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BIOCHEMICAL STUDIES ON ANTIPARASITIC DRUGS

by

Yu-Long Hong

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

1996

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ABSTRACT

BIOCHEMICAL STUDIES ON ANTIPARASITIC DRUGS

by

Yu-Long Hong

Adviser: Dr. Steven R. Meshnick.

Understanding the mechanisms of antiparasitic drugs is necessary for the development of new drugs. The modes of action of sulfa drugs and artemisinin are studied in this thesis.

P. carinii synthesizes folates de novo from exogenous p-aminobenzoic acid (pABA). The predominant forms of folates in *P. carinii* are pteroylpentaglutamates. Culture-derived *P. carinii* produces pteroylpentaglutamates at a four-fold higher specific activity than lung-derived organisms do, possibly because they contain less contaminating lung debris.

Forty-four sulfa drugs were screened against crude preparations of recombinant *P. carinii* dihydropteroate synthetase (DHPS). The apparent Michaelis-Menten constants (K_m) for p-aminobenzoic acid and 7,8-dihydro-6-hydromethylpterin pyrophosphate are 0.34 ± 0.02 and 2.5 ± 0.71 μM, respectively.

Several sulfa drugs inhibit DHPS approximately as well as sulfamethoxazole, as determined by the concentrations which cause 50% inhibition and/or by K_i values. For all sulfones and sulfonamides tested, unsubstituted p-amino groups are necessary for activity, and sulfonamides containing N1-heterocyclic substituent are found to be the most effective inhibitors.

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Folate biosynthesis in isolated intact *P. carinii* is approximately equally sensitive to inhibition by sulfamethoxazole, sulfathiazole, sulfachlorpyridazine, sulfamethoxypyridazine, and sulfisoxazole. Two of these drugs, sulfamethoxy-pyridazine and sulfisoxazole, are known to be less toxic than sulfamethoxazole and should be further evaluated for the treatment of *Pneumocystis carinii* pneumonia (PCP).

The recombinant *P. carinii* Fas was expressed as a glutathione S-transferase fusion protein in baculovirus-infected insect cells, and then purified by affinity chromatography using glutathione beads. The overall yield of DHPS activity was 4.6% with a purification of 39-fold.

In vitro, hemin and artemisinin were found to undergo a chemical reaction forming two major products which were isolated by high-performance liquid chromatography (HPLC). The *m/z*'s of the two products were 856 and 871.

[^{14}C]-Artemisinin was taken up by *Plasmodium falciparum* in culture and concentrated in hemozoin. Majority of the hemozoin-associated radioactivity comigrated with the synthetic adducts.

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1. INTRODUCTION

1.1. *Pneumocystis carinii* Pneumonia--historical background.

Pneumocystis carinii Pneumonia (PCP) is a fatal pulmonary disease which only occurs in immunocompromised hosts. The disease is caused by an opportunistic pathogen--*Pneumocystis carinii.*

P. carinii was first described in 1909 by Chagas in the lungs of guinea pigs infected with trypanosomes. However, he thought that these parasitic forms were just the sexual states in the life cycle of trypanosomes and not a different organism. In 1912, Delanöe and his wife in Paris found the identical forms of parasites in the lungs of rats that had not been infected with trypanosomes. Therefore they proposed that these organisms represented a new genus and species. They named the organism *P. carinii* in honor of Dr. Carini [reviewed by Walzer et al, 1989].

Although in the following years, *P. carinii* was found in the lungs of humans and many other animals, such as rabbits, monkeys and mice, the parasite was still not recognized as a pathogen. It was not until 1952, that Vanek and Jirovec established *P. carinii* as the cause of interstitial plasma cell pneumonia [Vanek et al, 1952]. Soon many reports substantiated the etiologic relationship of *P. carinii* to pneumonitis of infants.

During World War II in Europe, PCP was very common in children and premature infants who ware malnourished or debilitated and had very high mortality rate [Gajdusek, 1957]. After the war, with the improvement of nutritional conditions, PCP gradually disappeared from Europe.

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In 1950s corticosteroid therapy, immunosuppressive anticancer drugs and broad-spectrum antibiotics gained widespread usage. These developments in medicine caused immunodeficiency in patients who were treated with these drugs. PCP in immunocompromised child and adults began to emerge. In the late of 1950s, Ivady and Paldy reported the successful treatment of PCP with pentamidine, and the drug became generally accepted as the first anti-PCP agent in the world [Ivady et al, 1967].

Medical interest in *P. carinii* in the United States developed in the 1960s when the organism emerged as an important cause of pneumonia in cancer patients or organ transplant recipients being treated with immnosuppressive drugs [Robbins, 1967]. In 1970s, Hughes and colleagues found that the combination of trimethoprim and sulfamethoxazole (TMP-SMX) was highly effective in both the therapy and prophylaxis of PCP [Hughes et al, 1974]. Since TMP-SMX was less toxic than pentamidine, it immediately became the drug of choice. The widespread use of TMP-SMX led to a marked decline in the number of cases of PCP [Hughes, 1984a].

In 1980s, PCP became prevalent in patients with acquired immunodeficiency syndrome (AIDS) in the United States and many countries in Western Europe [Gottlieb et al, 1981]. From then on, with the pandemic of AIDS, the number of PCP cases has steadily increased. Tens of thousands of AIDS patients suffered and finally died of this disease. The high rate of adverse reaction, slow response to treatment and frequent relapse to TMP-SMX and pentamidine illustrated the new challenges [Haverkos et al, 1984]. This serious situation stimulated a new era of research in basic and clinical science in the world.

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1.2. *Pneumocystis carinii* Pneumonia and AIDS

PCP is the most common serious opportunistic infection among persons in the United States who are infected with the human immunodeficiency virus (HIV) [Center for Disease Control and Prevention, 1994]. It has been estimated that, without any PCP prophylaxis, 60 to 80% of patients with AIDS will eventually develop PCP at least one episode during their life-time [Kovacs et al, 1984 and Murray et al, 1987].

For adults with HIV infection, the risk of developing PCP rises dramatically as the peripheral blood $CD4^+$ lymphocyte count drops below 200/µl or less than 20% of the total peripheral blood lymphocyte number [Masur et al, 1989]. Among HIV-infected children, the risk for PCP is the highest among infants < 1 year old. According to the study done by Simonds and colleagues, about 61% of children were diagnosed with PCP in the first year of their lives. In contrast, only 19% of children 1 through 12 years old were diagnosed with PCP [Simonds et al, 1993].

Among adults and adolescents with AIDS diagnosed from 1981 through 1992 and reported to the Center for Disease Control and Prevention (CDC), 51% have had PCP. This number decreased from 61% among persons with AIDS diagnosed in 1985-1987 to 43% among those with AIDS diagnosed in 1991-1992 [Simonds et al, 1995]. PCP is the most common AIDS-defining infection among children: 37% of children 0 to 12 years old with AIDS reported to the CDC through 1992 had PCP [Simonds et al, 1993]. Although the incidence of PCP among adults infected with HIV has declined in recent years due to the use of effective chemoprophylaxis for PCP, no decline in PCP incidence has been observed among HIV-infected children. PCP remains the

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most common AIDS-defining opportunistic infection [Wall et al, 1993 and Simonds et al, 1995].

PCP is also the leading cause of death of in AIDS patients. The mortality rate of PCP in AIDS patients is between 24% and 45% [Walzer et al, 1989 and Haverkos et al, 1984]. The mortality rate is higher in children with AIDS. As of December 31, 1992, 67% of children with PCP have died [Simonds et al, 1993].

Since AIDS patients are characterized by profound defects in their immune systems, they are also susceptible to other opportunistic infection diseases, such as bacterial infections and fungal infections. Besides PCP, Toxoplasmosis and Cryptosporidiosis are also major opportunistic parasitic diseases in AIDS patients [Feinberg et al, 1991].

1.3. The biology of *Pneumocystis carinii*.

Knowledge of the basic biology of *P. carinii* is severely limited by the lack of a reliable in vitro culture system. Most of the basic biological information has come from ultrastructural studies of infected rodents and humans or short-term cultures of *P. carinii* isolated from infected lungs .

P. carinii is a unicellular eukaryote that appears to be ubiquitous. In the immunocompromised hosts, *P. carinii* is an opportunistic pathogen, but it harmlessly presents in the lungs of a wide variety of hosts, such as human, rats and mice. Although the organisms in different hosts are morphologically indistinguishable, it is likely that different species or strains exists. Direct sequencing of a PCR amplified gene encoding for the large subunit of mitochondrial ribosomal RNA of *P. carinii* derived from both rat and human

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samples showed that the natural infections in man and in the rat are due to different strains of *P. carinii* [Wakefield et al, 1990 and Sinclair et al, 1991].

P. carinii infection in humans and in animals has a world wide geographical distribution. Several seroepidemiolgical studies have shown that the vast majority of the human population have antibodies to *P. carinii* by the age of 4 years old [Pifer et al, 1978 and Meuwissen et al, 1977]. This age-related exposure to *P. carinii* also appears to occur in rodents [Walzer et al, 1981]. These studies imply that *P. carinii* is ubiquitously distributed in even healthy mammalian hosts.

The infective form of *P. carinii* has not been identified, but as with other protozoan, the thick-wall cyst is more resistant to adverse environmental conditions and seems to be a logical candidate; however, this hypothesis is still controversial, because the relatively large size of the cyst makes it more susceptible to airway defense systems and less likely to reach the alveoli.

1.3.1. The taxonomy of *P. carinii*.

Previous attempts to address the phylogenetic placement of *P. carinii* were usually based on the ultrastructure of the organism, and there was no general agreement on the classification. Some researchers thought that *P. carinii* was a protozoan. This classification was based predominantly on morphologic data and insensitivity to antifungal drugs. Some workers classified it as a fungus because it could be stained with fungal stains (e.g. methenamine silver) and also because of certain ultrastructural characteristics [reviewed by Walzer et al, 1989].

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The controversy over *P. carinii* classification was finally solved by the application of molecular biology. The sequences of ribosomal RNA (18S-like rRNA) [Edman et al, 1988 and Stringer et al, 1989], mitochondrial DNA [Pixley et al, 1991] and other genes, such as thymidylate synthetase (TS) and dihydrofolate reductase (DHFR) [Edman et al, 1989a and Edman et al, 1989b], have revealed a close relationship with higher fungi. These new genetic information changed the old concept that *P. carinii* is a protozoan.

1.3.2. ***Pneumocystis carinii*** **cell structure and life cycles.**

Most studies of the cell structure and life cycles of *P. carinii* have relied on histochemical and ultrastructural analysis of organisms found in human and rat lung sections or on short-term cultures of *P. carinii* isolated from infected lungs. The complete life cycle of *P. carinii* is still poorly understood due to the lack of long-term cell culture [Cushion, 1989].

There are three developmental forms found in the life cycle of *P. carinii* : trophozoites, precysts, and cysts. The trophozoite is the smallest of the stages and is also referred to as a trophozoite with a size range of 1-5 μm. The most prominent feature of the trophozoite is the nucleus. Ultrastructural observations infer a haploid status for this form. Cytoplasmic organelles include one mitochondrion [Matsumoto et al, 1986 and Yoshida, 1989].

The precyst is considered an intermediate stage between the trophic and cystic stages with a size range of 4-6 μm. The precyst is oval and contains one or more nuclei and mitochondria. There are three subtypes of the precyst: early, intermediate and late precyst .

The cyst is the largest form with a size range of 5-8 μm. The cyst

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commonly contains up to eight daughter cells, referred to an intracystic bodies or sporozoites. The cell wall of the cyst has a thickness of 70-120 nm. Cysts are thought to be a resistant stage in the parasite's life [Campbell, 1972]. Although the mode of transmission from one human to another has not been established, inhalation of cysts from the air seems to be a reasonable explanation .

A possible life cycle of *P. carinii* is shown in **Figure 1.1** [Bogitsh et al, 1990].

1.3.3. General metabolism of *Pneumocystis carinii*

P. carinii may obtain all the nutrients in the form of low molecular weight substances found in the alveolar fluid [Barton et al, 1969]. The first metabolic and synthetic activities study in *P. carinii* was reported by Pesanti and Cox. They found that *P. carinii* was able to metabolize [^{14}C]-glucose to CO_2 [Pesanti et al, 1981]. Lactate dehydrogenase (LDH), which converts lactate to pyruvate, has also been detected in cysts [Mazer et al, 1987]. This suggested that *P. carinii* has the potential for anaerobic glycolysis .

The presence of mitochondria in *P. carinii* [Itatani et al, 1988], the incorporation of radiolabeled pyruvate into CO_2 [Pesanti et al, 1981], and the detection of other TCA enzyme activities [Mazer et al, 1987 and Pesanti, 1989] suggested that the organism also has the aerobic metabolic capacities including TCA cycles and ß-oxidation. The organism is also sensitive to selective electron transport chain (ETC) inhibitors, such as cyanide and atovoquone [Pesanti , 1984, Hughes et al, 1990 and Ittarat et al, 1995]. All these information indicated the presence of a functional ETC in *P. carinii* mitochondria.

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Kovacs and coworkers demonstrated that *P. carinii* was capable of de novo folate synthesis by the incorporation of the precursor [^{3}H]-p-aminobenzoic acid (pABA) into reduced folates. This de novo pathway can be inhibited by pABA analogs [Kovacs et al, 1989]. This important metabolic pathway will be further discussed in the latter section. The dihydrofolate reductase and thymidylate synthase genes have also been found and sequenced [Edman, et al, 1989a and Edman, U et al, 1989b].

The susceptibility of *P. carinii* to α-difluoromethylornithine (DFMO) and other inosine analogs suggests the presence of polyamine and purine salvage pathways, respectively [Lipchik et al, 1991, Clarkson et al, 1990 and Merali et al, 1996]. The organism may use inosine or hypoxanthine as a purine source [Bartlett et al, 1986].

1.3.4 The interaction between *P. carinii* and host lung cells.

Although the mechanisms by which *P. carinii* invades host cells are not known, the attachment of pathogenic organisms to host cells has been demonstrated to be an essential event in the pathogenesis and propagation of many pathogens [Keusch, 1982 and Manocha et al, 1990]. This is also thought to be true for the pathogenesis of *P. carinii* infections. The interaction between *P. carinii* and host lung cells was described by electron microscopic studies demonstrating the tight binding of *P. carinii* to type I peumocytes in both rat [Yoneda et al, 1983] and human [Sueishi et al, 1977] lung tissues. The *P. carinii* outer membrane invaginates into the host cell membrane and enables the organism to maintain its position on the host cell and avoid alveolar clearance mechanisms.

The attachment of *P. carinii* to the feeder cells was also observed when

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culturing in media with cells. Limper and Martin showed that attachment of *P. carinii* inhibited the proliferation of A549 cells and this effect was reversed by blocking *P. carinii* attachment through inhibition of cytoskeletal function [Limper et al, 1990]. Such attachment of the organisms to foci on the A549 cell monolayers was also reported by Cushion and colleagues [Cushion et al, 1985].

The attachment to host lung cells or feeder cells appears to be required for the growth of *P. carinii.* The mechanism of attachment has not clearly established. Studies showed that both extracellular matrix protein, fibronectin, and lectin might be the mediators in the binding [Pottratz et al, 1990; Ezekowitz et al, 1991]. It is quite possible that multiple mechanisms of attachment may exist.

The understanding of the attachment mechanisms of *P. carinii* may provide important clues to the development of novel therapeutic strategies for PCP. Targeting specific therapy to disrupt the mechanism of *P. carinii* attachment might provide an effective and safe means to stop the propagation of these organism within the lungs.

1.3.5 Transmission of PCP

Infection with *P. carinii* appears to be acquired mainly by an airborne route. Hendley and Weller in 1971 demonstrated that both close and distant contact between chronic immunosuppressed *P. carinii*-free rats (naïve) rats and *P. carinii* infected rats resulted in infection of the naïve rats [Hendley et al, 1971]. Walzer and colleagues showed similar results and also successfully established that *P. carinii* could be transmitted by intrapulmonary injection of infected lung homogenates or prolonged exposure to other infected animals

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[Walzer et al, 1977]. Studies by Hughes and colleagues showed that the infection could not be transmitted when soil, water, or food was used as the source of organism [Hughes et al, 1982, 1987a &1987b] .

1.3.6 Cultivation of *P. carinii.*

Attempts to cultivate *P. carinii* in cell-free environments have been unsuccessful. It was not until 1977 that the propagation of *P. carinii* using cultures of primary embryonic chick epithelial lung cells was first reported by Pifer and coworkers [Pifer et al, 1977]. This culture is based on the facts that *P. carinii* infection in the lungs appears to require attachment of the organism to a specific alveolar lining cells, the type I pneumocytes [Walzer, 1991]. In the following years, numerous other cell types have been reported to support the modest growth of *P. carinii* in vitro. The increase in *P. carinii* number in the culture system could reach up to 10-fold [Cushion, 1989 and Sloand et al, 1993]. But there is a general agreement that no reproducible, continuous culture system for *P. carinii* currently exists.

The problems surrounding *P. carinii* culture are as follows: 1) The organisms isolated from host lungs may be contaminated with bacteria and fungi which have the potential to overgrow and destroy the culture; it may also contain biological active substances, such as cytokines, which have the potential to influence the culture. 2) The isolation procedure may affect the viability of *P. carinii* in the culture inoculum. 3) It is possible that only trophozoites can grow in vitro [Armstrong et al, 1989]. 4) Strain differences among *P. carinii* isolates may also determine the success or failure of a particular culture [Hong et al, 1990].

In addition to the above problems, simpler and more accurate ways are

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necessary to quantitate the organism and to determine its viability and metabolic activity. Lapinsky and colleagues employed flow cytometric analysis to obtain reproducible quantitation of both *P. carinii* cysts and trophozoites which were sampled from purified *P. carinii*-infected rat homogenates and bronchoalveolar lavage fluid [Lapinsy et al, 1991]. Comley and coworkers measured the [^{3}H]-p-aminobenzoic acid incorporation ability of *P. carinii* in a microtiter assay and used such activity as an index of viability [Comley et al, 1991]. Chen et al used an ATP bioluminescent assay to evaluate viability of *Pneumocystis carinii* isolated from infected rat lungs [Chen et al, 1994].

Even though the long-term in vitro cultivation of *P. carinii* has not been achieved, short-term *P. carinii* cultures have proved valuable in 2 ways: 1) it can provide organisms relatively free from host contamination, 2) it can be employed to test anti-pneumocystis drugs in vitro. Several culture models have been developed and successfully used to screen a variety of chemical compounds for anti-pneumocystis efficacy [Queener et al, 1987; Comley et al, 1991 and Chen et al, 1994].

1.4 Treatment of *Pneumocystis carinii* pneumonia

The present standard treatment and/ or prophylaxis regimens for PCP include either TMP-SMX, administered orally or intravenously, or pentamidine. They are used for the treatment of acute PCP in both non-AIDS and AIDS patients. In addition to TMP-SMX and pentamidine, there are several other agents currently under evaluation, they are used as alternative agents to treat mild to moderate PCP.

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1.4.1. Pentamidine and related compounds.

Pentamidine isethionate (**Figure 1.2**) is diamidine with broad-spectrum antiprotozoan activity, such as the treatment of African trypanosomiasis [Ivady et al, 1967]. Pentamidine is thought to prevent DNA replication by binding to DNA and inhibiting topoisomerase [Tidwell et al, 1989]. The exact mechanism of selective toxicity is still unknown.

NH ‖ C — NH$_2$ —O—$(CH_2)_5$—O—   NH ‖ C — NH$_2$

Figure 1.2. Chemical structure of pentamidine isethionate

Pentamidine was first used to treat PCP in 1958. It reduced the case fatality rate from 50 to 4% [Ivady et al, 1967]. Before the epidemic of AIDS, pentamidine therapy was successful in 63-77% of immunosuppressed patients who could tolerate therapy for 9 days. But it was associtated with a number of adverse reactions, including renal insufficiency, skin rashes and hypertension [Western et al, 1970]. Similar efficacies of pentamidine treatment were achieved in AIDS patients, the overall survival rates after initial treatment were between 61% and 95% [Sattler et al, 1988; Wharton et al, 1986 and Klein et al, 1992].

AIDS patients experience a high frequency (up to 40%) of adverse reactions to pentamidine [Klein et al, 1992]. The toxic effects in AIDS patients are similar to those seen in other immunocompromised hosts, but the frequency of neutropenia and possibly hypoglycemia is considerably higher. Kovacs et al.

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compared pentamidine treatment of PCP in AIDS patients with other immunodeficient patients. 11% of AIDS patients who received pentamidine as initial therapy required institution of an alternative agent because of adverse effects, compared with none of the patients with other causes of immunodeficiency [Kovacs et al, 1984].

In order to reduce the toxicity of parental pentamidine, aerosolized pentamidine (AP) is now used. Aerosol administration results in much higher administration of the drug in the lung than in other body sites and this reduces the toxicity of pentamidine. However, implicit in this approach is that aerosolized pentamidine would not be useful for the treatment of disseminated *P. carinii.* Thus, patients receiving AP may be predisposed to develop extrapulmonary disease. Recently prospective trials comparing AP to intravenous pentamidine found unacceptably high failure rates of 45% or greater in the AP-treated groups [Conte et al, 1990]. So it is not recommended to be used for PCP treatment based on current data. However, AP has been proved to be safe and effective for primary and secondary prophylaxis for PCP in AIDS patients [Antinori et al, 1995 and Carr et al, 1992].

1.4.2. Trimethoprim-Sulfamethoxazole (TMP-SMX).

Trimethoprim-Sulfamethoxazole (**Figure 1.3**) is a widely used first-line drug for prophylaxis and treatment of PCP. It is a combination of two antimicrobial agents that act at different sites to inhibit folate metabolism. The functions of these two drugs will be discussed in section 1.5.

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Trimethoprim (TMP)

Sulfamethoxazole (SMX)

Figure 1.3. Chemical Structures of Trimethoprim and Sulfamethoxazole.

The efficacy of this drug for treating PCP was first reported in 1974 by Hughes and colleagues [Hughes et al, 1974]. The efficacies of TMP-SMX therapy were from 67% to 86% [Sattler et al, 1988; Wharton et al, 1986 and Klein et al, 1992]. The antipneumocystis activity of TMP-SMX is probably due to SMX. Hughes and colleagues demonstratied that SMX provided effective prophylaxis for PCP without TMP [Hughes et al, 1996]. Walzer and colleagues also demonstrated that SMX administrated alone was highly effective in both treatment and prophylaxis in the rat PCP models [Walzer et al, 1992]. In addition, both in vitro and in vivo studies show that TMP has little anti-PCP activity [Hughes et al, 1984, and Allegra et al, 1987].

One of the most striking and poorly understood features of TMP-SMX therapy is the high incidence of adverse reactions occurring in patients with

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AIDS. The reported adverse reactions to TMP-SMX includes rash, fever, neutropenialn etc. A recent large prospective randomized treatment trial done by Klein and coworkers, 42% of the patients treated with TMP-SMX were changed to pentamidine because of failure to respond, and 34% were changed to alternative drug therapy because of toxicity [Klein et al, 1992].

TMP-SMX is also very effective in the primary and secondary prophylaxis against PCP in AIDS patients. More than 97% of efficacy was usually achieved [Antinori et al, 1995 and Carr et al, 1992].

1.4.3. Other agents.

Dapsone (**Figure 1.4**) , a sulfa drug, alone or in combination with TMP has been used to treat mild and moderate PCP in AIDS patients [Leoung et al, 1986 and Mills et al, 1988]. Recently, the combination of dapsone and pyrimethamine plus leucovorin has been evaluated for the primary prophylaxis against PCP in AIDS patients. The efficacies were from 89% to 92% [Podzamezer et al, 1995 and Mallolas et al, 1993]. Although dapsone-pyrimethamine is not as effective as TMP-SMX, it is better tolerated than TMP-SMX. It appears to be a safe and effective alternative.

Trimetrexate (**Figure 1.4**) is another alternative agent, it is a lipid-soluble derivative of methotrexate and is 1500-times more potent than TMP in vitro to inhibit protozoan DHFR [Allegra et al, 1987]. A high incidence of relapse is the major problem, and it must be used in combination with leucovorin and/or with a sulfa drug [Sattler et a, 1990].

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Dapsone

Trimetrexate

Figure 1.4. Chemical structure of dapsone and trimetrexate.

Atovaquone (hydroxynaphthoquinone BW 566C80, **Figure 1.5**) has potent anti-*P. carinii* activity in vitro and in animal models [Hughes et al, 1990 and Comley et al, 1995]. Its mechanism of action is thought to be by blocking mitochondrial electron transport [Ittarat et al, 1995]. Atovaquone is used to treat mild and moderate PCP in AIDS patients, who do not tolerate TMP-SMX. It is now being evaluated in the Phase II dose-range trial [Fallen et al, 1990]. The major adverse reactions are rash and fever which require discontinuation of therapy.

Figure 1.5. Chemical structure of atovoquone

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The combination of primaquine (**Figure 1.6**) with clindamycin also showed anti-PCP activity in vitro and in a rat model. Such a combination was safe and well tolerated in patients with AIDS-related PCP with efficacy rates of 70-100% [Toma, 1991].

CH_3O N N H_2N

Figure 1.6. Chemical structure of primaquine

Difluoromethylornithine (DFMO) was initially used as an anti-African trypanosome drug [Bacchi et al, 1980]. It inhibits the polyamine biosynthesis by acting as a selective inhibitor of ornithine decarboxylase. However, the reason for its selective toxicity against *P. carinii* is unknown since both host and parasite are dependent on the same enzyme for polyamine biosynthesis.

The anti-pneumocystis activity of DFMO has been demonstrated both in vitro [Cushion et al, 1985] and in rat PCP models [Clackson et al, 1988]. DFMO was also found to be active against PCP in AIDS patients [Smith et al, 1992]. A variety of reversible adverse reactions were reported during the treatment of AIDS patients with DFMO, including thrombocytopenia, leukepenia. DFMO is now considered as an alternative therapy for PCP in AIDS.

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1.5 Folate metabolism--overview.

Folates are essential nutirents for all living cells. The chemical structure of dihyrofolic acid is shown in **Figure 1.7**. The metabolically active forms of folates in the circulation are all substituted tetrahydropteroylpolyglutamates. The major metabolic function of folate is as a carrier of one-carbon fragment in a wide variety of biosynthetic and catabolic reactions, these may be formyl, formimino, methyl, methylene or methenyl residues. These active folates play an integral role in the biosynthesis of purines and pyrimidines, in the metabolisms of glycine,serine and histidine, in the formation of formylmethionyl-tRNA and initiation of protein synthesis in all species [Blakeley et al, 1984].

Figure 1.7. Chemical structure of dihydrofolic acid

Mammalian cells obtain their required folates by active transport from their environment. They do not have the machinery to synthesize folates de novo. In contrast, most of microorganisms lack such an active transport system and synthesize folates de novo. The key enzyme in de novo folate synthesis is dihydropteroate synthetase (DHPS), which catalyzes the condensation of p-aminobenzoic acid (pABA) with 6-hydroxymethyl dihydropterin pyrophosphate. Since mammalian cells do not possess this enzyme, it is a very attractive target for selective inhibition. Inhibition of folate biosynthesis can lead to the inhibition

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of DNA, RNA and protein biosynthesis in the microorganisms.

Since only tetrahydrofolates are metabolically active, the reaction byproduct of thymidylate synthase (TS)--dihydrofolate must be reduced back to tetrahydrofolates. This reduction is catalyzed by another important enzyme, dihydrofolate reductase (DHFR). Both mammals and microorganisms possess this enzyme, but fortunately, DHFRs are species-specific. Inhibitors of DHFRs differ markedly in their affinity from different species. This species-specificity makes DHFR another ideal target for chemotherapy.

Folates inside the living cells are in the form of pteroylpolyglutamates. The pteroylpolyglutamates have received attention not only because they play important roles in metabolism, but also because methotrexate, an antitumor drug, is also converted to polyglutamate forms. There are two major functions of pteroylpolyglutamated folates. First, polyglutamate tails add negative charges to folate so that they are retained inside the cells. Second, polyglutamated folates have lower K_ms than monoglutamated folate when binding to folate requiring enzymes, such as thymidylate synthase and dihydrofolate reductase.

Folate polyglutamation is found in all the living cells. The predominant form of polyglutamates are consistantly different in different orgainsms, such as pentaglutamates in rat liver, triglutamates in Bacillus subtilis [Kisliuk, 1981], and tetraglutamates in *P. falciparum* K1 strain [Krungkai et al, 1989].

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1.6. Folate biosynthesis in *Pneumocystis carinii.*

1.6.1 *Pneumocystis carinii* dihydropteroate synthetase

DHPS activity was first found in short-term *P. carinii* cultures [Kovacs et al, 1989]. The authors demonstrated that rat-derived *P. carinii* cultured in pABA- and folates-free medium could incorporate [^{3}H]-pABA into reduced folates. Inclusion of folate synthesis precursors increased the incorporation by 2 to 4 folds. The major forms of reduced [^{3}H]-folates were 10-formyltetrahydrofolate and tetrahydrofolate. In 1990, Merali et al also identified the DHPS activity in crude *P. carinii* extracts, and further evaluated the relative inhibitory activity of different drugs [Merali et al, 1990]. However, due to the limitation of organisms and low enzyme activity, the characterization of DHPS has been difficult.

The *P. carinii* DHPS has recently been sequenced, cloned and expressed by Volpe and coworkers [Volpe et al, 1992 and 1993]. The sequence contained 3 enzymatic activities involved in folate biosynthesis: dihydroneopterin aldolase, pterin pyrophosphate kinase (PPPK) and DHPS, respectively. The authors called this multifunctional gene *fas* (folic acid synthesis) (**Figure 1.8**).

1.6.2. *Pneumocystis carinii* dihydrofolate reductase.

Studies on the *P. carinii* DHFR were done much earlier than *P. carinii* DHPS. DHFR activity was first demonstrated by Allegra and colleagues in the cytosol of partially purified rat *P. carinii* [Allegra et al, 1987]. The enzyme was then partially purified by Kovacs [Kovacs et al, 1990]. The gene encoding *P. carinii* DHFR was cloned by Edman and coworkers in 1989 [Edman et al, 1989]. The predicted protein had an MW of 24 KDa. Unlike the *P. carinii*

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DHPS, the DHFR is a unifunctional enzyme. *P. carinii* DHFR and thymidylate synthetase (TS) were separated by methotrexate affinity column, from this point of view *P. carinii* is more like a fungus rather a protozoan, since the protozoan DHFR is a component of a large, bifunctional molecule that also contains TS activity [Garrett et al, 1984]. The K_m of the *P. carinii* DHFR is larger than that of rat DHFR (17.6 vs 4.0 μM). This difference suggested a structure difference between the active sites of the two enzyme.

1.7. Sulfa drugs

Sulfa drugs are analogs of the DHPS substrate, pABA. They were first discovered in the 1930s and were initially used as effective therapy in the prevention and treatment of bacterial infections in 1940s. Sulfa drugs have been found to have a relatively broad antimicrobial spectrum. Besides bacteria, fungi and protozoa are also found susceptible to sulfa drugs. About 30 sulfa drugs are now used in clinical practice [Anand, 1980].

Sulfa drugs are the most promising class of agents for the treatment and prevention of PCP. Sulfamethoxazole and dapsone are currently used as standard treatment and prophylaxis regimens against PCP in patients infected with HIV. Another sulfa drug, sulfadoxine, which is known active against *P. carinii* [Walzer et al, 1988], is now under clinical evalution for the prophlaxis of PCP.

Sulfa drugs are also known for their high incidence of adverse reactions. Up to 40% of persons cannot tolerate TMP-SMX [Simonds et al, 1995]. These side effects are probably due to sulfa, not TMP [Ball, 1986]. However, sulfa drugs vary in their abilities to induce adverse effects [Björkman et al, 1991], such as sulfafurazole (sulfisoxazole) and sulfapral (sulfamethoxypyridazine

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plus sulfamethizole) have fewer side effects than TMP-SMX. Thus, a sulfa drug with fewer adverse effects might be found to be effective against PCP. Because a wide variety of sulfonamides and related compounds (more than 15,000) have been synthesized [Korolkovas et al, 1976] and very few of them have been tested for the treatment or prevention of PCP, attention should be focused on alternative sulfa drugs as therapeutic agents which are more effective and less toxic than SMX.

The efficacies of sulfa drugs have been tested in different systems. First, latently infected rats have been used extensively to evaluate sulfa drug's efficacy. Hughes and colleagues were the first to demonstrate the effectiveness of TMP-SMX in both prophylaxis and therapy against *P. carinii* [Hughes et al, 1974]. The anti-pnuemocystis activities of variety of sulfa drugs including dapsone, sulfachlorpyridazine, sulfamethoxypyridazine, and sulfaquinoxaline were also tested by Hughes and colleagues in the rat models [Hughes et al, 1984 and 1996]. The same rat models were also used by Walzer and colleagues to demonstrate the efficacies of SMX, sulfadizine, sulfadoxine and SMX-TMP [Walzer et al, 1988 and 1992].

Second, short-term cultures have been widely used to evaluate sulfa drugs. Kovacs and colleagues studied the inhibitory effect of TMP-SMX on the de novo folate synthesis of *P. carinii* cultured on WI 38 feeder cells [Kovacs et al, 1989]. While Chen and Comely used cell-free culture systems to evaluate TMP-SMX [Chen et al, 1994 and Comely et al, 1991].

P. carinii DHPS extracted form *P. carinii* was also employed to test sulfa drugs. Merali et al compared 5 sulfa drugs for their inhibitory effects on *P. carinii* DHPS [Merali et al, 1990]. The efficacies of 8 sulfa drugs were measured by

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Voeller and colleagues for their inhibitory effcts on both *P. carinii* DHPS and de novo folate biosynthesis in intact organisms [Voeller et al, 1994].

Recently, the successful cloning and expression of recombinant *P. carinii* DHPS provided a new tool to identify effective and less toxic sulfa drugs. The availablity of large quantities of recombinant *P. carinii* DHPS allows large-scale screening of the extensive repositories of sulfa drugs.

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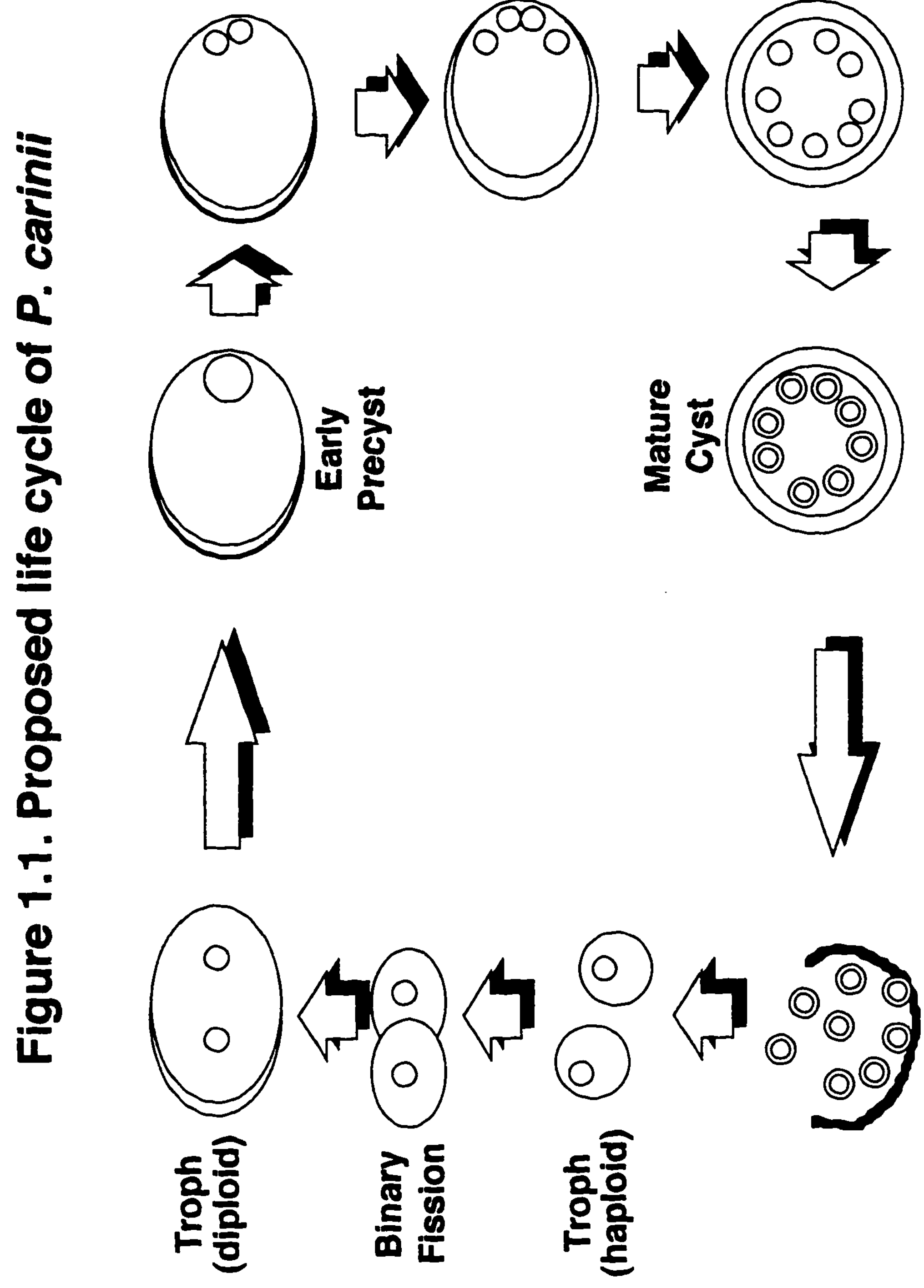

Figure 1.1. Proposed life cycle of *P. carinii*

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7,8-dihydroneopterin

$(CHOH)_2CH_2OH$

Dihydroneoptrin Aldolase

6-hydroxymethyl 7,8-dihydroneopterin

CH_2OH

ATP

AMP

PPPK

fas

6-hydroxymethyl 7,8-dihydroneopterin pyrophosphate

CH_2OP_2

pABA

PPi

DHPS

7,8-dihydropteroate

CH_2NH—⟨⟩—COOH

Glutamate+ATP

Dihydrofolate Synthetase

CH_2NH—⟨⟩—CNH—CHCOO⁻ $CH_2CH_2COO^-$

Dihydrofolate

Figure 1.8. De novo folate biosynthesis pathway in *P. carinii*.

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2. MATERIALS AND METHODS

2.1. Chemicals and Drugs:

[^{3}H]-p-aminobenzoic acid (50 Ci mmol^{-1}) was purchased from Moravek Biochemicals (Brea, CA). [^{14}C]-artemisinin (45.5 mCi mmol^{-1}) was a gift from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington DC. Artemisinin was purchased from Aldrich Chemical Company (Milwaukee, WI).

Folate and pABA-free RPMI 1640, Eagle's Minimum Essential Medium (MEM), Grace insect cell medium, Sf900-II SFM, heat-inactivated fetal bovine serum (FBS), *KpnI* (10 units/µl), gentamycin and amphotericin B (fungizone). were purchased from Gibco Life Technologies (Grand Island, NY).

7,8-Dihydroneopterin, p-aminobenzoyldiglutamate(pABA-glu$_2$), p-amino-benzoyltetraglutamate (pABA-glu$_4$) and p-aminobenzoylhexaglutamate (pABA-glu$_6$) were purchased from Dr. Schircks Laboratory (Wettswill, Switzerland).

Glutathione agarose bead, glutathione (reduced form), thrombin (3,500 units per mg protein), anhydrous diethyl ether, Tris/HCl, EDTANa$_2$, Zinc powder, $NaBH_4$, hemin, p-aminobenzoic acid (pABA), leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), saponin, dimethyl sulfoxide (DMSO), 1,4-dithiothreitol (DTT), dapsone, sulfamoxole, sulfadiazine, sulfamethoxazole, sulfaquinoxaline and tetracycline were all purchased from Sigma Chemical Co.(St.Louis, MO).

Sulfaisomidine and sulfisoxazole were purchased from Nutritional Biochemicals Corporation (Cleveland, OH). p,p'-difluoro, m,m'-dinitrodiphenyl sulfone was purchased from General Biochemicals (Chagrin Falls, OH).

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Sulfathiazole was provided by University of Michigan Hospital (Ann Arbor, MI). Sulfa drugs Ro.4-4393 (sulfadoxine), Ro.7-8307, Ro.5-2928, Ro.21-1182, Ro.5-5615, Ro.1-3354, Ro.1-4303 (sulfapyridine), Ro.1-9194, Ro.7-2844, Ro.4-0517 (sulfamethoxine), Ro.1-9623, Ro.5-0529 (sulfachlorpyridazine), Ro.02-0445, Ro.04-3476 were provided by Hoffman-La Roche Inc. (Nutley, NJ) and Diformyldapsone by Jocobus Pharmaceutical (Princeton, NJ). Sulfaguanidine, sulfamethoxypyridazine, sulfathiourea and sulfamerazine were provided by RHôNE-PONLENC RORER (Centre de Recherche de Vitry-Alfortville, France). Sulfa drugs NSC14652-J, NSC39345-X, NSC45751-M, NSC52105-S, NSC56605-K, NSC74428-I, NSC74587-G, NSC78438-Q, NSC107328-T, NSC142456-T, NSC163977-T, NSC205491-S, NSC229583-G, NSC270146-U, NSC279282-Z, 303757-F, NSC355394-H, NSC403439-F, NSC308795-G and 6-hydroxymethylpterin pyrophosphate ($PtCH_2OPP$) were kindly provided by Dr. Monamed Nasr, Division of AIDS, National Institute of Health.

2.2. Isolation and cultivation of parasites.

2.2.1. *Pneumocystis carinii*

Pneumocystis carinii was isolated from the lungs of infected PCP rat models. The rat model for PCP was maintained as described previously [Ittarat et al, 1995]. Male Sprague-Dawley rats (150 to 200 g) were immunosuppressed by the administration of dexmethasone (2 mg/L) and tetracycline (0.5 mg/ml) in their drinking water. Within 6 to 8 weeks, rats showing signs of severe pneumocystis pneumonia were sacrificed. The lungs were quickly removed, weighed and kept on ice. Touch preparations were made from the lungs and stained with Gram, Giemsa and Crystal violet stain. The experiment was continued if no fungi or bacteria were present. Then the lungs were minced and

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incubated with collagenase (20 mg per lung) at 37°C for 30 min. After the incubation, the mixture was filtered through a wire mesh filter to remove lung tissue and rinsed with MEM supplemented with 20% FBS (MEM-FBS) [Chen et al, 1994]. The filtrate was centrifuged at 1,100 X g for 10 min. The supernatant was discarded, and the pellet was resuspended in 30 ml MEM/FBS containing 100 mM DTT and centrifuged at 1,100 X g for 10 min. The resulting pellet was then resuspended in 15 ml 0.86% NH_4Cl, incubated at 37°C for 15 min, and then repelleted. The pellet was once again resuspended in MEM-FBS, filtered through an 8-μm-pore-size filter (Millipore Corporation, Marlborough, MS), and washed with 2 to 5 ml of MEM-FBS. The filtrate was centrifuged at 1,100 X *g* for 10 min, and the pellet was then resuspended in appropriate amount of MEM-FBS plus 100 μM dihydroneopterin.

2.2.2. *Plasmodium falciparum.*

Plasmodium falciparum (Strain FCR3) was cultivated in candle jars by the method of Trager and Jensen [Trager et al, 1976]. Culture media consisted of RPMI 1640 containing 0.2% $NaHCO_3$, 40 mM Hepes, 40 mg/L gentamycin and 10% human serum. Fresh type A+ blood was obtained from Interstate Blood Bank (Memphis, TN) and stored at 4°C for a maximum of three weeks. Parasite culture was maintained at parasitemia (percentage parasite infected red cells) of 1% to 10%.

P. falciparum was isolated from infected erythrocytes by saponin lysis [Fairfield et al, 1983]. Cultured parasite infected-red blood cells were washed three times with PBS, and then resuspended in PBS to four times the volume of the packed blood cells. One tenth volume of a saponin-saline solution containing 0.165% saponin and 0.85% NaCl was added drop by drop while the

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blood suspension was being vortexed gently. The solution of partially lysed red blood cells were then incubated in a 37°C water bath for 15 min. After cooling down on ice, the parasites were pelleted at 4°C and washed three times with PBS. The parasite pellets were immediately frozen at -70°C.

2.3. The studies of pteroylpolyglutamate synthesis in *P. carinii.*

2.3.1. The Uptake of [^{3}H]-pABA by *P. carinii* and *P. falciparum.*

P. falciparum (strain FCR3) cultures were preincubated for 12 hrs in folate- and pABA-free RPMI 1640 media, and then incubated for 24 hrs in the same medium plus dihydroneopterin (50 µM) and [^{3}H]-pABA (20 µCi). The parasites were then isolated by saponin lysis, and washed 3 times with phosphate-buffered saline (PBS).

Lung-derived *P. carinii* were partially purified from latently infected rats as described in section 2.2.1 and suspended in MEM containing 20% heat-inactivated FBS. Culture-derived *P. carinii* were harvested from spinner flask cultures as described [Lee et al, 1993], and freed of host cells by low-speed centrifugation. As controls, supernatants were obtained in the same manner from spinner flasks which were inoculated with lung homogenates from uninfected rats. To suspensions of approximately 10^7 organisms per ml, dihydroneopterin (50 µM, added from a 0.5 M stock solution in dimethylsulfoxide) and [^{3}H]-pABA (20 µCi), were added (with and without sulfamethoxazole, 1 µM) and then incubated for 6 hours at 35°C in a 5% CO_2 atmosphere. Organisms were then pelleted by centrifugation at 1,500 x g, and washed 3 times with cold PBS or Hank's buffer.

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Pelleted organisms were then suspended in PBS and lysed by sonication using 4 treatments of 5 seconds with a microprobe at maximum intensity (Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Long Island, NY). Aliquots were taken and assayed for protein and radioactivity in a Beckman LS 7000 Scintillation counter using Scintiverse BD scintillation fluid (Fisher Scientific, Fair Lawn, NJ). The remainder was stored at -70°C.

2.3.2. Cleavage of pteroylpolyglutamates.

In order to assay pteroylpolyglutamates, cell lysates were thawed, heated to 100°C for 5 min, and cooled to 4°C. Precipitated protein was removed by centrifugation (12,000 x g for 10 min) and the supernatant treated to remove the pterin ring as described by Shane [Shane et al, 1986]. Briefly, the supernatants were adjusted to pH 1 with 5 N HCl and left at 4°C overnight. The solutions were then adjusted to pH 6 with 5 N NaOH and n-octanol (1 drop) and $NaBH_4$ were immediately added. After 15 min, excess NaBH4 was destroyed by acidification. The solutions were adjusted to pH 12 and stored at room temperature for 4 h with occasional shaking. The solutions were then adjusted to pH 1 with 5 N HCl and stored overnight at 4°C. Zn dust suspension (1g Zn per 2 ml 0.5% gelatin) was then added and the mixtures shaken intermittently for 5 min. The mixtures were centrifuged and the supernatant collected for further HPLC analysis.

2.3.3. Identification of pABA-glutamates.

The number of glutamate units in above pterin-cleavaged samples was determined by comparison with authentic standards of pABA, pABA-glu_1, pABA-glu_2, pABA-glu_4 and pABA-glu_6 on a Rainin Instrument HPLC using a C18 hypersil 5 μm column (4.6 x 250mm) (Phenomenex, Torrance, CA) by the

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method of Selhub [Selhub, 1989], except that dithiothreitol was not used and a different gradient was used (Buffer B at 10% for 12 min, followed by a linear increase to 60% over the next 23 min. Buffer A is a solution of 25 mM NaCl, 5 mM PIC A, pH6.8, buffer B is the same as buffer A, but in 65% acetonitrile by volume). The radiolabeled elute was monitored on a Flo-One radioactive detector (Radiometric Instruments, Meriden, CT) and offset by 1.4 min to adjust for the time delay between this detector and the UV detector. Elution times of standards were monitored at 280 nm.

2.4. Inhibition of recombinant *P. carinii* DHPS by sulfa drugs.

2.4.1. Expression of recombinant *P. carinii* DHPS.

Spodoptera frugiperda (Sf9) cells and wild-type baculovirus were obtained from Richard Jove, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor. They were routinely cultured as monolayers at 27°C in Sf900-II serum-free medium containing 10% FBS, 50 µg gentamycin per ml, and 2.5 µg amphotericin B per ml. Recombinant virus expressing *P. carinii* DHPS was obtained from Dr. Chris Delves, Wellcome Research Laboratories, Beckenham, Kent, United Kingdom.

For protein expressions, insect cells were seeded in 150-cm^2 flasks at 1.8 X 10^7 cells per flask and infected with recombinant virus at a multiplicity of infection (MOI) of 10 [Volpe et al, 1993]. Infected cells were harvested at 96 hrs post infection (p.i.) by rapidly pipetting media across the monolayer, and pelleted by low speed centrifuge at 4°C at 1,100 X g for 10 min. The cell pellet was then washed three times with ice-colded PBS, resuspended in 3 volume (v:v) of lysis buffer (50 mM Tris/HCl pH8.0, 100 mM NaCl, 1 mM EDTA ,1 mM

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DTT and protease inhibitors--1μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM PMSF), and sonicated with a microprobe at intensity X 7 for 3 x 5 sec. (Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Long island, NY). After centrifugation at 12,000 X g at 4°C for 30 min, the cell pellet containing cell membrane and debris were discarded. The protein concentration in the supernatant was determined by the method of Bradford [Bradford, 1976] (Bio-Rad Laboratories, Richmond, CA).

2.4.2. DHPS Assay.

$PtCH_2OPP$ (10 mM in Tris-HCl, pH8.0) was stored as 10 μl aliquots at -70°C. Dihydro$PtCH_2OPP$ (H_2PtCH_2OPP) was prepared freshly by incubating 10 μl 10 mM $PtCH_2OPP$ with 90 μl mixture of 1.0 mg/ml sodium dithionite and 1.0 mg/ml potassium ascorbate (pH 8.0) at room temperature for at least 15 min immediately before use [Shiota et al, 1969].

The enzyme assay buffer contained 40 mM Tris/HCl pH 8.2, 5 mM $MgCl_2$, 10 mM DTT, 66 nM PABA (made up as a mixture 16 nM [^3H]-pABA and 50 nM unlabeled PABA), and 100 μM H_2PtCH_2OPP. The reaction was initiated by the addition of 20 to 100 μg Sf9 cell lysates in a final volume of 100 μl at 37°C. Reaction mixture without cell lysate or H_2PtCH_2OPP served as blanks.

After 1-hr incubations, the reactions were stopped by adding of 300 μl 1 M citrate-phosphate buffer, pH 3.8. The radioactive 7,8-dihydropteroate (H_2Pte) formed was separated from unreacted [^3H]-pABA by using a modification ether extraction method [Thijssen, 1973]. At pH 3.8, pABA, but not H_2Pte or folates, is extracted into the ether layer. Two hundred microliters of the pH 3.8 mixture were extracted with four 0.6-ml portions of anhydrous diethyl ether. An aliquot of

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the extracted water layer and the combined evaporated ether layers, redissolved in 0.2 ml 0.1 N NaOH, were put into scintillation vials containing 15 ml Scintivers BD scintillation fluid (Fisher Scientific, Fair Lawn, NJ). The radioactivity in the vials were counted in a Bechman LS 7000 Scintillation Counter. One unit of activity is defined as the amount of enzyme required to catalyzed the production of 1 pmol H_2Pte per h at 37°C.

2.4.3. Kinetic studies of crude recombinant DHPS.

The apparent K_m for pABA was determined by incubating the extract in mixture of a constant [^3H]-pABA (16 nM) and eight concentrations of unlabeled pABA varying from 1 nM to 5 μM. An apparent K_m for pABA was also determined with a lysate that had been dialyzed by three passages through a Centricon-30 (Amicon, Inc., Beverly, Mass). The apparent K_m for the other substrate, H_2PtCH_2OPP, was determined by using six concentrations varying from 1 to 40 μM, with the pABA concentration kept at 66 nM (a mixture of 16 nM [^3H]-pABA and 50 nM unlabeled pABA). Two independent experiments were performed for each determination. The apparent K_ms were calculated using Lineweaver-Burk double-reciprocal plots.

2.4.4. Determination of IC_{50} values and K_i values.

Stock solutions of each sulfa drug were prepared in DMSO and then diluted in water to provide a range of concentrations appropriate for testing the DHPS activity. The final concentration of DMSO in the assay mixture was $\leq 1\%$, a concentration which had no effect on enzyme activity. For determinations of 50% inhibitory concentrations (IC_{50} values), DHPS was assayed in the absence of inhibitor and in the presence of four inhibitor concentrations of up to 10 μM.

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The logarithm of drug concentration was plotted against percent inhibition, and the line was then drawn by linear regression. In all cases, r^2 was greater than 0.9.

The K_i values of sulfa drugs were determined by Dixon plots [Segal, 1976]. Two concentrations of [^{3}H]-pABA (16 nM and 80 nM) and four concentrations of drug (10, 40, 70 and 100 nM) were used.

2.4.5. [^{3}H]-folate biosynthesis in *P. carinii.*

P. carinii parasite suspension (obtained in section 2.2.1) was incubated with 0.2 μCi [^{3}H]-pABA in the presence or absence of drug at 37°C. After the incubation, the parasites were spun down and washed 3 times with fresh MEM. In all cases at least three incubations were performed in parallel: one with drug for 2 h, one without drug for 2 h, and a third in which the organisms were mixed with [^{3}H]-pABA and then immediately centrifuged and washed to measure nonspecific adsorption. The parasite pellet was then resuspended in 200 μl Tris-HCl buffer (pH8) containing 3% ß-mercaptoethanol and was lysed by sonication. The membrane and debris was discarded. 1.0 ml of 1 M citrate-1 M phosphate buffer (pH 3.8) was then added to the cytosol, and the pH of the solution was adjusted to 3.8 if necessary. This aqueous suspension was then extracted with diethyl ether four times to remove the unreacted [^{3}H]-pABA. The radioactivity in the aqueous layer, which contains H_2Pte and various forms of folic acid, was then counted. The total amount of [^{3}H]-folate synthesized by parasites was expressed as fmol/mg protein/hr.

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2.5. Partial purification of glutathione S-transferase (GST) and recombinant *P. carinii* Fas fusion protein (GST-Fas).

2.5.1. Construction of recombinant baculovirus expressing GST-Fas fusion protein.

2.5.1.1. Cloning of *P. carinii* fas cDNA into pAcGHLT-B baculovirus transfer vector.

P. carinii fas cDNA was amplified by using two oligonucleotides: a 5' primer (5'-GCGCGCGGTACCATGATATTTAAAAGT-3'), which contains bases complementary to nucleotides coding for aa 1-4 and plus a new *Kpn*I site (underlined) proximal to the initiation codon, and a 3' primer (5'-GCGGGTACCTTAATAAATTTCTTCTTTCCA-3'), which contains bases complementary to nucleotides coding for aa 735-740 and created another new *Kpn*I sites (underlined) distal to the stop codon. The new PCR product was named fas-1. Both primers were synthesized by University of Michigan Medical School Core facility (University of Michigan, Ann Arbor, MI). AmpliTaq DNA Polymerase, 10X PCR Buffer and 25 mM $MgCl_2$ were purchased from Perkin-Elmer (Perkin-Elmer Corporation, Foster City, CA). PCR reactions were performed under the following program: 1) 2 mins at 94°C; 2) 30 cycles of 1 min at 94°C, 2 mins at 55°C and 1 min at 72°C; 3) 7 mins at 72°C; 4) infinite hold at 4°C.

The amplified fas-1 fragment was then digested with *Kpn*I and ligated to *Kpn*I-digested baculovirus transfer vector pAcGHLT-B (**Figure 2.1**) (PharMingen, San Diego, CA) to construct pAcGHLT-B/fas-1. The plasmid DNA was then transformed into E. coli (strain INVαF', Invitrogen Co., San Diego, CA). The orientation of the insert was checked by *Pst*1 and *Hind*III digestion and

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compared with the restriction maps of the pAcGHLT-B and recombinant *P. carinii* fas gene. Three clones were selected. pAcGHLT-B/fas-1 was then purified by cesium chloride centrifugation.

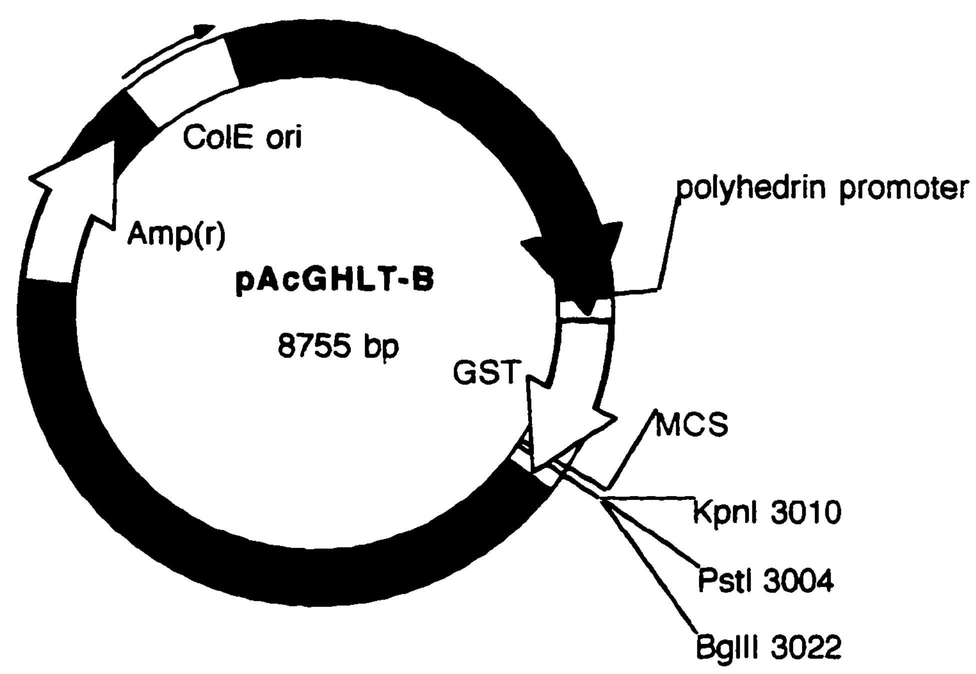

Figure 2.1. Map of pAcGHLT-B.

2.5.1.2. Generation of recombinant baculovirus by co-transfection.

The generation of recombinant baculovirus followed PharMingen's Baculovirus Expression Vector System Manual (Gruenwald, 2nd Edition). 2 X 10^6 Sf 9 cells were seeded onto a 25 cm^2 tissue culture flask. While cells were attaching to the plate, 0.5 μg virus BaculoGold DNA (PharMingen, San Diego, CA) was mixed with 5 μg pAcGHLT-B/fas-1 in a microcentrifuge tube and let sit at room temperature for five mins before 1 ml of Transfection buffer B (125 mM Hepes, pH 7.1, 125 mM $CaCl_2$, 140 mM NaCl) was added. The old medium in the plate was then replaced with 1 ml of Transfection Buffer A (Grace's Medium with 10% FBS). The 1 ml of Transfection Buffer B/DNA solution was then added

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with 10% FBS). The 1 ml of Transfection Buffer B/DNA solution was then added drop-by-drop to the co-transfection plate, and the plate was incubated at 27°C for 4 hrs. At the end of incubation, the medium was removed and replaced with 3 ml fresh Sf900-II serum-free medium containing 10% FBS, 50 μg gentamycin per ml, and 2.5 μg amphotericin B per ml. The plate was then incubated at 27°C for 5 days. After 5 days the supernatant of the co-transfection plate was collected and further amplified to produce a high-titer stock solution. The virus supernatant was stored in the dark at 4°C.

2.5.2. Expression and purification of GST-Fas fusion protein.

The purification of GST-Fas fusion protein followed the PharMingen's Baculovirus GST purification manual (Crossen et al, 2nd Edition). The infection procedure was the same as described in section 2.4.1, except that High Five insect cells (Invitrogen Inc., San Diego, CA) were used. High Five insect cells were cultured as monolayers in Excell-400 (JRH Bioscience, Lenenax, KS) medium supplied with 50 μg gentamycin per ml.

High Five insect cells infected with recombinant baculovirus stock expressing GST-Fas fusion protein were harvested after 3 days and lysed as described in section 2.4.1. Solid ammonium sulfate was added to bring the solution to 40% saturation with the salt. Precipitated protein was then recovered by centrifugation and redissolved in lysis buffer.

One fifth volume of glutathione agarose beads were added to the 40% fraction and incubated at 4°C on a rocking platform for 30 mins. The slurry was then centrifuged at 500 x g for 5 min to sediment the beads. The beads was then washed twice with 5 bead volumes of PBS and centrifuged another 5 min to sediment the beads again. GST-Fas fusion protein was then eluted by 5 mM

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reduced form of glutathione in 50 mM, pH8.0 Tris-HCl buffer. Free glutathione was removed by dialysis against 50 mM Tris-HCl (pH8.0) at 4°C. The product was stored at -80°C.

2.6. The studies of the interaction of artemisinin with malaria hemozoin.

2.6.1. Isolation of malarial pigment--hemozoin

Hemozoin was isolated from malaria culture by a modification of the method of Goldie [Goldie et al, 1990]. Parasite cultures were pooled and the cells were pelleted by centrifugation at 540 x g. The pellet was washed once with RPMI, and then lysed by the addition of an equal volume of hypotonic buffer (8 mM potassium phosphate, 9 mM $EDTANa_2$, pH 7.2) followed by sonication with a microprobe at maximum intensity for 15 s (Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Long island, NY). Crude hemozoin was then pelleted by centrifugation at 35,000 x g at 4°C for 10 min. The pellet was resuspended in 10 ml hypotonic buffer, sonicated and centrifuged at least thrice, until the supernatant became colorless. The pellet was then resuspended in 1 ml hypotonic buffer and carefully layered on top of 10 ml of 30% sucrose in hypotonic buffer and centrifuged at 35, 000 x g for 1 h at 4°C. The hemozoin, at the bottom of the gradient, was removed, washed three times with water, resuspended in 1 ml 1% SDS, and centrifuged at 13,000 x g. The pellet and pooled supernatants were stored at -80°C.

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2.6.2. The interaction of isolated hemozoin and [^{14}C]-artemisinin.

Hemozoin, which was isolated as above from 10 plates of untreated parasites, was incubated with 0.92 nmol [^{14}C]- artemisinin (45.5 mCi per mmol) in 1 ml of 10% methanol and 90% PBS at 37°C in the dark. As a control, 0.92 nmol artemisinin was dissolved in the same solution in the absence of hemozoin. 7 µl of supernatant were removed at 0, 1, 2 and 19 h, respectively. Heme was determined by the modified methods of Fuhrhop and Smith [Fuhrhop et al, 1975].

2.6.3. Identification of hemozoin and [^{14}C]-artemisinin reaction adducts.

Hemozoin, which was isolated from [^{14}C]-artemisinin-treated parasites or was incubated with [^{14}C]-artemisinin as above, was dissolved in 200-300 µl 0.1N KOH, and then precipitated by the addition of one drop of 1N HCl. After microfuging for 1 minute, the supernatant was discarded and the pellet was redissolved in a mixture of methanol, water and acetic acid (32:18:5). 20 µl were spotted onto a C18 reverse-phase fluorescence Thin-Layer Chromatography (TLC) plate (Whatman International Ltd., Maidstone, England) and developed at room temperature with water : acetic acid: methanol (2:1:7). Artemisinin, hemin and the artemisinin-heme adducts isolated from HPLC were used as standards. After each run, the reverse phase plate layer was divided into 0.5 cm fractions and scraped into scintillation vials. 100 µl of methanol was added to dissolve the drug followed by 10 ml of scintillation fluid. The radioactivity was then counted in a scintillation counter.

Alternately, hemozoin was dissolved in 0.2 N KOH, mixed with an equal volume of methanol : acetic acid (9:1) and analyzed by HPLC on a 300 x 3.9

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mm μBondapak C18 analytical column (Waters, Milford MA), eluting at a flow rate of 1 ml min^{-1} with methanol : water : acetic acid (57:34:9) for 30 min followed by methanol : acetic acid (9:1) for 10 min.

2.6.4. Synthesis and partial isolation of hemin\artemisinin adducts

A 10 mM hemin solution was prepared immediately prior to each experiment by first dissolving hemin in 1 N NaOH, diluting with Tris-HCl buffer (50 mM pH 8.5) and adjusting the pH to 8.5 with 1N HCl. Artemisinin was dissolved in methanol as a 10 mM solution and stored at -20°C. Equal volumes of hemin (10 mM) and artemisinin (10 mM) were mixed and incubated in the dark at 37°C for 20 to 24 h.

The products of the reaction were separated by using high-performance liquid chromatography (HPLC; Rainin Instrument Co. Inc., Woburn, MA) with a 300 x 22.5 mm Bondclone C18 semipreparative column (Phenomenex, Torrance, CA) and a flow rate of 15 ml min $^{-1}$ by a modification of the methods of Choe and Ortiz de Montellano [Choe et al, 1991]. The column was eluted with a mixture of methanol, water, acetic acid (32:18:5) and monitored at 400 nm. Before each injection, 600 μl of the reaction mixture was mixed with 400 μl of methanol and acetic acid (3:1), clarified by centrifugation and injected. The products collected from the column were concentrated by low pressure distillation using a Buchi Rotary Evaporator (Brinkmann Instruments, Inc., Westbury, NY) until almost dry. The products were redissolved in methanol and rechromatographed by using a mixture of methanol, water and acetic acid (7:3:1) as the elution buffer and concentrated as above. Finally the purified products were dissolved in methanol and stored at -20°C.

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2.6.5. Mass spectroscopy

The molecular weights of the isolated reaction products were determined by two mass spectroscopic methods. Electrospray ionization (ESI) mass spectra were obtained on a model 201 single quadrupole mass spectrometer (Vestec Corp. Houston, TX), with a 2000 *m/z* range and a Vestec electrospray source. Fast atom bombardment (FAB) ionization mass spectra was obtained on a model 70-250-S mass spectrometer (VG Analytical, Manchester, England), using a fast atom bombardment atom gun with a current of 1 mA and a voltage of 8 KV. Xenon was the gas ion source.

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3. PTEROYLPOLYGLUTAMATE SYNTHESIS BY LUNG- AND CULTURE-DERIVED *PNEUMOCYSTIS CARINII*

As discussed in the introduction section, polyglutamated forms of folate plays very important roles in the metabolism of thymidylate, methionine and purines. The length of glutamate chains varies in different species and different strains. The biosynthesis of pteroylpolyglutamates by *P. carinii* was previously reported by Comley and colleagues [Comley et al, 1991]. However the predominant form of pteroylpolyglutamates in *P. carinii* had not been identified. In order to answer this question, we studied the de novo folate biosynthesis in *P. carinii* from exogenous source of [^{3}H]-pABA, and identified the predominant forms of the de novo synthesized pteroylpolyglutamates in both lung- and culture-derived *P. carinii* [Hong et al, 1995].

3.1. The uptake of [^{3}H]-pABA and de novo folate biosynthesis in *P. carinii.*

Since parasites are cultured in the folate- and pABA-free medium, the addition of [^{3}H]-pABA into the medium becomes the only exogenous source of radiolabeled metabolic precursor. Thus, [^{3}H]-pABA is taken up by parasites and incorporated into the folate pools. The total amount of pABA uptake can be determined by the total radioactivity inside the organisms.

Our study shows that [^{3}H]-pABA is readily taken up by lung-derived *P. carinii.* The total uptake of pABA is 0.38 pmol per 10^7 cells (**Table 3.1**). Sulfamethoxazole, which inhibits dihydropteroate synthetase, completely

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inhibited the uptake of [^{3}H]-pABA by lung-derived organisms at the concentration of 1 µM (< 0.01 pmol per 10^7 cells).

[^{3}H]-pABA is also taken up by culture-derived *P. carinii* in the absence of feeder cells, as has been reported by others [Kovacs et al, 1989 and Comley et al, 1991]. [^{3}H]-pABA is not taken up by supernatants from control cultures, which were inoculated with lungs from uninfected rats. *P. carinii* harvested from the supernatants of 2-day culture takes up 0.5 pmol [^{3}H]-pABA per 10^7 cells, while *P. carinii* harvested from the 4-day cultures takes up more than twice as much as 2 day culture does (1.24 pmol per 10^7 cells).

Although 4-day cultured *P. carinii* takes up [^{3}H]-pABA more than 2-day culture does, the specific activities of the uptake of [^{3}H]-pABA are the same. The specific activities of cultured-derived *P. carinii* are 4-fold higher than that of lung-derived organisms (13.9 vs 3.3 pmol/mg of protein).

For both lung- and culture-derived organisms, >60% of the [^{3}H] pABA can be recovered as [^{3}H]-pABA-polyglutamates. The specific activity of folate synthesis in lung-derived *P. carinii* is 2.3 pmol/ mg of protein. Culture-derived *P. carinii* synthesize over 4-fold more folates than lung-derived organisms do. The synthesis of [^{3}H]-pABA-polyglutamates in 4-day culture (8.9 pmol per mg protein) is lower than that of 2-day culture (11.1 pmol per mg protein)(**Table 3.2**).

3.2. Identification of pteroylpolyglutamates in *P. carinii.*

In order to determine the length of γ-glutamate chains, a chemical method was used to cleave the folates, which are de novo biosynthesized from exogenous [^{3}H]-pABA, at the C,9-N,10 bond to yield the corresponding [^{3}H]-

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pABA-polyglutamates ([^{3}H]-pABAglu$_n$) derivatives. These can then be separated, according to the number of glutamate residues, by high performance liquid chromatography (HPLC) and quantitated by the radioactive marker.

Since *P. falciparum* also incorporates exogenous [^{3}H] pABA [Zhang et al, 1992 and Selhub, 1989], we first carried out an analysis of the pteroylpolyglutamates synthesized by this organism. pABA-tetraglutamate represents approximately 80% of the pABA-glutamates (**Table 3.3**). Tetraglutamates are predominant (**Figure 3.2**), when parasites are incubated with pABA for only 6 hours instead of 24, although representing only 45% of the total pteroylpolyglutamates (data not shown).

In contrast, both lung-derived and culture-derived *P. carinii* produce a more mixed population of polyglutamates with 31-42% pABA-pentaglutamates and 17-30% monoglutamates (**Figure 3.1** and **Table 3.3**).

3.3. Discussion

The synthesis of pteroylpolyglutamates by *P. carinii* was previously reported by Comley and coworkers. Our study confirms that pteroylpolyglutamates are synthesized by this organisms, and that the predominant form are pteroylpentaglutamates.

The identity of the major *P. carinii* folate as pteroylpentaglutamates can only be inferred, since pABA-glu$_5$ was not used as a standard. However, in six experiments, the predominate *P. carinii*-derived radioactive peak had retention times of 29.5-30.5 min, in between the retention times for pABA-glu$_4$ and pABA-glu$_6$, which were 27.2-28.4 min (n=5) and 31.0-33.2 min (n=5), respectively. Thus, the *P. carinii* product elutes where pABA-glu$_5$ would be expected to elute.

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In this study, *P. falciparum* was found to produce primarily pteroyltetraglutamates. Krungkrai and coworkers [Krungkrai et al, 1989] have previously measured the polyglutamates of *P. falciparum* and found pentaglutamates to be the most common form. There are two possible explanations for the different patterns observed. First, these investigators used the K1 strain, while FCR3 was used here, and there could be differences between strains. Secondly, there may have been differences in how folate-depleted the organisms were in the two studies; if so, *P. falciparum* might resemble *Lactobacillus casei* whose polyglutamation status is dependent on the concentration of folate in the media [reviewed by Cossins, 1984].

Culture-derived organisms take up [^{3}H]-pABA and produce pteroylpolyglutamates at 4X higher specific activities than lung-derived organisms (**Table 3.1**). This is consistent with our previous observation that lung-derived organisms are contaminated with lung debris, and that cultivation of organisms in the presence of feeder cells removes this debris to yield purer organisms [Ittarat et al, 1995 and Lee et al, 1993]. These data also confirm that *P. carinii* remains metabolically active when cultivated with feeder cells for at least 4 days.

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Table 3.1 Uptake of [^{3}H]-pABA by *P. carinii* preparations

Experiment	Total uptake (pmol (10^7 cells)$^{-1}$)	Specific Activity (pmol (mg protein)$^{-1}$)	Folate synthesis (pmol (mg protein)$^{-1}$)
Lung-derived *P. carinii* [a]	0.38	3.3	2.3
Lung-derived *P. carinii* + sulfamethoxazole	0.007	0.07	
2-day culture-derived *P. carinii*	0.5	13.9	11.1
2-day controls	0.004	0.36	
4-day culture-derived *P. carinii*	1.24	13.9	8.9
4-day controls	0.01	2.1	

[a] Average of two experiments.

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Table 3.2 Polyglutamate content of organisms

(in pmol per mg protein)

Organisms	pABA-glu$_1$	pABA-glu$_2$	pABA-glu$_3$	pABA-glu$_4$	pABA-glu$_5$	pABA-glu$_6$
P. falciparum	0.52	0.22	1.1	9.1	0.26	0
Lung-derived *P. carinii*	0.44, 0.48	0.19, 0	0.41, 0.35	0.33, 0.46	0.89, 0.54	0.37, 0
2-day cultured *P. carinii*	1.8	0.86	1.2	0.69	4.7	1.8
4-day cultured *P. carinii*	2.57	0.64	0.51	1.45	3.23	0.42

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Table 3.3 Distribution of polyglutamates (as percent of total) in *P. falciparum* and *P. carinii*

Organisms	pABA-glu$_1$	pABA-glu$_2$	pABA-glu$_3$	pABA-glu$_4$	pABA-glu$_5$	pABA-glu$_6$
P. falciparum	4.6	2	9.8	81.3	2.3	0
Lung-derived *P. carinii* [a]	21.3	3.6	17.2	18.6	31.4	7.1
2-day cultured *P. carinii*	17	7.7	10.7	6.2	42.4	16
4-day cultured *P. carinii*	29.2	7.2	5.7	16.5	36.8	4.7

[a] Average of two experiments

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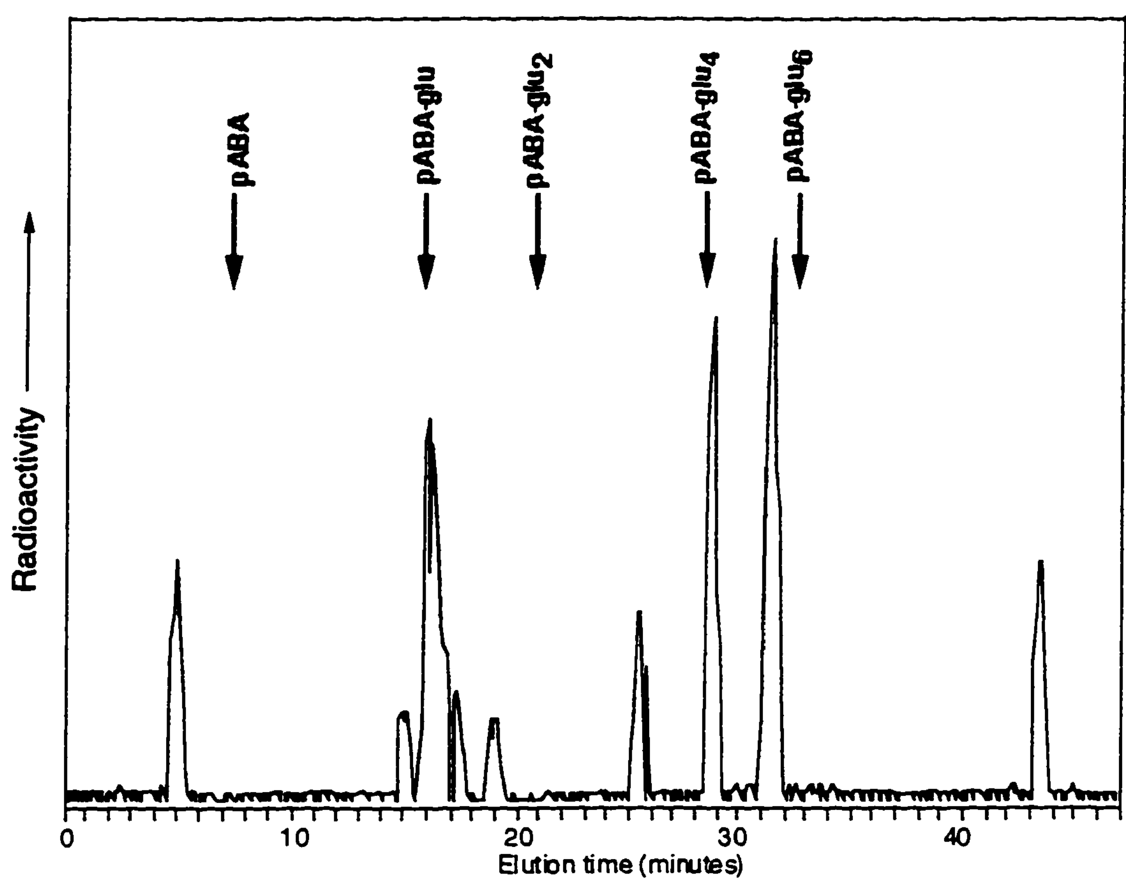

Figure. 3.1. High-performance liquid chromatography (HPLC) analysis of pABA-glu(n) formed by lung-derived *P. carinii* after incubation with [^{3}H]-pABA.

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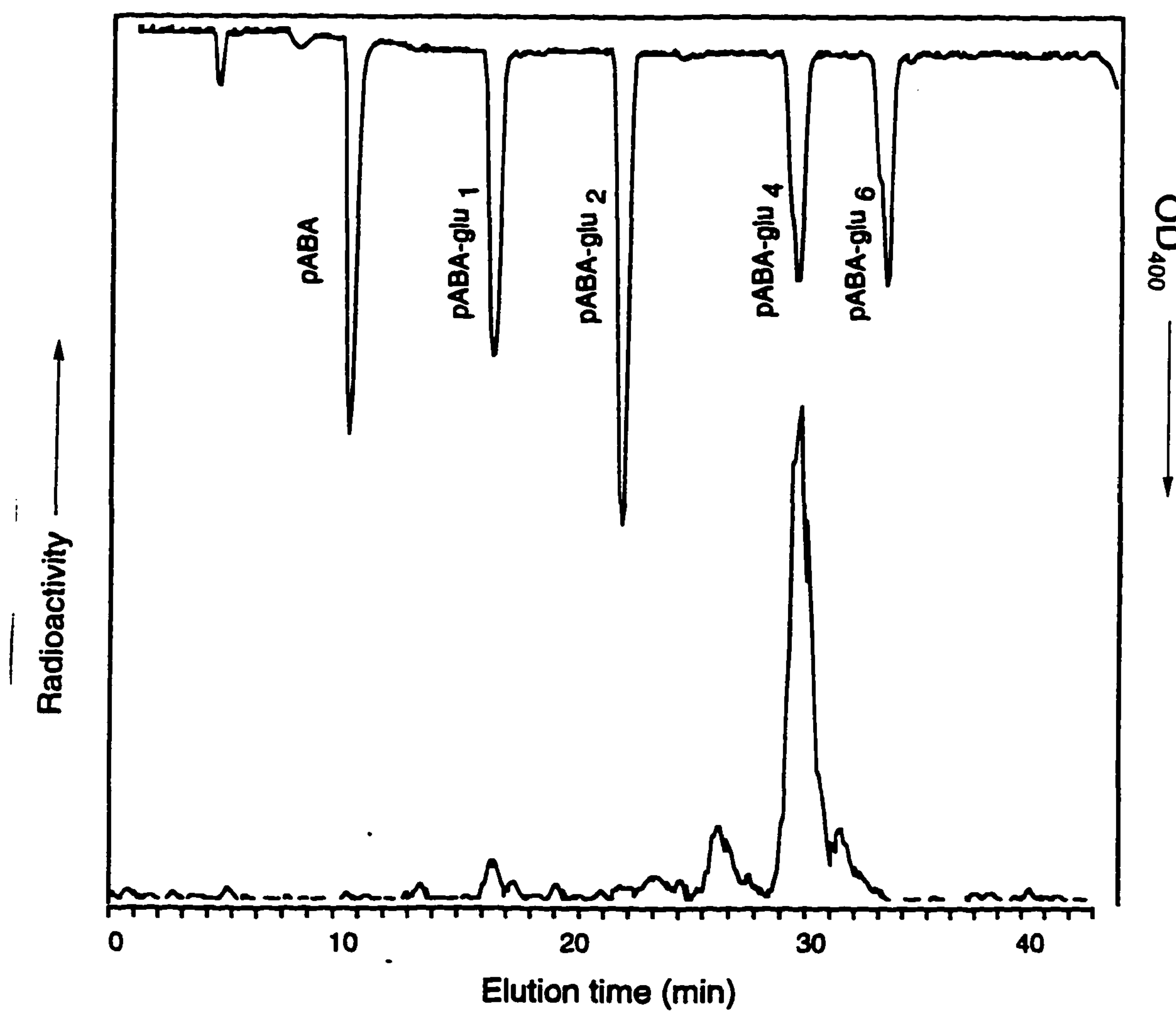

Figure. 3.2. High-performance liquid chromatography (HPLC) analysis of pABA-glu(n) formed by *P. falciparum* after incubation with [^{3}H]-pABA.

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4. INHIBITION OF RECOMBINANT *PNEUMOCYSTIS CARINII* DIHYDROPTEROATE SYNTHETASE BY SULFA DRUGS

As discussed in the introduction, the testing of new sulfa drugs has been greatly hampered by the facts that the inability to culture *P. carinii* continuously in vitro [Sloand et al, 1993] and the complexities of the various animal models [Walzer, 1991]. Therefore, an enzyme-based screen represents a more practical approach to the identification of effective sulfa drugs. However, previous studies using homogenates of isolated organisms were limited by difficulties in obtaining large amounts of organisms. There are no such limitations when a recombinant enzyme is used. In this section, we report a new sulfa drug screening method based on the recombinant *P. carinii* DHPS. The most potent compounds were further tested in isolated intact *P. carinii* [Hong et al, 1995].

4.1. Recombinant *P. carinii* DHPS.

DHPS activity was detected in the cytosol of recombinant baculovirus infected Sf9 cells. The specific activity of DHPS in the crude preparation was 4.04 ± 0.07 pmol / mg of protein per h (n=3) at 37°C. On the other hand, the cytosol of wild-type-baculovirus-infected Sf 9 cells had no detectable activity (< 0.3 pmol/mg of protein per h). An *E. coli* lysate was used as positive control and was found to have a specific activity of 5.1 ± 0.52 pmol /mg of protein per h (n=2). The amount of protein expressed in the insect cells was so little that no band with the expected apparent molecular weight of 71.5 KDa could be seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

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In the presence of a DHPS-containing lysate, the amount of the product (H_2Pte) increases with incubation time, while the substrate pABA disappears at approximately the same rate. Data from one of two equivalent experiments are shown in **Figure 4.1**. In these experiments, the reaction was linear for the first hour, indicating that the 1-h incubation used in the experiments described below are valid for enzyme kinetic studies.

The K_m values for both substrates were calculated by Lineweaver Burk plots. The apparent K_m values for pABA and H_2PtCH_2OPP substrates were 0.34 ± 0.02 and 2.50 ± 0.71 μM, respectively. The apparent K_m valures for pABA when determined in dialyzed lysates was not significantly different (0.15 μM).

4.2. The Inhibition of recombinant *P. carinii* DHPS by sulfa drugs

After assay conditions had been established, 44 sulfa drugs, including dapsone and sulfamethoxazole, were screened. Under the assay conditions used, sulfa drugs appear to inhibit the enzyme in a linear fashion. One micromolar sulfamethoxazole caused complete inhibition at 20, 40, and 60 min (data not shown).

The IC_{50} values of these compounds, grouped according to the chemical structures, are listed in **Table 4.1** to **Table 4.7**. Of the compounds tested, sulfamethoxazole is one of the most potent inhibitors, with an IC_{50} of 23 nM (**Table 4.4**). The IC_{50} values of dapsone was much higher (150 nM) (**Table 4.1**). Seven of the drugs tested had IC_{50} values similar to that of sulfamethoxazole: sulfathiazole (13 nM) (**Table 4.4**), sulfachlorpyridazine (18 nM) (**Table 4.3**), sulfamethoxypyridazine (17 nM) (**Table 4.3**), sulfathiourea

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(22 nM) (**Table 4.6**), sulfadimethoxine (25 nM) (**Table 4.3**), sulfisoxazole (40 nM) (**Table 4.4**), and sulfaquinoxaline (30 nM) (**Table 4.6**).

The K_i values of sulfamethoxazole and four other sulfa drugs, determined by Dixon plots, are shown in **Table 4.8**. The Ki value of sulfamethoxazole, 7.5 nM, is not appreciably different from those of sulfathiazole, sulfachlorpyridazine, sulfamethoxypyridazine, and sulfathiourea.

In order for a sulfa drugs to be effective, it must not only inhibit recombinant DHPS but also be taken up by intact organisms. To determine this, we measure the effects of selected sulfa drugs on folate metabolism in intact, isolated *P. carinii*. The rate of folate synthesis in *P. carinii* was 23.8 ± 7.5 fmol /mg of protein per h (n=9) in the absence of sulfa drugs. At 50 nM, sulfamethoxazole inhibited folate biosynthesis in situ by approximately half. Sulfachlorpyridazine, sulfamethoxypyridazine, sulfisoxazole, and sulfathiazole all inhibited folate biosynthesis to a similar extent at this concentration (**Table 4.9**). One sulfa drug, sulfamethoxypyridazine, was significantly more effective than sulfamethoxazole as determined by Student's test ($P < 0.05$).

4.3. Discussion.

In this study, a crude preparation of recombinant DHPS was used to screen the inhibitory activities of 44 sulfa drugs. Many of these compounds had IC_{50}s similar to or lower than that of sulfamethoxazole. At least four of them inhibited folate metabolism in intact organisms to an extent similar to that of sulfamethoxazole.

The K_m values reported here for pABA (0.34 ± 0.02 μM) and H_2PtCH_2OPP (2.50 ± 0.71 μM) are lower than those previously reported with *P.*

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carinii [Merali et al, 1990 and Voeller et al, 1994]. The K_m value for H_2PtCH_2OPP is similar to that found in one study (3.0 μM) [Voeller et al, 1994], but 40-fold lower than that found in the other (81 μM) [Merali et al, 1990]; the K_m value for pABA is > 25-fold lower than those found in either study (8.7 μM [Voeller et al, 1994] and 71 μM [Merali et al, 1990]). There are several factors that might contribute to these differences. First, all three studies used different enzyme assay methods. Second, the assays performed in the present study were carried out with extracts containing recombinant DHPS, whereas the other determinations were made with extracts from isolated *P. carinii.* Since all three studies measured DHPS activities in crude extracts, it is possible that other compounds of the extracts could have competed for binding with one or both of the substrates. Thus, the true K_m values of this enzyme will not be known until they are measured with purified enzyme.

The concentration of H_2PtCH_2OPP (100μM) used in our DHPS assay was chosen to be consistent with previous investigations [Merali et al, 1990; Volpe et al, 1993]. In contrast, a relatively low concentration of pABA (66nM) was used in our DHPS assay. The pABA concentrations used by other investigators varied between 2.2 μM [Chio et al, 1996] and 50 μM [Merali et al, 1990; Volpe et al, 1993]. A low concentration was used in our assay in order to increase the specific activity of the product. Lower concentration of [^{3}H]-pABA could result in lower value of IC_{50} for each sulfa drug. However, the purpose of this experiment was to develop a simple method to determine the relative activities of sulfa drugs against DHPS, and the "real" values are not necessary.

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The goal of this project was to develop a simple and rapid method to screen large numbers of sulfa drugs. In order to accomplish this, enzyme assays were performed on crude extracts from infected S. furgiperda 9 cells rather than on purified enzyme. This was done under the assumption that the critical factor was the relative potencies of the various drugs and not the absolute inhibitory efficacy. In addition, we utilized a simple and rapid ether extraction enzyme assay method. Since the [^{3}H]-pABA was of high specific activity, only 0.1 μCi was needed for each enzyme assay, giving measurement of around 10,000 cpm after subtracting the value of the blank. Furthermore, the backgroud radioactivity for this assay was never very high. For every assay performed, the radioactivity counts in the aqueous layer after incubation in the presence of extract were always approximately four times higher than those found in the aqueous layers after control incubations. Thus, this procedure can potentially be applied to screening large numbers of drugs.

The chemical structure of the compounds affect their inhibitory abilities in several obvious way. First, unsubstituted p-amino groups are essential for antipneumocystis activity for both the sulfones and sulfonamides. For example, dapsone, with two free p-amino groups, has an IC_{50} value of 0.15 μM. In contrast, diformyldapsone, which has both its amino groups blocked, has an IC_{50} value of > 10 μM (**Table 4.1**). Also, NSC 52105-S(**Table 4.7**) differs from sulfapyridine (**Table 4.3**) by the substituent on the p-amino groups; its IC_{50} value, is 30-fold higher (5.9 vs 0.18 μM). Furthermore, all of the p-amino-substituted derivatives in **Table 4.7** have very low inhibitory activities, with IC_{50} values higher than 6 μM. These results suggest that if the sulfa drugs are structure analogs of pABA, then the p-amino groups of both pABA and the sulfa drugs are involved in binding to the active site of DHPS.

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Of all the classes of sulfa drugs tested, those with heterocyclic substitutions on N1 are the most potent. The phenyl derivatives in **Table 4.5** have IC_{50} values in a range from 0.18 to 0.80 μM, while most of the heterocyclic sulfonamides have IC_{50} values lower than 0.1 μM (**Tables 4.2** to **4.4**). Four of the five most-effective drugs (**Table 4.8**) belong to these group.

Changes or substitutions in the heterocycle ring could affect the inhibitory activity. For example, sulfadimethoxine and sulfadoxine are identical except that they contain a single methoxy substituent in different positions (**Table 4.2**). Yet, sulfadoxine has IC_{50} value which is 30-fold higher than that of sulfadimethoxine. Sulfapyridine and Ro.211182 (**Table 4.3**) both have pyridinyl substituent but in different orientations, and their IC_{50} values differ by more than 56-fold. Similarly, Ro.19194 and sulfamerazine (**Table 4.3**) also have the same methylpyrimidinyl substituent but at different orientations. Their IC_{50} values are different by 35-fold.

In **Table 4.5**, sulfaguanidine, sulfathiourea and NSC78438-Q are different from each other by just a atom, but their IC_{50} values are quite different, 3.2, 0.3 and 0.022 μM, respectively.

A second substituent on N^1 does not have a consistent effect on inhibitory activity. Ro.55615 is identical to Ro.43476 except for an extra methoxyl group on N1, yet its activity is more than 185-fold lower (**Table 4.2**). In other cases, the N1 substituent has no effect. For example, sulfamethoxazole, Ro.72844, and Ro.52928 (**Table 4.4**) have almost the same IC_{50} values regardless of the presence or absence of an N1 substituent.

Sulfachlorpyridazine, sulfamethoxypyridazine, sulfisoxazole, and sulfathiazole compare well with sulfamethoxazole not only in their abilities to

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inhibit recombinant DHPS, but also in their abilities to inhibit de novo folate biosynthesis in *P. carinii.* Sulfamethoxypyridazine may even be a better inhibitor than sulfamethoxazole in intact cells.

Sulfamethoxazole is probably responsible for the adverse effects that occur during co-trimoxozole administration [Ball, 1989]. A sulfa drug which is less toxic than sulfamethoxazole would be of great use. Two of the drugs which are equivalent to sulfamethoxazole in potency. Sulfisoxazole and sulfamethoxypyridazine, have been shown to be less toxic. In a large study carried out in Sweden, the rates of serious adverse reactions to co-trimoxazole (sulfamethoxazole plus trimethoprim), sulfafurazole (sulfisoxazole), and sulfapral (sulfamethoxypyridazine plus sulfamethiazole) were found to be 33, 8 and 6 per 100,000 users, respectively [Björkman et al, 1991]. Thus, sulfamethoxypyridazine and sulfisoxazole should be further evaluated for the treatment and prophylaxis for PCP.

In the present work, a small number of available sulfa drugs were tested. However, we have established a new rapid, high-processivity in vitro screening system and this system can now be used to screen a large number of compounds.

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Table 4.1. Structure-Activity Relationship for Dapsone Analogs

Compounds	R_1	R_2	R_3	R_4	IC_{50}(μM)
Dapsone	$-NH_2$	$-NH_2$	-H	-H	0.15
Diformyldapsone	-NHCOH	-NHCOH	-H	-H	>10
Difluorodinitrophenylsulfone	-F	-F	$-NO_2$	$-NO_2$	>10

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Table 4.2. Structure-activity relationships of 4-Pyrimidinyl derivatives

Compound	R1	R2	R3	R4	R5	IC_{50}(μM)
Sulfadoxine	-H	-H	$-CH_3O$	$-CH_3O$	-H	0.740
Sulfisomidine	-CH3	-H	-CH3	-H	-H	0.370
Sulfadimethoxine	-CH3O	-H	-CH3O	-H	-H	0.025
Ro.43476	-H	-H	-CH3O	-H	-H	0.054
Ro.55615	-H	-H	-CH3O	-H	-CH3O	>10
Ro.78307	=O	-H	-CH3O	-H	-H	0.094

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Table 4.3. Structure-activity Relationships of pyridinyl, 3-pyridazinyl and 2,5-pyrimidinyl derivatives

RNH-SO_2-(C$_6$H$_4$)-NH_2

Compounds	R_1	IC_{50}(μM)
Sulfapyridine	N	0.180
Ro.211182	N—	>10
Sulfachlorpyridazine	Cl— N=N	0.018
Sulfamethoxypyridazine	CH_3O— N=N	0.017
Ro. 19194	N N=	2.7
Sulfadiazine	N =N	0.19
Sulfamerazine	N =N	0.078

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Table 4.4. Structure-activity relationships of heterocyclic derivatives

$R_1-N(R_2)-SO_2-C_6H_4-NH_2$

Compounds	R1	R2	IC50(μM)
Sulfisoxasole		-H	0.040
Ro.72844		2-propoxyacetyl	0.023
Ro.52928		acetyl	0.020
Sulfamethoxazole		-H	0.023
Sulfamoxole		-H	0.089
Sulfathiazole		-H	0.013

Table 4.5. Structure-activity relationships of phenyl derivatives

$RNHSO_2$–(benzene ring)–NH_2

Compounds	R	IC_{50}(μM)
NSC 14652-J	NH_2–(ring)–$NHSO_2$–(ring)–	0.18
NSC 39345-X	HO_2C–(ring)–	0.26
NSC 56605-K	CF_3, CF_3 (ring)–	0.80
NSC 229583-G	OH (ring)–	0.23

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Table 4.6. Structure-activity relationships of miscellaneous derivatives

$RNHSO_2$–⟨benzene⟩–NH_2

Compounds	R	$IC_{50}(\mu M)$
NSC 403439-F	MeO, N (quinoline ring)	0.38
Sulfaquinoxaline	N, N (quinoxaline ring)	0.030
NSC 74428-I	NH_2NH–⟨benzene⟩–C(=O)–	>10
NSC 74587-G	⟨benzene⟩–C(=O)–	0.32
Ro.13354	H	4.2
Sulfathiourea	NH_2-C(=S)–	0.022
Sulfaguanidine	NH_2-C(=NH)–	3.200
NSC-78438-Q	NH_2-C(=O)–	0.30

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Table 4.7. Sturcture-activity relationship of substituted p-amino derivatives

R_2NHSO_2–(C₆H₄)–NHR_1

Compounds	R1	R2	$IC_{50}(\mu M)$
NSC 52105-S	$—CSNH—$ (benzene ring) $—CO_2H$	(pyridine ring, N) —	5.9
NSC 107328-T	Me, N, N (pyrimidine ring), Me $—NHC(=NH)—$	$—CONH—$ (benzene ring)	>10
NSC 142656-T	$—C(=O)—CH_3$	$—CONHCH_2CH_2CHMe_2$	>10
NSC 163977-T	(benzene ring) $—CO—$, CO_2H	$—C(=O)—CH_3$	>10
NSC 205491-S	$—C(=O)—CH_3$	— (benzene ring) $—C(=O)—CH_3$	>10
NSC 270146-U	(furan ring, O) $—C(=O)—$	$—C(=O)—CH_3$	>10
NSC 279282-Z	$—N$, O, O, N, N (ring with two benzene rings)	$—C(=O)—CH_3$	>10
NSC 303757-F	$=N$, Me, N, N, O, $C(=O)$ (pyridine ring, N)	$—C(=O)—CH_3$	>10
NSC 355394-H	$—C(=O)—CH_3$	— (ring with O, $=O$, N, H)	>10
NSC 308795-G	$—C(=O)—CH(CH_3)CH_3$	— (benzene ring) OH, NH_2	>10

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Table 4.8. K_i values of most effective sulfonamides

Compounds	Ki(nM)	IC_{50}(nM)
sulfamethoxazole	7.5	23
sulfathiozole	10.5	13
sulfachloropyridazine	10.0	18
sulfamethoxypyridazine	12.5	17
sulfathiourea	16.5	22

Table 4.9. Inhibition of folate biosynthesis in situ

Compounds*	% Inhibition (Mean ± SD)	n[a]
Sulfamethoxazole	48.6 ± 11.9	4
Sulfachlorpyridazine	29.5 ± 16.6	3
Sulfamethoxypyridazine	75.0 ± 8.1	3
Sulfisoxazole	39.2 ± 36.7	3
Sulfathiazole	60.9 ± 25.1	3

[a] Number of experiments.

* 50 nM final concentration.

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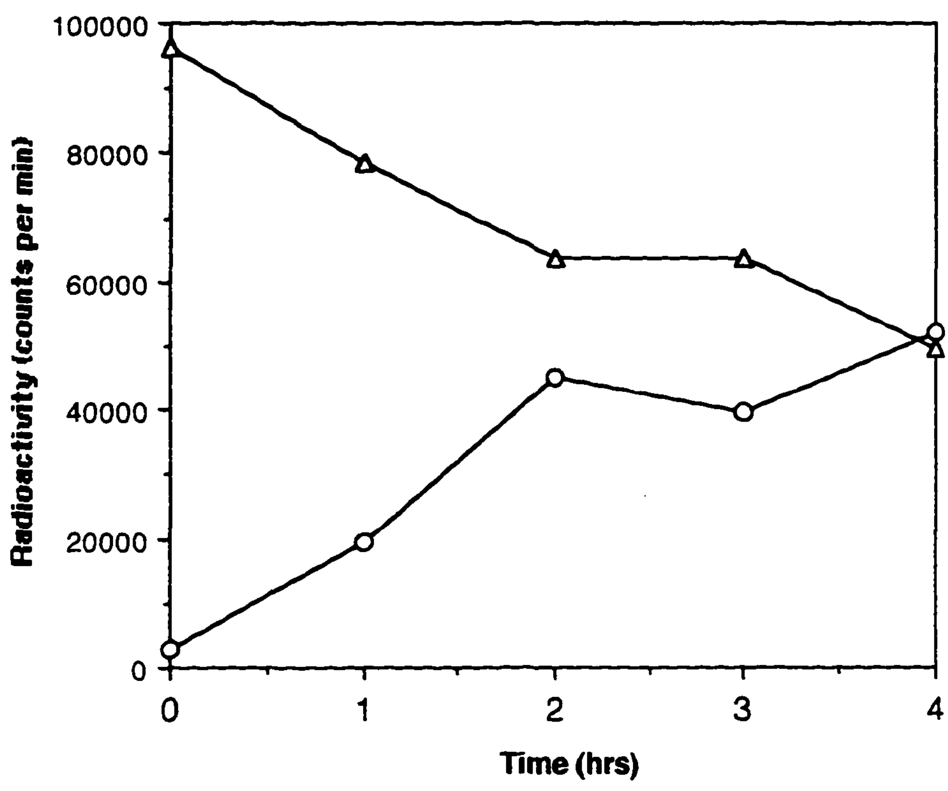

Figure 4.1. Changes in the concentration of H2Pte (--O--) and pABA (--Δ--) with time. Aliquots of a recombinant DHPS-containing lysate (40 μg) were incubated in 100 μl of assay buffer for 0 to 4 h at 37°C and then extracted with diethyl ether as described in Material and Methods.

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5. PARTIAL PURIFICATION OF THE GST-FAS FUSION PROTEIN

As described in section 4, crude extracts of recombinant DHPS were successfully used to screen 44 sulfa drugs based on their relative potencies. However, since other components of the extracts might compete for binding with one or both of the substrates, the absolute inhibitory efficacy of each sulfa drug can't be determined, and our knowledge about the structure-activity relationships of sulfa drugs is also limited by the impurity of the crude extracts. So a purer recombinant DHPS is needed for future sulfa drug design.

Our previous attempts to purify recombinant *P. carinii* Fas by using a folate affinity column (folate sepharose column) and 10 dye columns (Cibacron Blue 3GA; Reactive Blue 72 and 4; Reactive Yellow 86 and 3; Reactive Green 5 and 19; Reactive Brown 10; Reactive Red 120) were not successful. Since the amount of Fas protein expressed in insect cells is low, most of DHPS activities were lost during the long-time procedures which include washes, elutions, dialysis and concentrations. So we switched to GST fusion protein method. The recombinant *P. carinii* DHPS was expressed as fusion protein, GST-Fas, and then purified by affinity chromatography using glutathione agarose.

5.1. Purification of GST-fused recombinant *P. carinii* Fas.

DHPS activity was detected in the cytosol of both GST-fused recombinant baculovirus-infected Sf9 cells and High Five insect cells. The specific activities of DHPS in the Sf9 cell and High Five cell crude preparations were 1.45 $\pm$ 0.05 (n=2) and 19.3 $\pm$ 0.01 (n=2) pmol/ mg of protein per h, respectively.

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When the crude High Five cells extract was fractionated with 40% of ammonium sulfate, 34 % of the total protein and 71.5 % of the total DHPS activity were recovered. The specific DHPS activity increased by 2.1-fold (**Table 5.1**). After the 40% ammonium sulfate fraction was further purified by glutathione agarose, the specific activity of GST-fused Fas in the 50 mM glutathione elute was 742.9 pmol/ mg of protein per h. A summary of the purification of GST-fused Fas is shown in **Table 5.1**. The overall yield of activity was 4.6% with a purification of 39-fold.

The amount of GST-Fas expressed in High Five cells was still so little that there was no overexpressed protein band with the expected apparent molecular weight of 100 KDa could be seen by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After glutathione agarose purification, a protein band of 100 KDa showed, although there are several other protein bands (**Figure 5.1**). The High Five cells infected with recombinant baculovirus expressing GST-XYLE, a positive control protein, showed an overexpressed 60 KDa band. Pure GST-XYLE was easily purified by glutathione agarose (**Figure 5.2**).

Attempts were then made to remove the GST peptide from Fas using thrombin followed by incubation with GSH-agarose beads. The cleavage efficiency was low. Only 40% of total DHPS activities was recovered and the specific activity was reduced (data not shown). Since DHPS is not very stable at room temparature, long incubation time might cause the loss of DHPS activity.

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5.2 Discussion.

In this study, we successfully expressed recombinant *P. carinii* Fas as a GST fusion protein. The biological activity of the DHPS was retained . Nearly 40-fold purification of GST-Fas was achieved. The overall yield of DHPS activity was approximately 5%.

The major reason for the low yield purification is probably that the level of GST-Fas expression is too low, even though High Five cells are higher expressers. Thus, the binding of low concentration of GST-Fas to the glutathione agarose was blocked by the high concentration of other proteins in the crude lysate.

The 40% ammonium sulfate fractionation removed nearly 70% of total protein and retained 70% of total DHPS activity, this step slightly increased the purification yield from previous 1-2% (data not shown) to the current 5%.

The efficiency of thrombin cleavage on the partial purified GST-Fas is low. The thrombin cleavage site might be blocked by 72 KDa Fas and 26 KDa GST domains and longer incubation time is needed (up to 12 h). However, since DHPS is not stable at room temparature, longer incubation time is not recommended. The addition of 10 μM heparin might help to inprove the cleavage [Chang, 1985].

In order to achieve high yield of purification of Fas, high level of expression of Fas is needed.

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Table 5.1. Purification of GST-Fas

Fraction	Volume (ml)	Protein (mg/ml)	Total (mg)	Yield (%)	Specific Act. (pmol/mg)	Total (units)	Yield (%)	Purification
Crude lysate	17	5.5	93.5		19.2	1795		
$(NH_4)_2SO_4$	4.5	7.1	31.8	34	40.4	1284	71.5	2.1
GSH-agarose	0.5	0.21	0.11	0.1	742.9	81.7	4.6	39

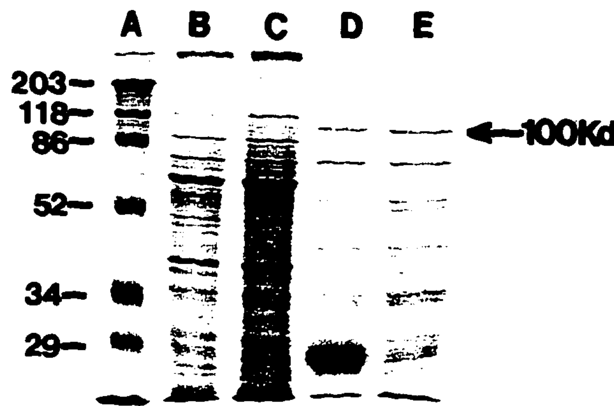

Figure 5.1. Purification of GST-Fas expressed in baculovirus-High Five cells by using GSH-agarose bead. 10-30 μg samples were subject to 12% SDS/PAGE electrophoresis, and stained with Coomassie brilliant blue. Lane A, protein standards; Lane B, cell lysate; Lane C, flow through; Lane D, 5 mM GSH eluate (first binding); Lane E, 5 mM GSH eluate (second binding).

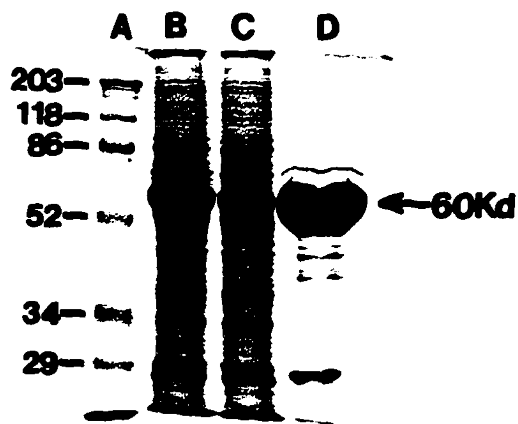

Figure 5.2. Purification of GST-XYLE expressed in baculovirus-High Five cells by using GSH-agarose bead. 10-30 μg samples were subject to 12% SDS/PAGE electrophoresis, and stained with Coomassie brilliant blue. Lane A, protein standards; Lane B, cell lysate; Lane C, flow through; Lane D, 5 mM GSH eluate.

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6. THE INTERACTION OF ARTEMISININ WITH MALARIA HEMOZOIN

Artemisinin, which is derived from an ancient Chinese herbal remedy for fever, is a sesquiterpene endoperoxide [Klayman, 1985 and Qinghaosu Research Group, 1979]. Several artemisinin derivatives, especially artemether and artesunate have been widely used in Asia, particularly China, where over one million patients have been treated [Hien et al, 1993]. In addition, a large number of synthetic endoperoxides have been synthesized and found to have antimalarial activity.

Malaria parasites live inside erythrocytes and consume hemoglobin. When heme molecules are released, they polymerize and accumulate in intraparasitic granules known as malaria pigment or hemozoin [Goldberg et al, 1990 and Slater et al, 1991]. We have previously proposed that this accumulation of heme sensitizes malaria parasite to the toxic effects of artemisinin and its derivatives, because it catalyzes the decomposition of the drug into free radicals which act as alkylating agents [Meshnick et al, 1991; Zhang et al, 1992; Meshnick et al, 1993 and Yang et al, 1993]. In this section we show that artemisinin concentrates in hemozoin where it is covalently bound to heme [Hong et al, 1992].

6.1. Fate of radioactivity in [^{14}C]-artemisinin-treated parasites

When malaria parasites were incubated in the presence of [^{14}C] artemisinin, more than 40% of the added drug was taken up by the cultures (data not shown). Of the cell-associated radioactivity, only 25% was found in the pooled washes and gradient, which contained more than 99% of the total

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protein (**Table 6.1**). The remainder, 75 % of the radioactivity, was associated with the isolated parasite hemozoin. About 1/3 of the hemozoin-associated radioactivity could be extracted with 1% SDS, which solublized >95% of the pelleted protein (**Table 6.1**). The SDS-washed hemozoin pellet contained the equivalent of 2.3 mmols of artemisinin per mol of heme.

6.2. Synthesis and partial isolation of hemin\artemisinin adduct.

In order to understand the interaction of hemin and artemisinin, the two compounds were mixed and allowed to react in solution. Two reaction product peaks were found by HPLC which eluted after the heme peak (**Figure 6.1**). These peaks were not seen when either reactant was incubated in the same buffer but in the absence of the second reactant (data not shown). The molecular weights of the adducts were then determined by ESI and FAB mass spectroscopy. Both mass spectra gave identical *m/z*'s for heme (616), adduct A (856), and adduct B (871) (**Figure 6.2a** and **6.2b**). Hemin, artemisinin and the adducts could also be separated by reverse-phase TLC (**Figure 6.3**). [The Rf of artemisinin is 0.41, and the Rf of hemin is 0.33. The adducts have Rfs of 0.16 and 0.21]. Neither adduct is fluorescent, suggesting that they still contain iron atoms (data not shown). Unfortunately, however, we were unable to successfully remove the iron from these adducts in order to be able to run NMRs.

6.3. Identification of hemozoin/artemisinin adducts in vitro and in situ.

TLC and HPLC were then used to determine whether the above adducts were also formed in vivo. The TLC system used could easily distinguish

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between artemisinin, heme and the adducts, although not easily between the two adducts (**Figure 6.3**) When hemozoin was isolated from [^{14}C]-artemisinin treated parasites and analyzed by TLC, 77% of the total radioactivity comigrated with the synthetic adducts (**Figure 6.4**). Similar results were found using HPLC (**Figure 6.5**), which also could not distinguish easily between the two products. (**Figure 6.1**)

Artemisinin also reacted with isolated hemozoin. When [^{14}C] artemisinin and hemozoin were incubated together, [^{14}C] artemisinin disappears from solution in a time-dependent manner (**Figure 6.6**). TLCs show a radioactive band, representing 20% of the hemozoin-associated radioactivity which comigrated with the adducts (not shown). This is the equivalent of incorporating 0.5 mmols of artemisinin per mol of heme.

6.4. Discussion

Artemisinin is taken up by malaria parasites and concentrated in hemozoin, possibly by forming adducts. In aqueous solution, artemisinin was found to react with hemin, yielding two major products with molecular weights of 856 and 871. Compounds with identical mobilities on TLC and HPLC were found in hemozoin, when either whole infected red cells of isolated hemozoin was incubated with drug. Thus, artemisinin-heme adducts appear to form in vivo.

The data presented here is consistent with a two-step mode of action of artemisinin, in which the first step consists of the heme-catalyzed cleavage of the endoperoxide bridge to form a free radical, and the second step consists of the alkylation of heme and proteins by the drug-derived free radical [Meshnick

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et al, 1994] or other transient drug-derived intermediate [Posner et al, 1992]. Other evidence consistent with this mechanism includes observations that the endoperoxide bridge is necessary for biological activity [Brossi et al, 1988 and China Cooperative Group, et al 1982], and that free radical scavengers and iron chelators antagonize the drug [Krungkrai et al, 1987;Levander et al, 1989 and Meshnick et al, 1989]. The ability of heme and iron to catalyze the decomposition of artemisinin has been demonstrated by cyclic voltammetry [Zhang et al, 1992], by electron paramagnetic resonance spectroscopy [Meshnick et al, 1993], and by the isolation and characterization of the decomposition products [Posner et al, 1992]. Evidence for alkylation of heme [Meshnick et al, 1991] and proteins [Yang et al, 1993] has also been presented previously. This paper is the first time that the heme-artemisinin adducts have been isolated and partially characterized. The synthetic adducts are likely to be identical to the products formed when parasites are treated with [^{14}C] labeled drug because they coelute on both silica TLC and reverse-phase HPLC. The importance of this process is underscored by the observation that about 1/2 of the cell-associated radiolabeled drug can be found in SDS-washed, protein-free hemozoin.

In a previous publication, we showed that a crude reaction mixture of heme and artemisinin, when analyzed by FAB mass spectrometry, gave peaks with *m/z*'s of 840, 868, 886 and 914 [Meshnick et al, 1991]. In contrast, the purified peaks shown here give *m/z*'s of 856 and 871 by both ESI and FAB mass spectrometry. The differences between the two observations may be due to differences in the reaction conditions, as well as to the possibility that the impure reaction mixture contained components which were not observed by HPLC since the elute was monitored at 400 nm, a wavelength which is

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characteristic of compounds containing porphyrin rings [Fuhrhop et al, 1975]. Interestingly the MW of adduct B, 871, is 18 less than that of the sum of artemisinin (282.4) and heme (616.5) suggesting a dehydration.

More artemisinin concentrates in hemozoin obtained from [^{14}C] artemisinin-treated infected red cells (2.3 mmols/mol heme) than by exposing isolated hemozoin to the labeled drug (0.5 mmols/mol heme). There are two possible reasons for this. First, the heme-catalyzed decomposition of artemisinin is reductive [Yang et al, 1993 and China Cooperative Group, 1982], suggesting that ferrous heme (Fe^{+2}) is more reactive than ferric heme (Fe^{+3}). Heme is more likely to be in the reduced state in vivo than in solution because of the presence of intracellular reducing agents such as glutathione. Second, in vivo, heme may react with artemisinin in the brief time period between when it is liberated from hemoglobin and polymerized to hemozoin [Goldberg et al, 1990]. Once incorporated into hemozoin, only the heme molecules on the surface of the granules are likely to be reactive with artemisinin, since the iron atoms of the interior molecules may be sequestered by iron-carboxylate bonds [Slater et al, 1991].

What role does the formation heme-artemisinin adducts play in the drug-mediated killing of malaria parasites? The heme-artemisinin adduct is not toxic to in vitro cultures of P. falciparum when added to the media [Meshnick et al, 1991]. When parasites are grown in resealed red cells containing an excess of heme, they appear to be protected from artemisinin, presumably because the adducts form in the red cell cytoplasm (unpublished results). However, the CR strain of P. berghei, which lacks hemozoin, is resistant to artemisinin [Peters et al, 1986]. Furthermore, 3/4 of the cell-associated drug winds up in hemozoin, of which 2/3 (1/2 of the total) remains even after SDS

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extraction of protein. Thus, the formation of artemisinin-heme adducts play important but as yet undefined roles in the mode of action of this drug.

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Table 6.1. Fate of radioactivity in [^{14}C]-artemisinin treated parasites*

Fraction	counts min^{-1}	%	protein (mg)	%
Pooled supernatants	84635	25	2081.3	99.58
Hemozoin				
SDS-extract	90665	27	8.3	0.40
SDS-pellet	165000	48	0.5	0.02
Total	340300		2090.1	

* cultured for 8 hours in the presence of [^{14}C]-artemisinin (2.7 nCi ml^{-1}).

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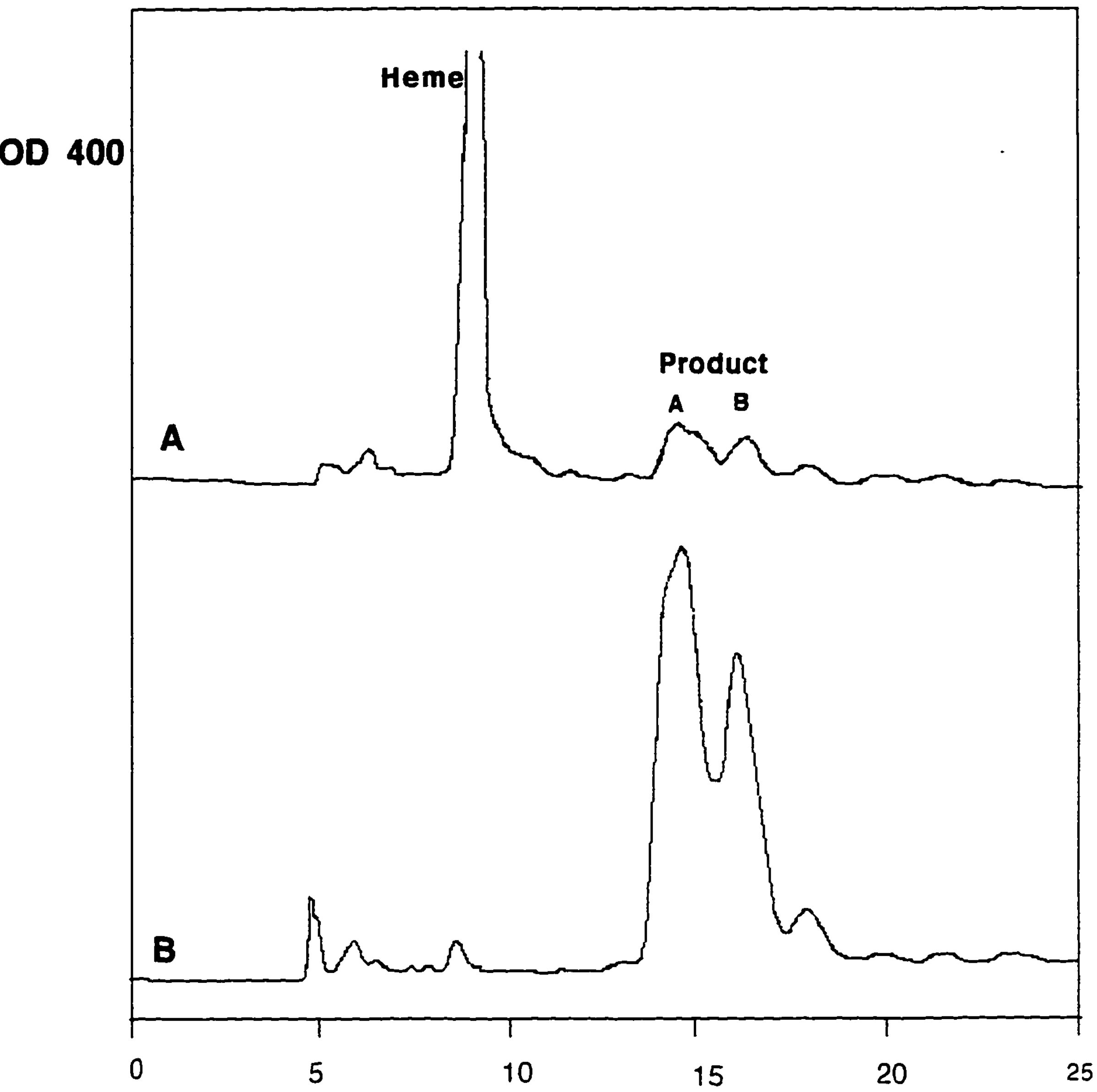

Figure 6.1. HPLC separation of hemin and artemisinin reaction products after incubating 20 h at 37°C in the dark. HPLC was performed on a semipreparative column as described in the Materials and Methods, eluting with methanol: water : acetic acid (7: 3: 1). (A). Reaction Mixture; (B) Rechromatography of pooled products from A.

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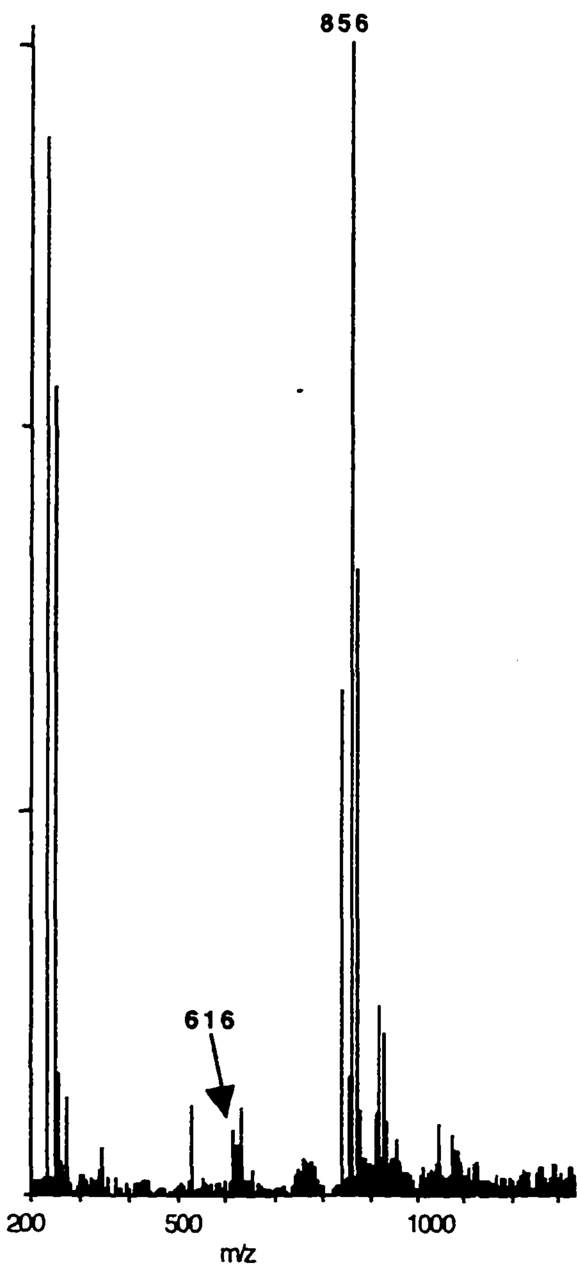

Figure 6.2a. Electrospray mass spectra of HPLC-purified hemin-artemisinin reaction product A.

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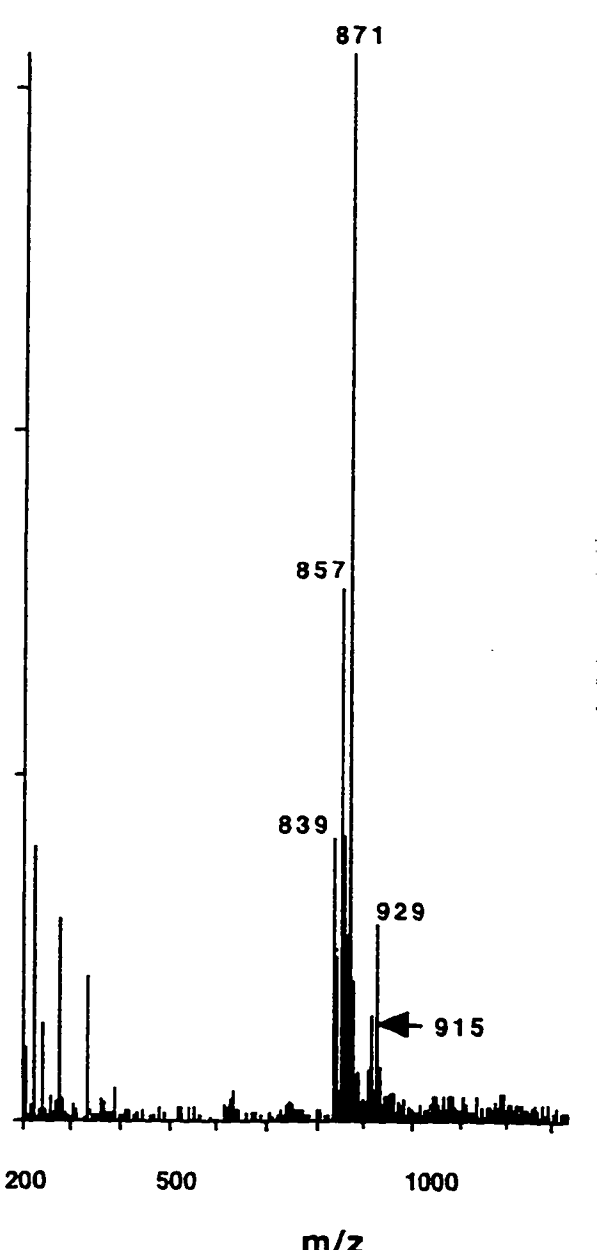

Figure 6.2b. Electrospray mass spectra of HPLC-purified hemin-artemisinin reaction product B.

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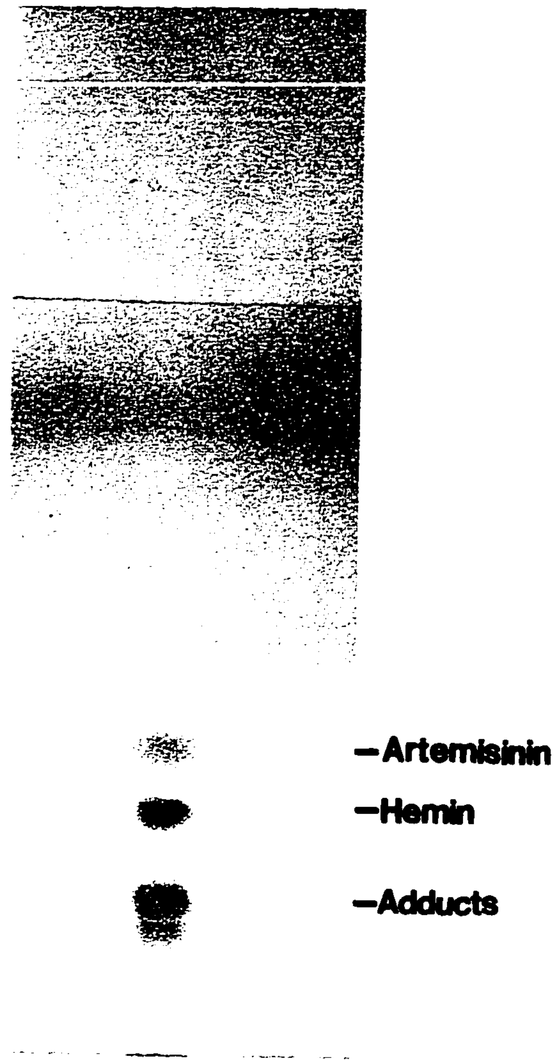

Figure 6.3. TLC of artemisinin, hemin and the isolated hemin/artemisinin adducts visualized by exposure to iodine vapor.

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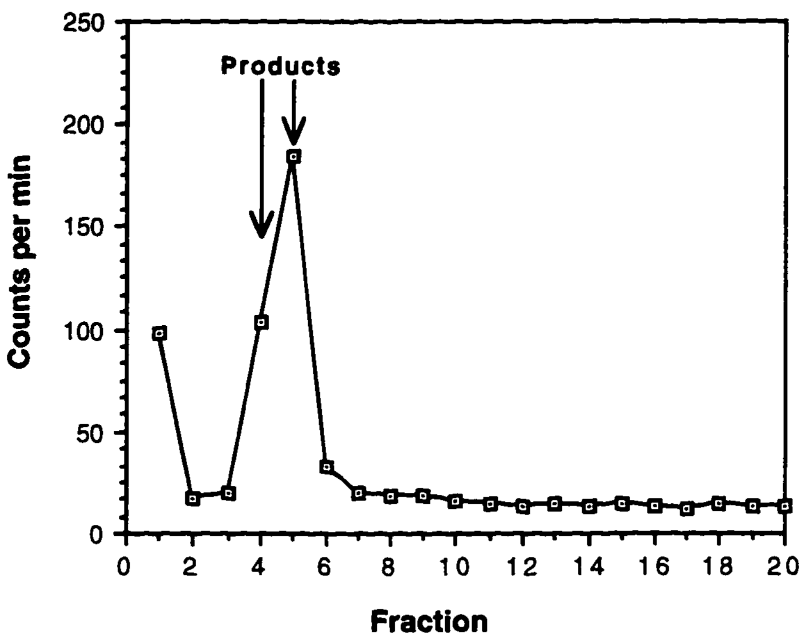

Figure 6.4. Radioactivity profile of TLC run on hemozoin isolated from [^{14}C]-artemisinin-treated parasites. The parasites were cultured in the presence of [^{14}C]-artemisinin (1.8 nCi per ml) for 6 h before isolation. Origin of plate on left, solvent front on right.

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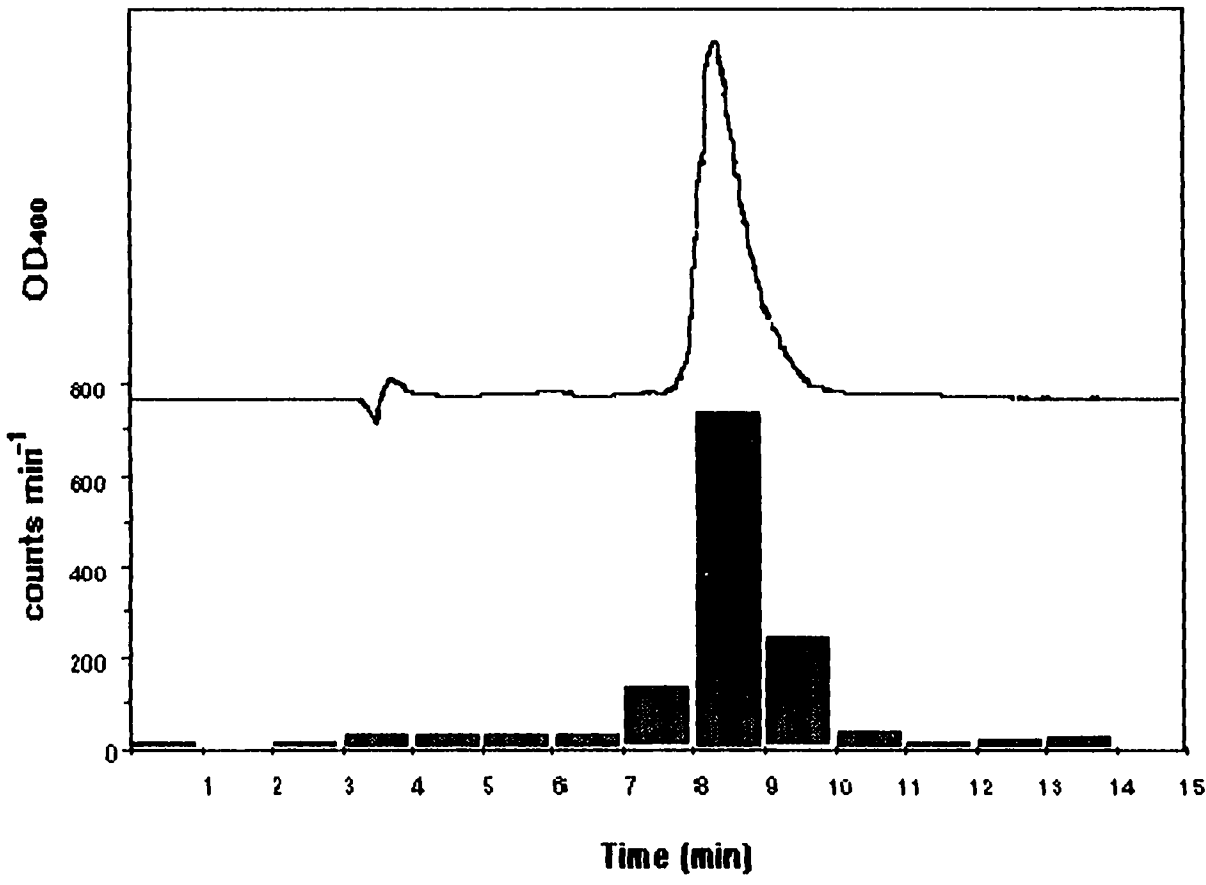

Figure. 6.5. HPLC profile of purified product A (top) and of radioactivity eluted from an HPLC separation of hemozoin isolated from [14C]-artemisinin treated parasites (bottom).

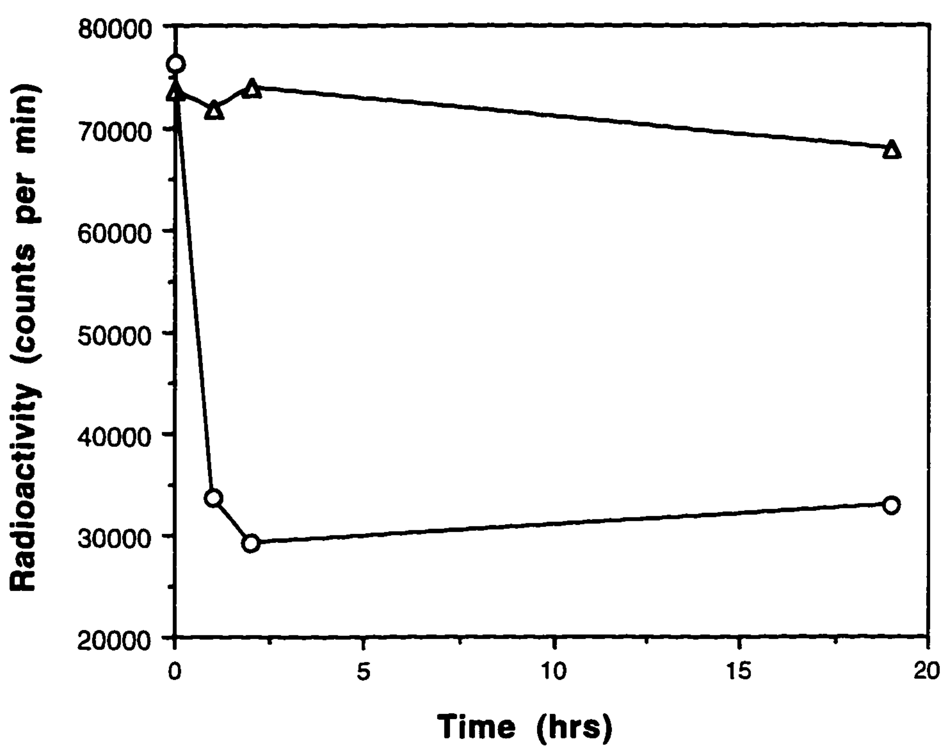

Figure 6.6. Radioactivity in the supernatant during the incubation of [^{14}C]-artemisinin in the presence (--O--) and absence (--Δ--) of isolated hemozoin.

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7. SUMMARY AND FUTURE DIRECTIONS

7.1 Summary.

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

1. Both lung-derived and culture-derived *P. carinii* synthesize pteroylpolyglutamate de novo from exogenous pABA, and the predominant forms are the pteroylpentaglutamates.

2. Culture-derived *P. carinii* remains metabolically active when cultured with feeder cells for at least 4 days. They produce pteroylpolyglutamates at 4 X higher specific activities than lung-derived organisms.

3. Crude preparations of recombinant *P. carinii* DHPS could be successfully used to screen the relative efficacies of 44 sulfa drugs. This in vitro screening system can now be used to screen a large number of compounds.

4. Sulfachlorpyridazine, sulfathiazole, sulfamethoxypyridazine and sulfisoxazole compare well with sulfamethoxazole not only in their abilities to inhibit recombinant DHPS but also in their abilities to inhibit de novo folate biosynthesis in *P. carinii.*

5. In vitro, hemin and artemisinin are found to undergo a chemical reaction forming two major products. The m/z values of them are 856 and 871.

6. [^{14}C]-Artemisinin is taken up by malaria parasites and concentrated in hemozoin. The major of the hemozoin-associated radioactivity comigrates with the synthetic hemin/artemisinin products in both TLC and HPLC of hemozoin

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isolated from [^{14}C]-artemisinin-treated parasites. Thus, artemisinin appears to react covently with heme in malaria hemozoin both in vitro and in situ.

7.2. Future directions.

1. Since sulfamethoxypyridazine and sulfisoxazole are known to be less toxic than sulfamethoxazole, they should be further evaluated in animal models for the treatment and prophylaxis of PCP.

2. More efforts are needed for the purification of recombinant *P. carinii* Fas. The priority is to increase the level of GST-Fas expression.

3. The pure recombinant Fas will then be used for enzyme kinetic studies, crystallography and site-directed mutagenesis. Then we will learn the 3-dimensional structure of active sites of DHPS and design new sulfa drugs that are more effective and less toxic.

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