography procedure. Fifty microliters of each reaction mixture, after denaturing and pelleting proteins, was spotted along a line toward one end of a piece of 15 centimeter 3 MM Whatman paper. The samples were then eluted with 0.1 M potassium phosphate pH 7.0, descending for about one and a half hours. The trails of samples on the paper were visualized under flourescent light. Each trail was then cut into ten equal parts between the origin and the solvent front. Each piece of filter paper was put in a 20 ml scitillation vial with 15 ml Aquasol-2 and counted in an LKB betarack liquid scitillation counter. Two radioactive peaks emerged: [^{14}C-] PAB eluted near the solvent front, while [^{14}C-] dihydropteroate remained at the origin.

2.6 Transport measurements

2.6.1 Equilibrium transport

For the purpose of comparing the uptake of sulfamethoxazole by parasite-infected red cells and normal red cells, and for the purpose of determining the effects of sulfa drugs

on PAB uptake, the transport of sulfamethoxazole and PAB into normal and infected red cells was measured under equilibrium condition. Prior to such measurements, *P. falciparum* infected red blood cells and uninfected red blood cells were incubated for three days in PFF RS.

For the measurements of sulfamethoxazole uptake by infected red cells, infected red cells were concentrated by gelatin flotation. The fraction of uninfected red cells was used as an internal control. The parasitemia of both fractions were determined. The concentrated infected red cells were then diluted with uninfected red cells to indicated parasitemia. Cells were washed three times with warm PFF RPMI 1640. One ml suspensions (5% hematocrit in PFF RPMI 1640) of these infected red cells were then incubated with 200 nM of [^{3}H-] sulfamethoxazole (14.2 mCi per μmole, a gift from Dr. Peter Sorter, Hoffmann-LaRoche, Nutly, NJ) in duplicates. At the end of each incubation, cells were spun down at 4^o C for 15 seconds using an Eppendorf microfuge (Brinkmann Instruments Co. Westbury, NY) and pellets were quickly washed three times with cold buffer (60 mM NaCl, 60 mM KCl, 1 mM NaH_2PO_4, 7 mM K_2HPO_4, 10 mM $NaHCO_4$, 11 mM dextrose, pH 7.4) containing 200 nM unlabelled sulfamethoxazole. The red cell pellets were lysed by adding 1 ml water, which released the radio label from both red cells and parasites, and the lysates were transfered to 20 ml scintillation vials. Hemoglobin was then bleached by treating the hemolysates first with one drop of 1 M NaOH and then with 0.5 ml H_2O_2. Scintillation fluid Aquasol-2 (18 ml) was added to each vial and radioactivity counted in a liquid scintillation counter. For the parasitemia dependence of sulfamethoxazole uptake, 15 minute incubation times were used because the uptake saturates at this time, and the uptake measurements were therefore at an equilibrium condition. Aliquots of blood cell suspensions used in the uptake experiment were assayed for hemoglobin contents (see 2.4.5). The number of red cells in each uptake experiment

was calculated by assuming $5x10^6$ red cells are equal to 360 µg hemoglobin (Beutler, 1984)

The equilibrium [^{14}C-] PAB uptake were measured similarly except the infected red cells were not concentrated by gelatin flotation and the washing buffer contained 10 µM PAB instead of sulfamethoxazole.

2.6.2 The initial rates of PAB transport

In order to study the kinetics and the mechanism of PAB transport, its initial rate was measured.

The association of [^{3}H-]PAB with parasite-infected or normal red cells was measured in duplicate in PFF RPMI 1640 medium pH 7.1 at 36°C unless otherwise specified. Infected and uninfected cells were washed 3 times in warm PFF RPMI, and incubated for 10-15 minutes at 36°C between each washing to allow for export of intracellular PAB (see 4.3). Transport experiments were initiated by mixing washed red cells with PFF RPMI containing [^{3}H-] PAB (and additional reagent where indicated) to a final hematocrit of 5 % in 200 µl. To terminate the transport, cells were spun down through a dibutylphthalate layer (Ellenberger and Beverley, 1987) for 15 seconds in an eppendorf microfuge at 4°C. The top layer of transport media was removed and tube walls washed once with PFF RPMI lacking [^{3}H-] PAB. After the dibutylthalate layers were removed, the red cells pellets were lysed in 200 µl water, and transfered to 20 ml scintillation vials. The lysates were then treated by 1 M NaOH and 0.5 ml 30 % H_2O_2 to bleach hemoglobin. Scintillation fluid Aquasol-2 (18 ml) was added to each vial and radioactivity counted .

2.6.3 Counter-transport

In order to assess the effect of intracellular unlabelled PAB on PAB uptake, counter-transport, the uptake in the presence of efflux, was measured. For these measurements, red cells were either loaded with unlabelled PAB by incubating with 500 μM unlabelled PAB in PFF RPMI for 2 hours at 36°C or depleted of intracellular PAB by washing and incubation in media free of PAB. Immediately prior to the transport experiments, PAB-loaded or PAB-depleted red cells were quickly washed twice in PFF RPMI. Transport experiments were initiated within 30 seconds after washed red cells were suspended in fresh PFF RPMI. Transport measurements were made similarly as in measuring initial rate except the incubation times were as indicated.

2.6.4 PAB release from red cells

To measure PAB release from red cells, red cells were first incubated with 2 μM [^{14}C-] PAB for 2 hours. Immediately before the measurements, these cells were quickly washed three times with PFF RPMI. At 0 time, cells were suspended at 5-10 % parasitemia in PFF RPMI in aliquots. At each time point, aliquots of cells were spun down, washed with cold PFF RPMI containing 2 μM unlabelled PAB. The radioactivity remaining associated with these cells was counted in the same manner as above.

PAB release was also measured similarly using infected red cells incubated with [^{14}C-] PAB for 16 hours to measure PAB metabolism (metabolized PAB was not released as efficiently as was PAB). To determine the effects of sulfamethoxazole on PAB metabolism, the same measurements were also made using infected red cells treated with 200 nM of sulfamethoxazole for 24 hours.

2.6.5 pH Dependence of the initial rate of PAB transport

PAB transport into red cells was measured at different pH values in PFF RPMI. PFF RPMI media of different pH (adjusted by adding HCl or NaOH) were mixed with equal volumes of red cells of 20% hematocrit in PFF RPMI pH 7.1. Transport experiments were then initiated by mixing 100 µl of each of above red cell suspensions with 100 µl of PFF RPMI pH 7.1 containing 10 nM [^{3}H-] PAB (with or without 200 µM unlabelled PAB). The final pH for each transport experiment was then determined by measuring the pHs of solutions mixing one volume of the PFF RPMI used for adjusting pH and three volumes of PFF RPMI pH 7.1.

2.6.6 Inhibitors of the PAB transport

For inhibitor studies, all the inhibitors were first incubated with infected or uninfected red cells for 20 min, after which [^{3}H-] PAB was added to initiate transport. Where DMSO was used as solvent for stock solutions of inhibitors, the concentration of DMSO in transport media never exceeded 1 %, which did not effect PAB transport. The non-saturable PAB association (measured in presence of 100 µM unlabelled PAB) was then subtracted from the total PAB association (in absence of unlabelled PAB) to represent PAB transport into the red cells in the presence of inhibitors.

2.6.7 ATP depletion

Red cells were depleted of cellular ATP by incubating red cells (25 % hematocrit) with 7.5 mM iodoacetamide and 7.5 mM sodium fluoride (Plagemann et al., 1985). Transport experiments were carried out immediately following each incubation period. Portions of the same treated red cells were also immediately frozen at -70^{o}C for ATP assay.

2.7 Determination of K_is, K_ms, and K_ds

Michaelis-Menten constants (K_m) were determined by using five substrate concentrations per experiment and averaging K_ms calculated from at least three experiments. IC_{50}s were determined from plots of DHPS activities versus five drug concentrations under standard reaction condition. IC_{50}s of dapsone, sulfamoxole, sulfamethoxazole and sulfaquinoxoline were determined by measuring the enzyme activities in the presence of 0, 50, 100, 200 and 500 μM of each of these drugs respectively. The drug concentrations used to determine the IC_{50}s for sulfadoxine and sulfadiazine were 0, 200, 500, 1000 and 2000 μM. Lineweaver-Burke plots were made for each drug, and K_is were determined by replotting apparent K_ms versus drug concentrations. Plots were drawn by linear regression analysis using Lotus 1-2-3 version 2.

Dissociation constant (K_d) for PAB transport was determined by measuring the PAB transport at five different extracellular PAB concentrations. The Lineweaver-Burke plots were then drawn by linear regression analysis using Lotus 1-2-3, version 2.

The inhibitory constant (K_i) of PAS inhibition of PAB transport was determined by measuring the apparent K_ds of PAB transport in presence of 0, 200, and 1000 nM of PAS. Lineweaver-Burk plots were made to determine the mechanism of inhibition. Inhibitory constants (K_is) were determined by replotting apparent K_ms versus PAS concentrations. All plots were drawn by linear regression analysis using Lotus 1-2-3, version 2.

3. THE INHIBITION OF *P. FALCIPARUM* DIHYDROPTROATE SYNTHETASE AND GROWTH *IN VITRO* BY SULFA DRUGS.

As discussed in the introduction, sulfa drugs are key antimalarial agents, particularly in the therapy and prophylaxis of chloroquine-resistant *P. falciparum*. Studying the effects of various sulfa drugs on *P. falciparum* , both in terms of dihydroperoate synthetase (DHPS) inhibition and preventing parasite growth, can aid the development of new and better antifolate antimalarials. We have here studied the inhibition of *P. falciparum* DHPS by a number of sulfa drugs, in comparison with their efficacies of inhibiting parasite growth *in vitro* .

3.1 The inhibition of *P. falciparum* parasite growth

Using the *in vitro* sensitivity assay, we compared the antimalarial effects of dapsone, sulfamoxole, sulfamethoxazole, sulfaquinoxaline, sulfadiazine, and sulfadoxine. All of these sulfa drugs exhibited antimalarial activity *in vitro* . Figure 3.1 shows the inhibition of parasite growth (measured by [^{3}H-] hypoxanthine incoorpration) by sulfadoxine. Similar effects on parasite growth were observed for other sulfa drugs (data not shown). The 50 % lethal concentrations (LC_{50}s) of the drugs were determined from these curves and they ranged from 31 to 520 nM (Table 1). Dapsone, sulfamoxole and sulfamethoxazole were most effective, sulfadiazine and sulfadoxine had intermediate activities and sulfaquinoxaline was least effective.

3.2 *P. falciparum* DHPS

In order to assay the malarial DHPS activity, one of the substrates of the synthetase reaction, 6-hydroxylmethyl-dihydropterin pyrophosphate, was synthesized by reacting 6-hydroxylmethyl-dihydropterin with pyrophosphoric acid. After being separated from the reactants, the reaction products were separated on a DEAE-cellulose column on FPLC. As shown in the elution profile (Figure 3.2), the reaction had three products which eluted at 56, 74, and 90 mM LiCl respectively. When each of these peaks was pooled and assayed for both pteridine and inorganic mono-phosphate concentrations (after alkaline phosphatase treatment), it was found that the monophosphate/pterindine ratios of these three fractions were 1, 2, and 3 respectively. This indicates that the three fractions of the products were mono-, pyro-, and tri- phosphates of 6-hydroxymethylpterin. Dihydropteroate synthetase assays using *E. coli* lysate (*E. coli* lysate has a DHPS activity 40 times higher than that of *P. falciparum* lysate) further confirmed this peak assignment because the second fraction had substrate activity while the other two fractions had minimum activity.

Using the synthesized 6-hydroxylmethyl-dihydropterin pyrophosphate, *P. falciparum* extracts were assayed to contain an average of 10 μunits of DHPS activity per mg protein. The DHPS activity showed Michaelis-Menton kinetics for both substrates and apparent K_m's for this activity were 17.2 ± 1.3 μM for 6-hydroxymethyldihydropterin pyrophosphate and 8.1 ± 2.9 μM for PABA.

3.3 The inhibition of *P. falciparum* DHPS

The inhibitory effects of the six sulfa drugs on *P. falciparum* DHPS were studied. The five sulfonamides and dapsone inhibited DHPS with IC_{50}s in the range of 30 to 500

μM (Table 1). Dapsone, sulfamoxole and sulfaquinoxaline were much better DHPS inhibitors than were sulfadiazine and sulfadoxine, which have roughly 10 times higher IC_{50}s. Sulfamethoxazole had intermediate activity. The DHPS inhibition by these drugs were all competitive with PAB (Figure 3.3). Their K_is are listed in Table 1.

When IC_{50}s and K_is of these drugs were compared with their LC_{50}s, however, no correlation was found between the ability of a drug to inhibit the parasite DHPS and its *in vitro* antimalarial activity (Figure 3.4, R^2 = 0.009 and 0.002 for IC_{50}s and K_is respectively). Furthermore, the drugs appeared to inhibit parasite growth *in vitro* at concentrations 100-1000 fold lower than their K_is and IC_{50}s (Table 1).

3.4. Discussion

The sensitivity of cultured *P. falciparum* to sulfadoxine, the sulfa component of fansidar, has been previously examined. The LD_{50} of sulfadoxine reported here (360 nM) is in the same range as reported for other fansidar sensitive species (900 nM and 290 nM; Brockelman et al., 1982 and Ferone et al., 1973 respectively). In addition to sulfadoxine, we studied the sensitivities of *P. falciparum in vitro* to an additional five sulfa drugs, some of which, sulfamethoxazole and sulfamoxole for example, are much more effective in inhibiting parasite growth (Table 1).

DHPS has been studied in *E. coli* (McCullough et al., 1973), *Lactobacillus plantarum* (Shiota et al., 1963) , *P. chabaudi* (Walter et al., 1980), *P. berghei* (Ferone et al., 1973; McCullough, 1974) and most recently in *Pneumocystis carinii* (Merali et al., 1990). The specific activities of *P. falciparum* crude extracts reported here (10 μU per mg protein) is much lower than that for *E. coli* (400 μU per mg protein) and *L. plantarum* (720 uU per mg protein) but in the same range of that of *P. chabaudi* (16 μU per mg protein) and *P.*

carinii (14 μU per mg protein). The K_ms for PAB and 6-hydroxy-methylpterin pyrophosphate reported here (8.1 and 17.2 μM, respectively) are higher than that reported in *P. berghei* (2.8 μM and 1.4 μM, respectively), which were measured using partially purified enzyme.

As expected, all sulfa drugs studied inhibit DHPS by competing with PAB (Figure 3.3). But for the six sulfa drugs examined, there is no correlation between IC_{50} of DHPS and LC_{50} of parasite growth. Neverthless, if sulfaquinoxaline and sulfadiazine are excluded, then there was a good correlation for the remaining drugs (R^2 = 0.980 and 0.444 for IC_{50}s and K_is respectively). As will be shown in next chapter, the fact that sulfadiazine is a good inhibitor of parasite growth but not a good inhibitor of DHPS is not due to its ability to inhibit PAB uptake.

The observation that the sulfa drug concentrations that inhibit parasite growth and that inhibit the parasite DHPS differ by a 2-3 order of magnitude is surprising. There are three possible explainations for this. Firstly, sulfa drugs may be concentrated by parasites, so that the intraparasitic concentrations of sulfa drugs are much higher than that in the media. Secondly, sulfa drugs may act on additional targets other than DHPS. Since sulfa drugs structurally resemble PAB, a logical candidate for such targets is PAB uptake by infected red cells. Finally, the intraparasitic chemical environment might be so different than that in the enzyme assay solution that DHPS is much more sensitive to the sulfa drug inhibitions *in vivo* . The evaluations of the first two of these possibilities will be presented in the next chapter.

Drugs	LC_{50}s (nM)	IC_{50}s (μM)	K_is (μM)
dapsone	31 ± 14	33	6 ± 2
sulfamoxole	61 ± 17	55	7 ± 3
sulfamethoxazole	60 ± 21	100	36 ± 19
sulfaquinoxaline	519 ± 443	40	15 ± 3
sulfadiazine	125 ± 25	500	89 ± 18
sulfadoxine	357 ± 33	400	39 ± 24

Table 3.1: Inhibitin of *P. falciparum* DHPS (IC_{50}s and K_is) and parasite growth *in vitro* (LC_{50}s). LC_{50}s, IC_{50}s, and K_is were determined as described in methods. LC_{50}s and K_is are shown as means ± deviations; IC_{50}s are shown as averages of 3 determinations.

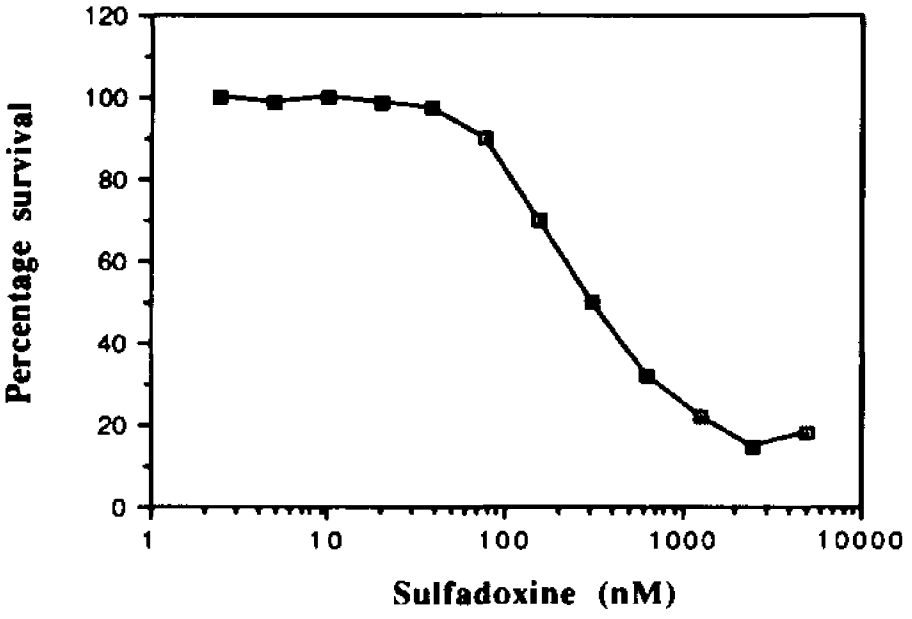


Figure 3.1 : The inhibition of *P. falciparum* growth *in vitro* by sulfadoxine. *P. falciparum* growth *in vitro* was measured by [^{3}H-] hypoxanthine incorporation. Data shown are means of three measurements.

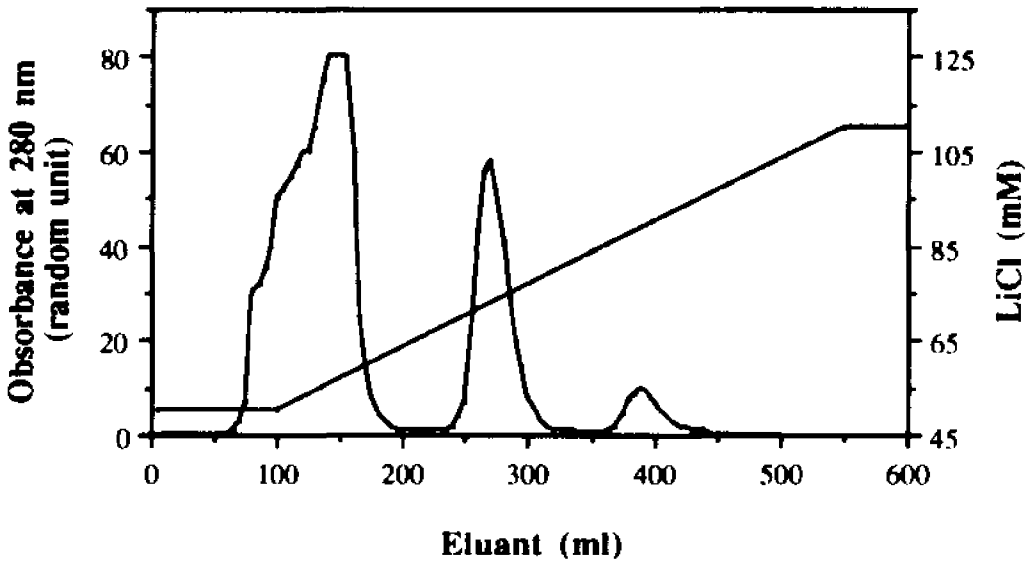


Figure 3.2 : The separation of 6-hydroxymethyl dihydropterin phosphates by a DEAE 650-S colume on HPLC. The colume was eluted by a linear gradient of 50 to 110 mM LiCl at a flow rate of 8 ml per minute.

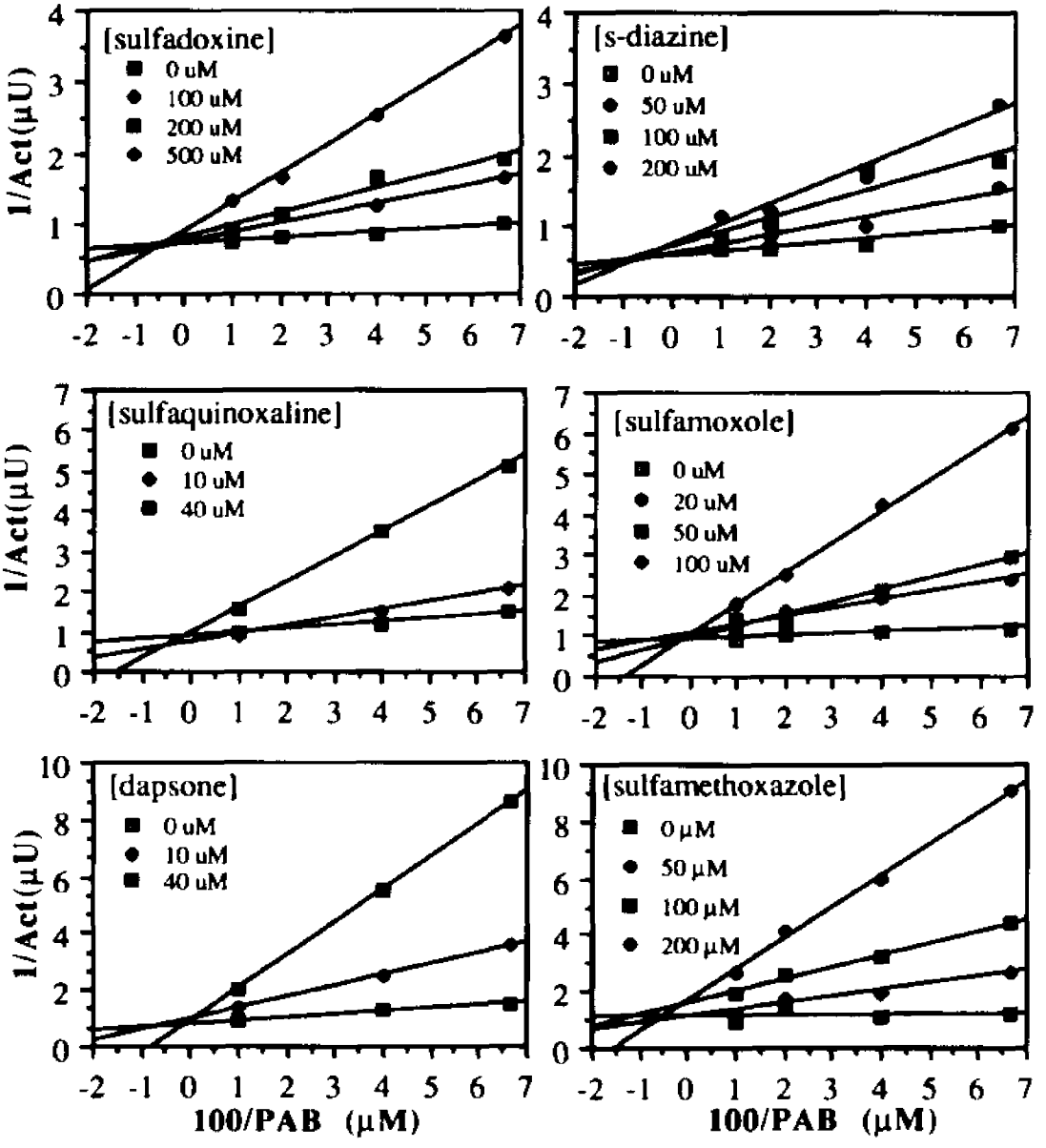


Figure 3.3 : Lineweaver-Burk plots of the inhibitions of *P. falciparum* DHPS by six sulfa drugs. DHPS activities were measured under standard assay conditions in the presence of various drug concentrations.

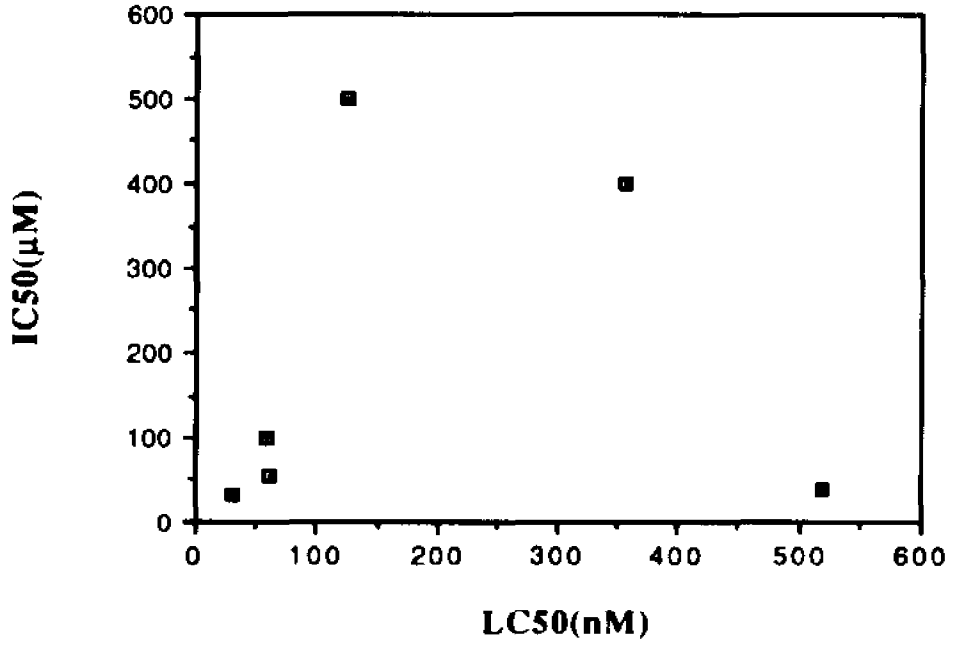


Figure 3.4 : Correlation between LD_{50} (inhibition of DHPS) and LC_{50} (inhibition of *P. falciparum* parasite growth) of six sulfa drugs.

4. DIFFERENCES BETWEEN THE IC_{50}S AND THE LC_{50}S

As shown in the previous section, sulfa drugs inhibit the growth of *P. falciparum in vitro* at concentrations 100 to 1000 times lower than those which inhibit parasite DHPS. We examined possible mechanisms underline this observation, and found that the concentration of sulfa drugs by malaria parasites may explain why sulfa drugs can inhibit parasites by inhibiting DHPS.

4.1 Parasites concentrate sulfa drugs

Intraerythrocytic parasites are separated from the culture media by three membranes, namely, the red cell membrane, the parasitophorous vacuole membrane, and the parasite plasma membrane. The concentration of a sulfa drug inside the parasites at a given extracellular drug concentration is not known. An ideal approach to the assessment of intraparasitic drug concentration would be to isolate parasites from the host red cells, and determine the amount of radiolabelled drug associated with parasites. However, methods of isolating parasites from red cells often result in the leakage of parasite membrane (Sherman, 1989), which makes such an approach unreliable. Thus, we chose to use intact parasite-infected red cells. By comparing the uptake of sulfa drugs in parasite-infected red cells of differen parasitemia, we estimated the the sulfa drug concentration inside parasites.

The uptake of [^{3}H-] sulfamethoxazole by red cells saturates at about 15 min (Data not shown). This [^{3}H-] sulfamethoxazole uptake by parasite-infected red cells of different parasitemia was then measured after 15 min of incubation, an equilibrium condition. Infected red blood cells were found to take up significantly more sulfamethoxazole than

normal red blood cells (Figure 4.1). The degree of uptake was proportional to the parasitemia (R^2= 0.88).

If we assume the concentration of sulfamethoxazole in the erythrocytic cytoplasm of infected red cells is the same as in the cytoplasm of uninfected red cells, and that mature parasites are spherical with a radius of 2.5 μm (Dieckmann, 1986), it can be calculated that the intraparasitic concentration of sulfamethoxazole was 4.3 μM, which is roughly 20 times higher than the extracellular sulfamethoxazole concentration (200 nM).

4.2 Sulfa drugs do not inhibit PAB uptake

Since malaria parasites utilize PAB for the synthesis of folate-type coenzymes, and sulfa drugs structurally resemble PAB, sulfa drugs might also inhibit parasite growth by inhibiting PAB uptake. To determine the effect of sulfa drugs on the uptake of PAB by red cells, PAB uptake by parasites was measured under equilibrium conditions. As shown in Figure 4.2, uptake of 1 to 10 μM of PAB was unaffected by the presence of 100 μM of sulfamethoxazole. Sulfadiazine and sulfadoxine were also tested and did not effect the PAB uptake either (data not shown). In contrast, 100 μM unlabelled PAB sufficently blocked the uptake, demonstrating that the assay could detect inhibition of uptake. Furthermore, as will be shown in next chapter, this is also true when the initial rate of the PAB transport is measured. Therefore, PAB transport into parasite-infected red cells is not an additional target for sulfa drug action.

4.3 Sulfa drugs impair folate synthesis

As discussed in introduction, in *Streptococcus pneumoniae* and *Leishmania major*, targets other than the folate synthesis pathway have been suggested for sulfa drugs. In order to confirm that sulfa drugs indeed act on the folate synthesis pathway in malaria, we tested the effect of sulfamethoxazole on both the utilization of PAB and the total folate synthesis in malaria.

The metabolism of [^{14}C-] PAB by parasite-infected red cells could be measured because we found that metabolized [^{14}C-] PAB is selectively retained by the red cells. Normal red cells do not metabolize PAB. Ninety percent of [^{14}C-] PAB accumulated inside normal red cells after incubation with 2 μM of [^{14}C-] PAB for 2 hours was released into media lacking PAB in a hour (Fig. 4.3). Similar result was obtained when normal red cells were incubated with 2 μM of [^{14}C-] PAB for 16 hours (Figure 4.4b). Malaria parasite-infected red cells (concentrated by gelatin flotation), on the other hand, could only release a portion (28%) of the accumulated labelled PAB (Figure 4.4a), indicating that metabolites of [^{14}C-] PAB in parasites can not be released as efficiently under these experimantal conditions. This confirms that PAB is indeed metabolized in infected red cells.

To measure the effect of sulfa drugs on PAB metabolism, parasite infected red cells were preincubated for 24 hours in media containing 200 nM of sulfamethoxazole, which was also included in the subsequent incubation with [^{14}C-] PAB. In parasite infected red cells, the drug treatment resulted in a increased PAB release (52%; Figure 4.4c) compared to without drug treatment (28%; Figure 4.4a), indicating a decreased PAB metabolism. In contrast, the drug treatment did not affect the PAB release by control cells (Figure 4.4b and d). This demostrated that sulfamethoxazole interupted a PAB utilizing pathway, which by all our knowledge, is the folate synthesis pathway

Folate and its derivatives synthesized from labelled PAB can be detected by using folate binding proteins. Folate binding proteins have high affinities for dihydropteroate, dihydrofolate, tetrahydrofolate, and tetrahydrofolate derivatives, but have minimum affinity for PAB (Kamen and Caston, 1986). Therefore, [^{3}-H] PAB and [^{3}-H] folates in a sample can be separated by first incubating the sample with folate binding protein and then seperating the protein fraction from the fraction containing small molecules by gel filtration. Using this method, folate derivatives synthesized in parasite-infected red cells were measured after incubating these cells with [^{3}H-] PAB for 24 hours. Malaria parasite infected red cells were separated from uninfected red cells by gelatin flotation (30 % parasitemia). The fraction of uninfected red cells from gelatin flotation (parasitemia < 3 %) was used as an internal control. As shown in Figure 4.5, in parasite infected red cells, 5.2 % of [^{3}H-] PAB was converted to folates while in uninfected red cells (internal control), only 0.5 % of the [^{14}C-] PAB was converted to folates. When the same measurements were made using infected red cells treated with 200 nM sulfamethoxazole for 24 hours prior to the incubation with [^{3}H-] PAB, [^{3}H-] folates was only 2.2 % of [^{3}H-] PAB added in infected red cells. While in uninfected red cells (internal control), it is the same as in the absence of the drug treatment. It was confirmed that sulfa drugs indeed interupted folate synthesis in malaria parasites. This method of separating [^{3}H-] PAB and [^{3}H-] folates using folate binding proteins was later optimized by Merali (Zhang et al., 1991).

4.4 Discussion:

We have shown that infected red cells take up significantly more sulfamethoxazole than uninfected red cells. Sulfamethoxazole uptake was proportional to parasitemia, indicating that the presense of parasites is responsible for this increased drug uptake. An

intraparasitic drug concentration was estimated from these data. What is the importance of this observation? Compared to an extracellular concentration of 200 nM, the estimated intraparasitic concentration of 4.3 μM is much closer to the K_i of sulfamethoxazole for DHPS (36 μM). Thus, the concentration of sulfonamides by parasites may explain why their LC_{50}s are so much lower than their IC_{50}s and K_is. There is still a 10 fold difference in the estimated intraparasitic drug concentration and the K_i for DHPS. However, the assumed average parasite size (2.5 μm radius) may have been too large. If we assume these parasites have sizes of 1.5 μm radius instead, the intraparasitic drug concentration can be calculated to be another 5 fold higher. Other factors such as differece in the enzyme assay buffer and parasite cytosol, may also attribute to the observed difference between LC_{50}s and K_is.

Another possible reason that LC_{50}s for parasite growth and IC_{50}s for DHPS of sulfa drugs are so different is that sulfa drugs may inhibit a target other than DHPS. We have shown here that the equilibrium transport of PAB into parasite infected red cells is not inhibited by sulfa drugs. Therefore, the PAB transport is not an alternative target of sulfa drugs. Could there be a sulfa drug target outside of folate synthesis pathway? Sulfamethoxazole, at a concentration inhibitory of parasite growth (200 nM), inhibited both the PAB utilization (PAB is utilized for folate synthesis) and total folate synthesis. Taken together, these data indicate that folate synthesis, instead of another process such as PAB transport, is the target of sulfa drug action in malaria. And the observation that sulfa drugs inhibit parasite growth at concentrations much lower than those inhibiting DHPS can probably be explained by the concentration of sulfa drugs in parasites.

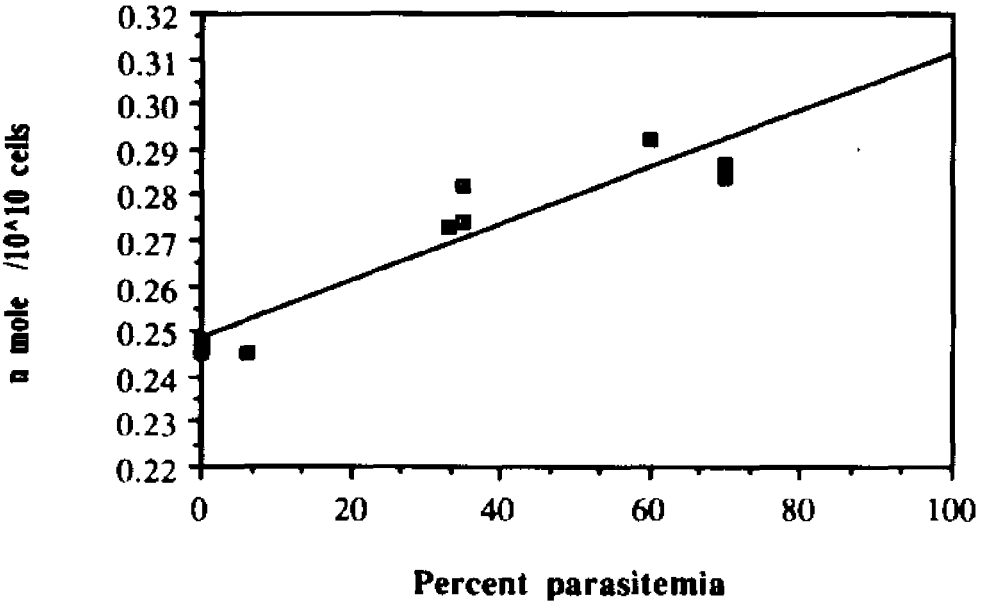


Figure 4.1 : Parasitemia dependence of [^{3}H-] sulfamethoxazole uptake by infected red cells. Radioactivities associated with red cells were measured after incubating 5 % of the red cells of different parasitemia levels with 200 nM [^{3}H-] sulfamethoxazole for 15 min at 37°C.

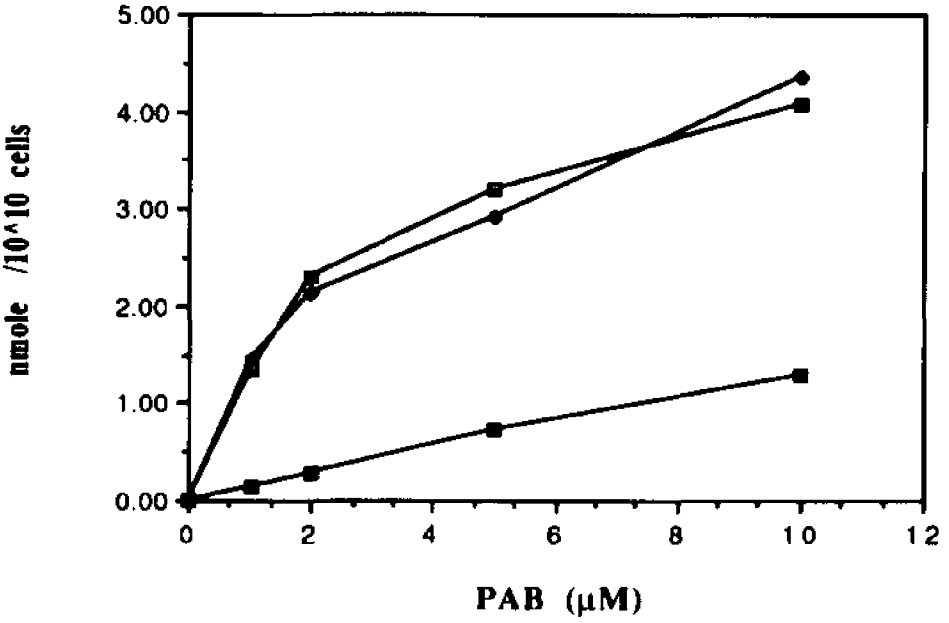


Figure 4.2 : Competition of [^{14}C-] PAB uptake by unlabelled PAB and sulfamethoxazole. ■ , [^{14}C-] PAB alone; ◆ , [^{14}C-] PAB plus 100 μM sulfamethoxazole; ■ , [^{14}C-] PAB plus 100 μM unlabelled PAB.

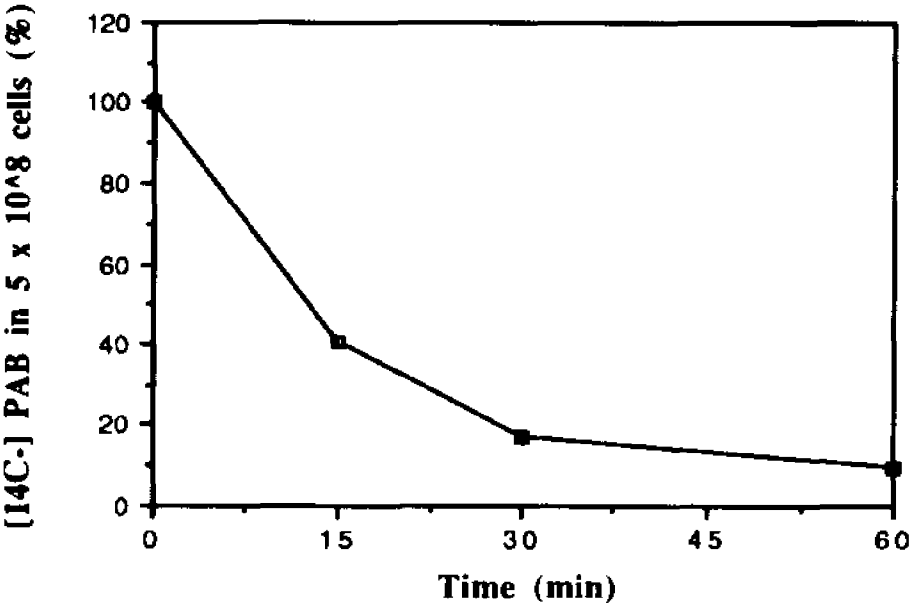


Figure 4.3 : The time course of [^{14}C-] PAB release by normal red cells. The radioactivities remain associated with normal red cells preincubated with [^{14}C-] PAB for 2 hours were measured after incubating these cells in media free of PAB for indicated time.

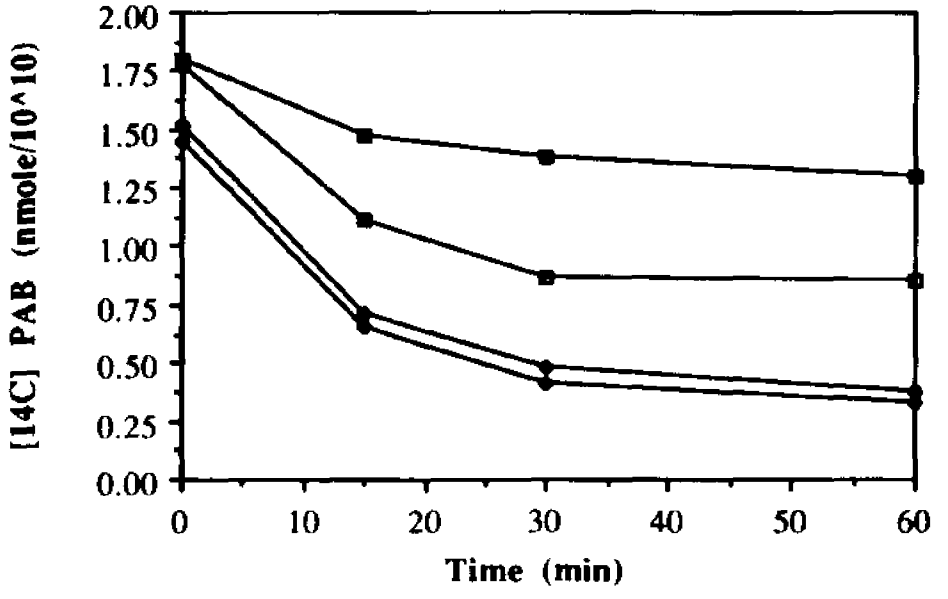


Figure 4.4 : [^{14}C-] PAB release from infected red cells preincubated with [^{14}C-] PAB for 16 hours. [^{14}C-] PAB release was measured using a). infected red cells (■ ; 30 % parasitemia; separated by gelatin flotation), b). control cells (◆ ; internal control; parasitemis < 3 %), c). infected red cells treated with 200 nM sulfamethoxazole for 24 hours prior to the incubations with [^{14}C-] PAB (■), and d). control cells treated with sulfamethoxazole (◆).

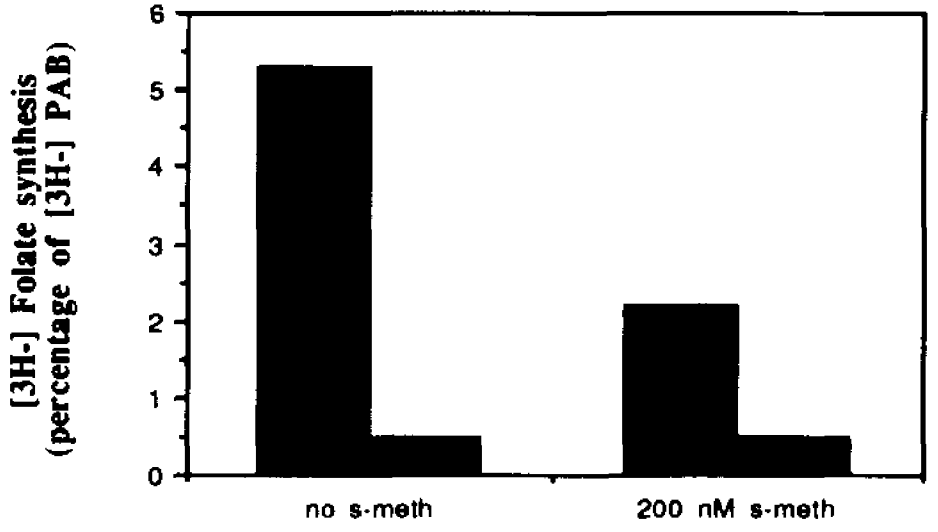


Figure 4.5 : The folate synthesis in *P.falciparum* infected red cells. [^{14}C-] folate was measured in infected red cells (solid bar; 30 % parasitemia; separated by gelatin flotation) and control cells (hatched bar; internal control; parasitemia < 3 %) with or without sulfamethoxazole treatment.

5. PAB IS TRANSPORTED INTO PARASITE-INFECTED RED CELLS

As discussed in the previous section, the malaria parasite depends on its *de novo* folate synthesis pathway for the supply of folate-type coenzymes, and one of the precursers of this pathway, PAB, is of extracellular nature. Little is known, however, about how parasites obtain PAB, and if this process can be interrupted. Thus, we examined PAB transport in normal and parasite-infected red cells.

5.1 Time course of PAB association with normal and infected red cells

The total PAB association with normal red cells increases with time and plateaus after about 3 min (Figure 5.1). Nonspecific [^{3}H-] PAB association, measured in the presence of an excess of unlabelled PAB (100 μM), is not time-dependent (Figure 5.1). The specific association, determined by subtracting the non-specific association of PAB from the total PAB association, is linear for approximately 60 seconds (Figure 5.1). The initial rate of PAB association with red cells is 29 fmol/10^8 cells/min. The association of PAB with parasite-infected red cells in the presence and absence of cold PAB follows a similar time course, and yields an initial rate of specific PAB association of 32 fmol/10^8 cells/min (data not shown).

5.2 Saturable and non-saturable PAB association

The concentration-dependent association of PAB with normal and parasite-infected red cells was measured using 15 second incubations (Figure 5.2), a time period during which uptake is linear (Figure 5.1). Radiolabelled PAB becomes associated with both normal and parasite-infected red cells in a concentration-dependent manner. The total association is not saturable, however, since it does not plateau at PAB concentrations as high as 2 mM (data not shown), suggesting that it includes a non-specific component. In order to distinguish a specific, saturable PAB transport mechanism from non-specific association, the initial association of labelled PAB was measured in the presence and absence of an excess of unlabelled PAB (100 μM). As shown in Figure 5.2, [^{3}H-] PAB association in the absence of cold PAB is non-saturable, but when [^{3}H-] PAB association in the presence of 100 μM cold PAB is subtracted from this, the concentration-response is saturable. The apparent K_d for this association is 83 ± 31 nM for normal red cells and 111 ± 40 nM for infected red cells. When K_ds are determined using 60 second incubations, the values are almost identical (83 ± 5 nM and 101 ± 1 nM, respectively, data not shown).

5.3 Counter-transport

When total [^{3}H-] PAB association is measured (Figure 5.3), uninfected red cells preloaded with cold PAB accumulate significantly more labelled PAB than uninfected red cells depleted of PAB. This confirms that labelled PAB is indeed transported into red cells; the increased accumulation represents decreased efflux of labelled PAB due to its intracellular dilution by unlabelled PAB (Kaczorowski, 1979). For the non-saturable component of the PAB association (measured in the presence of 100 μM of unlabelled

PAB), however, no difference is observed between red cells loaded with PAB and red cells depleted of PAB (Figure 5.3). This confirms that the saturable component of PAB association, but not the non-saturable component, represents PAB transport into red cells.

5.4 pH dependence

The saturable PAB transport and the non-saturable PAB association in uninfected red cells have a different pH dependence (Figure 5.4). Saturable PAB transport peaks at pH 6.5. Non-saturable PAB association increases almost linearly as pH decreases, suggesting that it favors the protonated form of PAB.

5.5 Discussion

As shown here, there appear to be two components in the PAB association with normal and parasite-infected red cells. One, which is non-saturable, is not altered when unlabelled PAB is added to the media. This is not suprising because PAB is amphipathic and is known to associate with membranes. Indeed, PAB has been used to label red cell membranes (Premechandra et al., 1989). The other component of the total PAB association with red cells, revealed by subtracting the non-saturable component from the total PAB association, is a saturable process, with a very high affinity for PAB (K_d = 83 nM).

The counter-transport experiment (Figure 5.3) demonstrates that the saturable component of PAB association with red cells represents specific transport into the red cells while the non-saturable does not. The different pH dependence confirms the presence of two processes.

These data indicate that PAB is transported into normal and parasite-infected red cells. This transport process is saturable, has a high affinity for PAB, and can be differentiated from the non-specific association of PAB with red cells. Furthermore, PAB transport into normal and parasite-infected red cells have a similar time course, concentration dependence, affinity for PAB, and as will be shown in the next chapter, energy dependence and inhibitor sensitivities. This suggests that among the three membranes PAB must be transported across in infected red cells, the transport across the red cell membrane (which is the only membrane involved in the case of normal red cells) is the rate-controlling step.

In malaria parasites, extracellular PAB is utilized by their *de novo* folate synthesis pathway to synthesize folates. The transport of PAB into parasites, therefore, represents the first step in the folate synthesis. Inhibition of the PAB transport into parasites could be detrimental for parasites. Furthermore, this type of inhibitor is expected to be synergistic with antifolates. If found, they could be used in combination with antifolates so that to lower the effective doses of antifolates and to reduce or eliminate their side effects.

Encouraged by its potential as a chemotherapeutic target, we characterized this PAB transport system and searched for inhibitors. These will be presented in the next chapter.

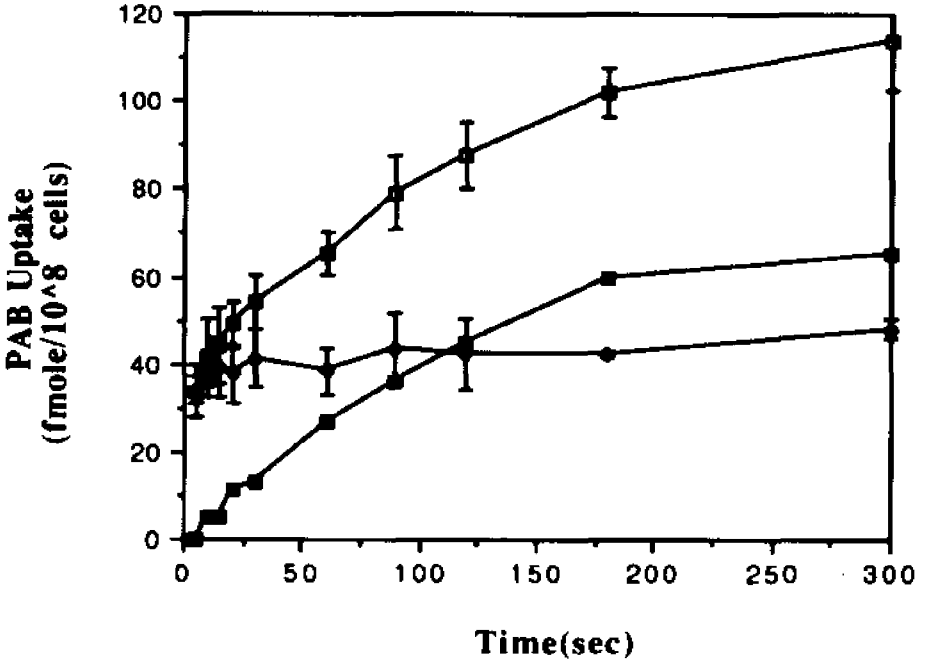


Figure 5.1 : The time course of [^{3}H-] PAB association with uninfected red cells. [^{3}H-] PAB association with red cells were measured in the absence (■) or presence (◆) of 100 μM unlabelled PAB in an extracellular [^{3}H-] PAB concentration of 5 nM as described in the Materials and Methods. The difference between the two curves is also shown (■). Data shown are the means of 6 readings ± standard deviations.

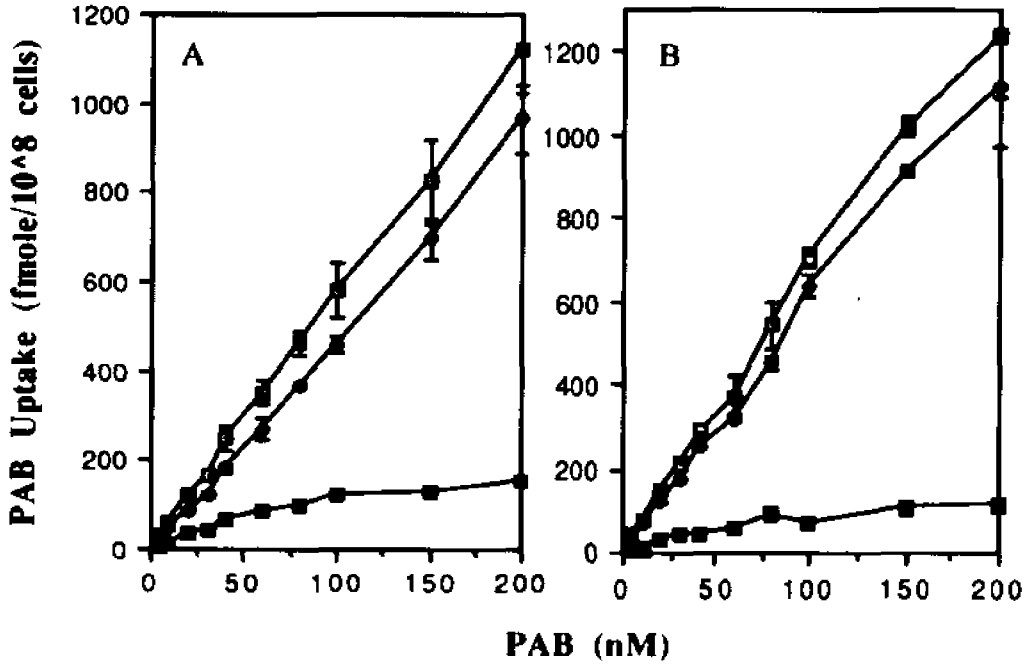


Figure 5.2 : Concentration-response of the association of [^{3}H-] PAB with uninfected (**A**) and infected (**B**) red cells. [^{3}H-] PAB associated with red cells were measured after 15-second incubations in the absence (◘) or presence (◆) of 100 μM unlabelled PAB as described in the Materials and Methods. The difference between the two curves is also shown (◘). Data shown are the means of 3 readings ± standard deviations.

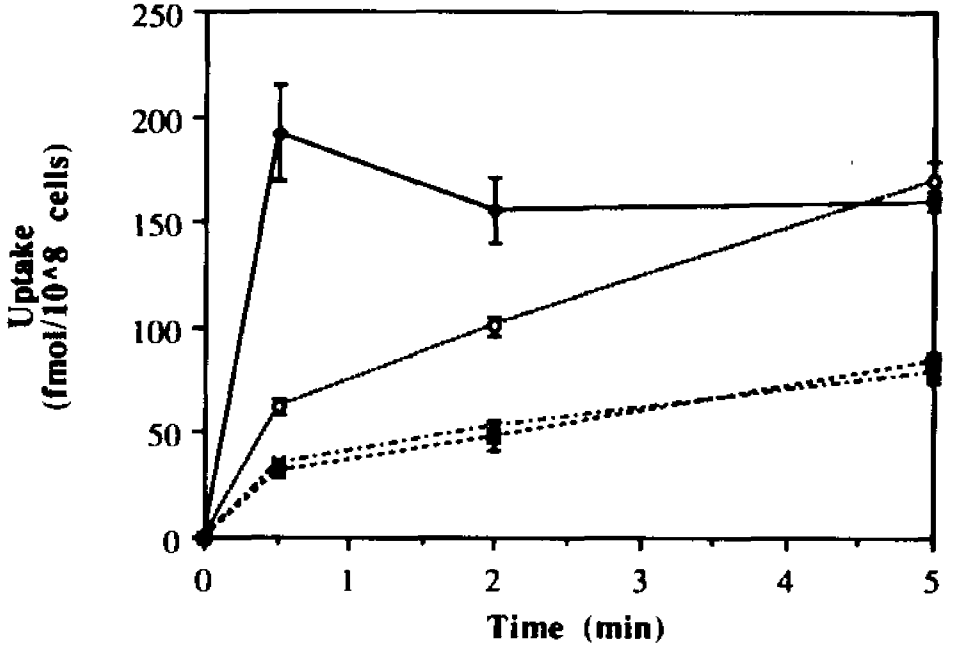


Figure 5.3 : Counter-transport of [^{3}H-] PAB into red cells. The association of [^{3}H-] PAB (5 nM) was measured using red cells preloaded with unlabelled PAB (◆), and depleted of PAB (o) in PFF RPMI. Association was also measured in red cells preloaded with unlabelled PAB (x), and depleted of PAB (■) in PFF RPMI containing 100 µM unlabelled PAB. Data shown are the means of 4 readings ± standard deviations.

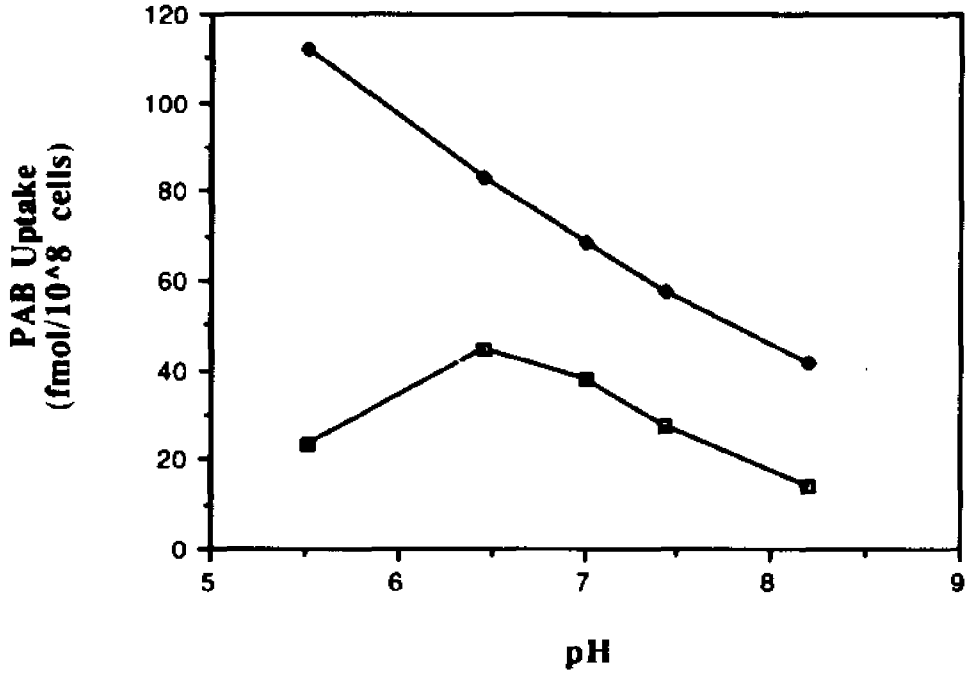


Figure 5.4 : pH-dependence of the PAB transport (■) and non-saturable PAB association (◆).

6. MECHANISM OF PAB TRANSPORT IN NORMAL AND PARASITE-INFECTED RED CELLS.

In order to understand the mechanism of the PAB transport system described in the last chapter, and to assess its potential as a chemotherapeutic target, we would like to know the following about the transporter: what is its energy dependence? Is it one of the known red cell transport systems? What other substrates does it transport? This last question is important for two reasons. First, knowing the substrate specificity would give a clue about the binding site of the transporter. Second, in order to be a chemotherapeutic target, the transporter should not be essential for red cell functions. And finally, we wanted to know if any inhibitors for this transporter exist.

6.1 Energy dependence

At 4°C or when 20 mM sodium azide was included in the media, the saturable PAB transport in uninfected red cells is abolished (Figure 6.1a). In contrast, the nonsaturable PAB association with red cells is affected by neither temperature nor azide (data not shown). Under these conditions, the nonsaturable PAB association was slightly higher in the presence of unlabelled PAB than in its absence for some unknown reason; this is responsible for the negative values shown in Figure 6.1. Similar results are obtained with parasite-infected red cells (Figure 6.1b). PAB transport, therefore, appears to be an energy-dependent process.

In order to identify the energy source used in PAB transport, the effects of nigericin, monensin and carbonylcyanide m-chlorophenylhydrazone (CCCP), which are ionophores,

and iodoacetamide (IAA), a glycolysis inhibitor, were measured. As shown in Figure 6.2, sodium ionophores nigericin and monencin have little effect on PAB transport in uninfected red cells. Also consistent with this is the observation that sodium concentration in the media does not effect the PAB transport (data not shown). In contrast, the proton ionophore, CCCP, and the glycolysis inhibitor, IAA, inhibit PAB transport. None of the inhibitors tested affects the non-saturable association (data not shown).

6.2 Correlation with intracellular ATP level

A combination of IAA and potassium fluoride depletes red cells of intracellular ATP without affecting some transport systems, such as nucleotide transport (Plagemann et.al. 1985). This combination, in contrast, inhibits PAB transport in red cells. Using uninfected red cells treated by this procedure, we measured PAB transport under ATP-depletion conditions. As shown in Figure 6.3, the PAB transport in red cells decreases as the intracellular ATP level decreases, with a good correlation between PAB transport and cellular ATP level (R^2 = 0.98). This shows intracellular ATP is required for PAB transport.

6.3 PAS inhibits PAB transport

In order to characterize the PAB transporter, we tested the effects of a variety of reagents on the PAB transport into red cells. These reagents tested can be divided into three catageries: a). substrates and inhibitors of known red cell transporters, b). other plausible red cell nutrients, and c). structural analoques of PAB.

p-Amino salicylic acid (PAS), a structure analog of PAB, was found to be a potent inhibitor of PAB transport. The IC_{50} of the PAS inhibition of PAB transport is 100 nM (Figure 6.4). As shown in Lineweaver-Burke plot (Figure 6.5), the inhibition is competitive with PAB and the inhibition constant (K_i) is 378 ± 90 nM.

6.4 Other inhibitors of PAB transport

There are three known red cell transporters which could facilitate PAB transport. These include the anion exchanger, the monocarboxylate transporter, and the glucose transporter. Due to the lack of structural similarity between its substrates and PAB, it is very unlikely that the glucose transporter could mediate PAB transport. This was confirmed by the experimental results that neither the presence or absence of glucose nor the presence of forskolin, a glucose trnasport inhibitor (Lavis et al., 1987), affected the PAB transport. Similarly, two substrates of the monocarboxylate transporter, lactate and pyruvate, were tested and found not to affect PAB transport. Therefore, PAB transport in red cells is clearly not mediated by these two red cell transporters.

The anion exchanger of red cells, although better known for exchanging bicarbonate and chloride anions as well as other inorganic anions, also mediates the transports of some organic anions (Jennings, 1989). Oxalic acid, a substrate for the anion exchanger (Aubert and Motais, 1975), was tested for its effect on PAB transport into red cells. At 2 μM, which is 400 times higher than the concentration of PAB, oxalic acid inhibits PAB transport by 30%. Furthermore, phloretin, flufanamic acid and 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid (DITS), which are inhibitors of the anion exchanger (Band 3), also inhibit PAB transport partially (Figure 6.5 A). Similar results were obtained when

parasite-infected red cells were used (Figure 6.5 B). The concentrations used (200 μM) completely inhibit inorganic anion exchange (Eaton et al., 1980; Ku et al., 1979). Interestingly, the combination of these anion exhanger inhibitors and 200 nM PAS results in a much greater degree of inhibition in both types of cells (Figure 6.5 A and B). In addition, the fact that each of these reagents has similar effects on PAB transport in normal and infected red cells confirms the conclusion made in the last chapter that the same transport system is operative in both normal and parasite-infected red cells.

Other reagents which were tested and found not to effect PAB transport include taurine, tyrosine, phenylalanine, folate, folinic acid, sulfamethoxazole.

6.5 Discussion

We showed here that the PAB transport into red cells is abolished when temperature is lowered to 4°C. This suggests that PAB transport is most likely an energy dependent process, because at 4°C, metabolic processes which supply energy to cells are inactivated. It could also be possible that the changed properties of membrane phospholipids at this temperature inhibited the PAB transport. However, both the sensitivities of PAB transport to glycolysis inhibitor and proton ionophores and the correlation between PAB tranport and intracellulat ATP level are consistent with the former assessment.

Sensitivity of a transporter to azide usually indicates its dependence on metabolic energy, because azide inhibits oxidative phosphorylation which supplies cells with ATP. In this case, however, the sensitivity of PAB transport in red cells to azide is difficult to interpret because red cells do not have an electron transfer chain and depend exclusively on glycolysis for ATP generation. Since the PAB transport is sensitive to glycolysis inhibitors and proton ionophores but insensitive to sodium ionophores and sodium concentration

gradient, its energy source is not a sodium gradient as is for many transporters in eucaryotic cells, but could be either proton gradient or ATP. The correlation between PAB transport capacity and intracellular ATP level confirms this assessment but does not differentiate the two possibilities, because the proton gradient is maintained by ATP in red cells.

Neither the substrates nor the inhibitors of the glucose transporter and monocarboxylate transporter inhibited PAB transport, indicating that these transporters are unrelated to PAB transport. However, both the substrate and the inhibitors of the anion exchanger partially inhibited PAB transport. Could PAB transport be carried out by the anion exchanger? A portion of the total PAB transport, which is inhibited by the three anion exchanger inhibitors and also oxalic acid, is probably carried out by the anion exchanger. The three anion exchanger inhibitors are structurally unrelated and inhibit the anion exchange by binding to different sites. Therefore it is quite unlikely that the three inhibitors all happen to also inhibit an unrelated transporter. Furthermore, this would also be consistent with the energy dependence of PAB transport since the anion exchanger must be phosphorylated by ATP to function (Bursaux et al., 1984). The portion of the total PAB transport which is not inhibited by these reagents, however, is most likely an independent transporter. This transporter is specific for PAB and energy dependent. Thus, PAB may resemble lactic acid, which is transported into red cells by both the anion exchanger and the monocarboxylate transporter (De Bruijne et al., 1983).

PAS effectively inhibits PAB transport by competing with PAB. This offers a new avenue for antimalarial drug design, since the transport of PAB is essential neither for red cells nor for other mammalial cells. A PAB transport inhibitor such as PAS has not been tested for *in vitro* antimalarial activity before. Any effective PAB transport inhibitor can be

expected to inhibit parasite growth synergistically with inhibitors of the folate synthesis pathway, such as sulfa drugs and DHFR inhibitors.

Both PAS and anion exchanger inhibitors have potential as antimalarial agents. PAS, which has been widely used in the treatment of tuberculosis, has a maximum therapeutic serum concentration of up to 1,000 fold higher than its K_i for the inhibition of PAB uptake (378 nM) (Mandell et al., 1985). A complete inhibition of PAB transport into red cells can therefore be achieved clinically. Anion exchanger inhibitors, on the other hand, have been previously found to inhibit malarial growth *in vitro* , and their mechanism of action was suggested to be the inhibition of permeation pathway (Cabantchik et al., 1983). If inhibiting PAB transport is also a mechanism of action of these anion exchanger inhibitors, a combination of PAS and anion exchanger inhibitors would be more effective at inhibiting parasite growth.

Although exogenous PAB is clearly utilized by the parasites, it remains to be determined to what extent would it be detrimental for parasites to deprive them of exogenous PAB. There are other possible sources of PAB for the intraerythrocytic parasites including intraerythrocytic PAB and *de novo* PAB synthesis (Dieckmann and Jung, 1986). It is important to characterize the *de novo* PAB synthesis pathway and its importance for malarial folate synthesis and parasite survival. Inhibitors of PAB synthesis and PAB transport may have to be used together in order to block the availability of PAB to parasites completely.

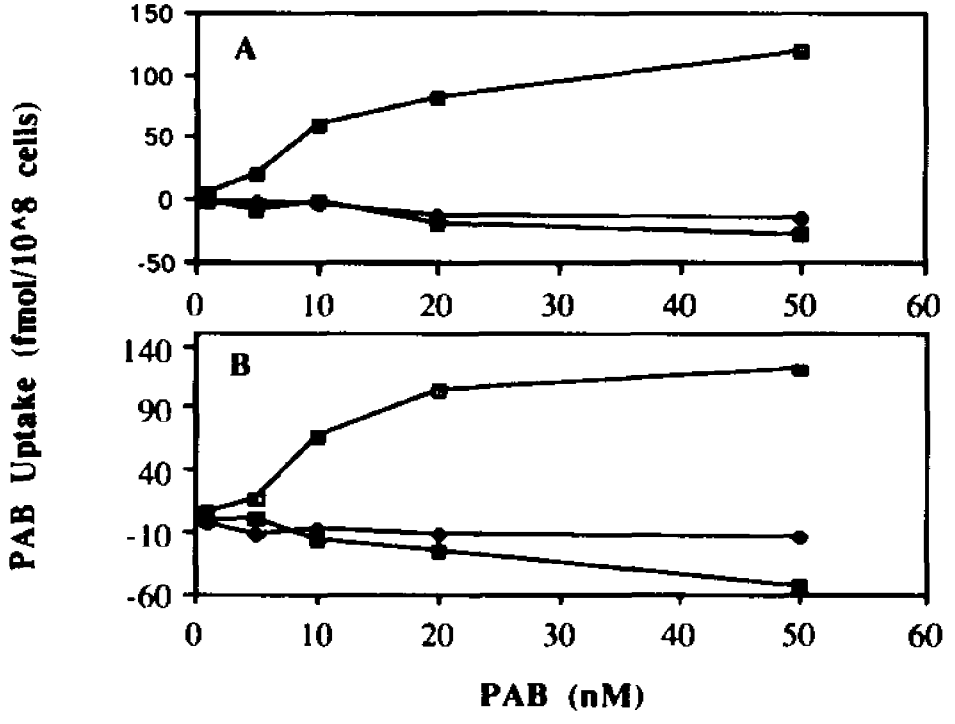


Figure 6.1 : Energy dependence of PAB transport. [^{3}H-] PAB transport into uninfected (**A**) and infected (**B**) red cells was measured at 36°C (■), at 4°C (◆), and at 36°C in the presence of 20 mM azide (◘). Data shown are averages of 4 readings.

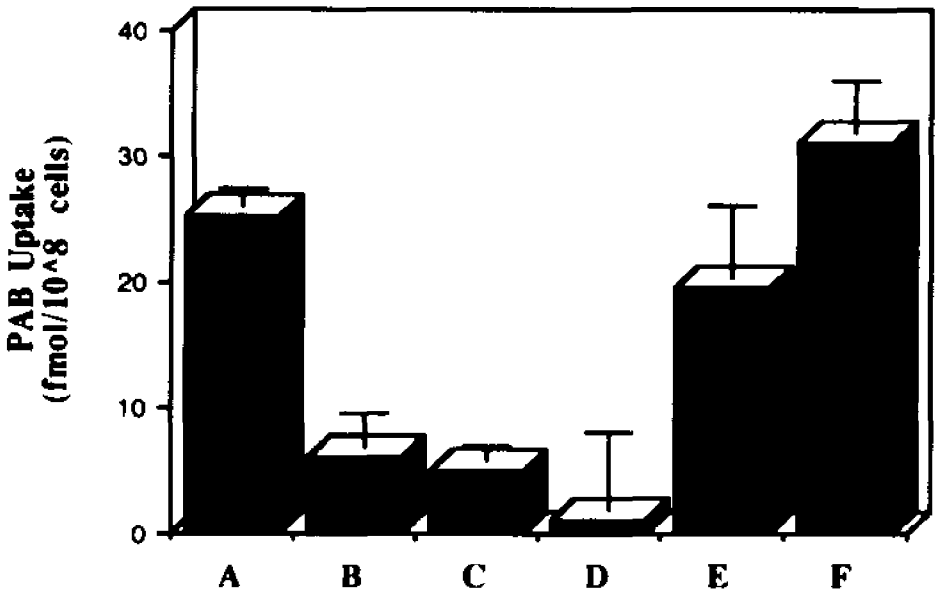


Figure 6.2 : The effects of ionophores and glycolosis inhibitor on [^{3}H-] PAB transport into uninfected red cells. A: no inhibitor, B: 1 mM IAA. C: 1 μM CCCP, D: 10 μM CCCP, E: 1 μM nigericin, and F: 1μM monensin. Data shown are the means of 4 readings ± standard deviations.

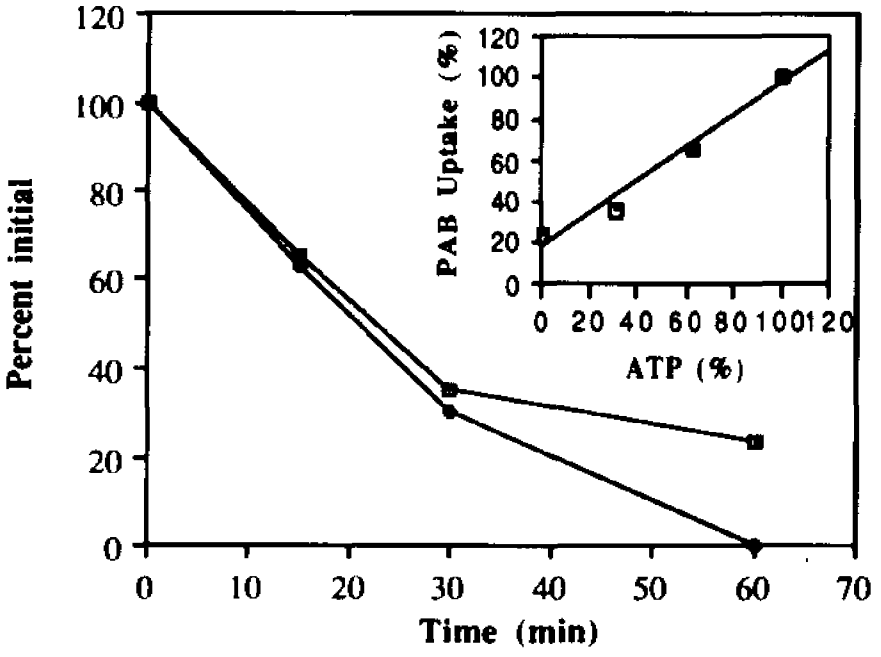


Figure 6.3 : [^{3}H-] PAB transport and ATP levels in fluoride- and IAA-treated uninfected red cells. ATP levels (◆) and PAB uptakes (■) are shown as percentage of initial amount. Insert shows PAB uptake plotted against ATP level ($R^2 = 0.98$)

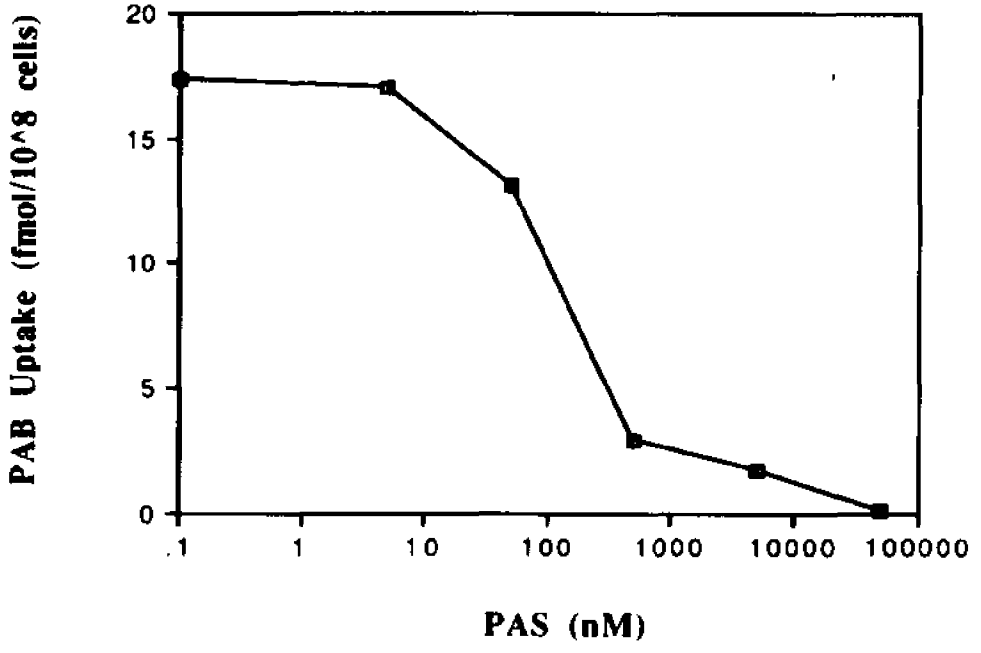


Figure 6.4 : The inhibition of [^{3}H-] PAB transport by PAS. Uninfected red cells were incubated with 5 nM [^{3}H-] PAB for one minute at 36°C in the presence of varying concentration of PAS, and the uptake was measured as described in the Methods.

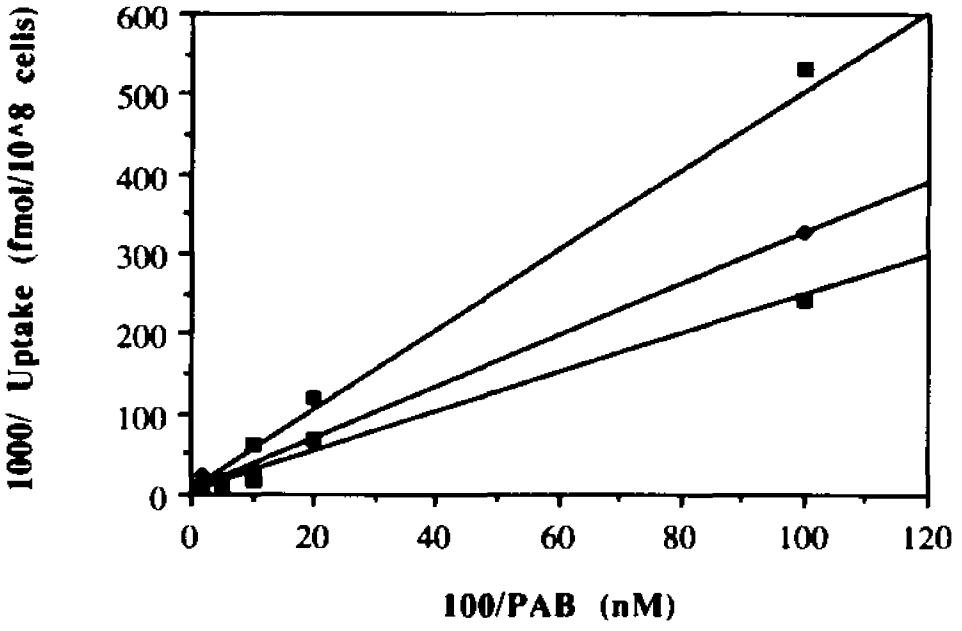


Figure 6.5 : Lineweaver-Burk plots of the inhibition of PAB transport by PAS. PAB transport into uninfectd red cells at indicated extracellular [^{14}C-] PAB concentrations was measured in the absence of PAS (■), in the presence of 200 nM PAS (◆), and in the presence of 1μM PAS (●).

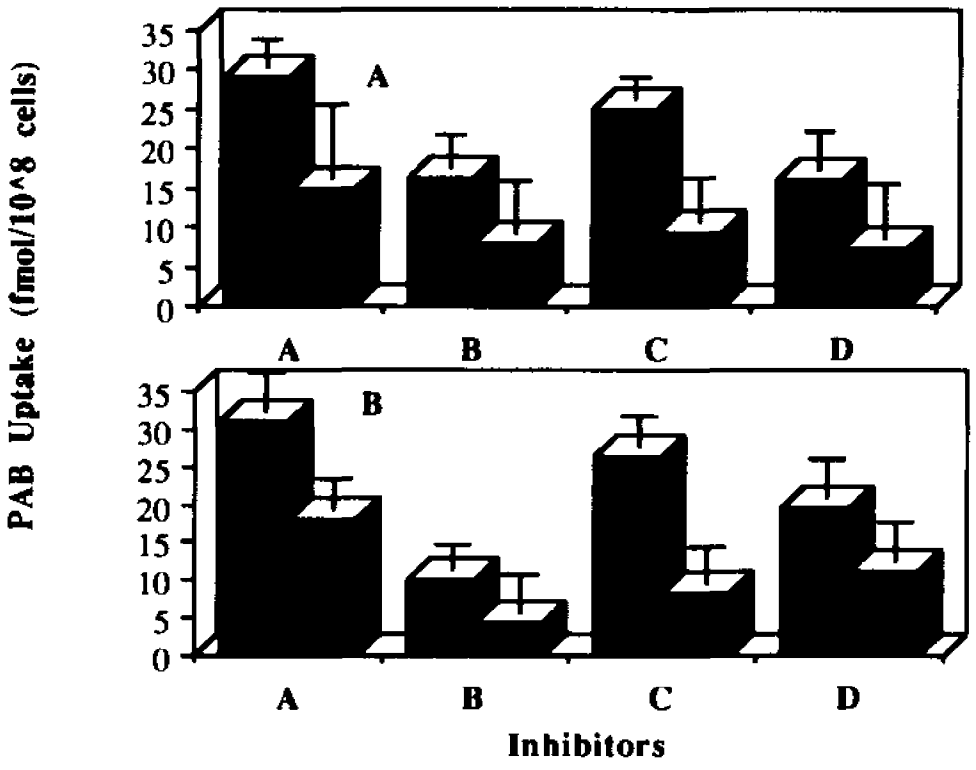


Figure 6.6 : Effects of inhibitors on [^{3}H-] PAB transport in uninfected (Graph **A**) and infected (Graph **B**) red cells. A: no anion exchanger inhibitor, B: 200 μM phloritin, C: 200 μM flufanamic acid, D: 200 μM DITS. Solid bars are uptake in the absence of PAS. Hatched bars are uptake in the presence of 200 nM PAS. Data shown are the means of 4 readings ± standard deviations.

7. SUMMARY AND FUTURE DIRECTIONS

7.1 Summary

The major findings in this thesis can be summarized as the following:

1. Dapsone, sulfamoxole, sulfamethoxazole, sulfaquinoxaline, sulfadiazine, and sulfadoxine inhibit the growth of *P. falciparum* in vitro (LC_{50}) at 31 to 519 nM and inhibit DHPS (IC_{50}) at 33 to 500 μM.

2. These drugs do not inhibit an alternative target, PAB transport into infected red cells, but they do impair folate synthesis. It is suggested that sulfa drugs exert their antimalarial effects by inhibitig DHPS, and they can do so at LC_{50}s 100 - 1000 times lower than IC_{50}s because they are concentrated in parasites by an estimated 20 times.

3. PAB is transported into normal and parasite infected red cells by a specific, saturable process which can be differentiated from a non-saturable association of PAB with red cells. This transport system of red cells is rate-controlling in the PAB transport in infected red cells, because infected red cells have similar time course, affinity, energy dependence, and inhibitor sensitivities as normal red cells.

4. The PAB transport in red cells depends on cellular ATP but not sodium gradient. It appears to be mediatd by two separate transporters, the anion exchanger and a specific transporter which was hitherto not known. This is suggested because a portion of the total PAB transport is inhibited by both the substrate and the inhibitors of the anion exchanger, while the other portion is not inhibited by substrates or inhibitors of any known red cell transporter.

5. PAB transport is competitively inhibited by PAS. The therapeutic potential of this observation should be further evaluated.

7.2. Future directions

1. In order to understand the mode of action of sulfa drugs, it is important to know more acurately the intraparasitic concentration of these drugs. Now Trager et al. (1990) have developed the method of cultivation *P. falciparum* parasites in the absence of intact red cells, it might be possible to measure the uptake of sulfa drugs by parasites directly so that the intracellular drug concentration can be assessed.

2. The specific PAB transporter of red cells studied is hitherto not known. We have shown it is not one of the three red cell transporters examined and it does not transport aromatic amino acids and monocarboxylates. It would be interesting to know the physiological function of transporter and its protein structure.

3. PAS inhibit PAB transport effectively. Structural analogs of PAS should be screened and better PAB transport inhibitors may be found.

4. The relative importance of exogenous PAB, as compared to de novo PAB synthesis, is not well understood. The PAB metabolism and PAS sensitivities of PAB auxotrophic mutant malaria strains (McConkey and McCutchan, 1991) should be studied.

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