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Folate metabolism and chemotherapy in *Pneumocystis carinii* and *Plasmodium falciparum*

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FOLATE METABOLISM AND CHEMOTHERAPY IN *PNEUMOCYSTIS CARINII* AND *PLASMODIUM FALCIPARUM*

by

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ABSTRACT

Folate Metabolism and Chemotherapy in *Pneumocystis carinii* and *Plasmodium falciparium*

by

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De novo folate biosynthesis is required for growth of *Pneumocystis carinii*, a microbe which causes serious respiratory infection in immunocompromised patients, and *Plasmodium falciparum*, a malarial parasite. In the studies reported here, we examined three steps of the folate biosynthetic pathway as targets for chemotherapy. These were the synthesis of para-aminobenzoic acid (PABA), synthesis of dihydropteroate, and synthesis of folates.

Using a new reversed-phase HPLC assay procedure, dihydropteroate synthetase (DHPS), activity was detected from *P. carinii* (14 units/mg). Concentrations required for 50% inhibition (IC_{50}) and the Michaelis-Menton inhibitory constants (K_i) for several sulfa drugs were determined. Dapsone (IC_{50} = 215μM, K_i = 9 μM) was the most potent inhibitor, whereas sulfadiazine was the weakest. Sulfaquinoxaline, sulfamethoxazole, and sulfadoxine all displayed intermediate inhibition, with IC_{50} of 400-600 μM and K_i of 60-150 μM.

A culture system using mink lung cells (MV1-LU) as supporting cells maintained *P. carinii* growth for up to 5 days. Because quantitation of *P.*

carinii by staining and counting is difficult and inaccurate, we developed a new reliable *in vitro* assay for quatitation of parasite growth. This assay measured the incorporation of [^{3}H]-PABA into folate. The folate synthesized by the microbe was bound by folate binding protein and unincoporated PABA was removed by binding to charcoal-coated dextran. Using this assay, it was determined that the microbe accumulates 1.8 ± 0.6 fmol of radioactive folate/mg protein. Several sulfa drugs were tested for their ability to inhibit folate synthesis; it was again determined that dapsone was the most potent inhibitor.

The sensitivity of *Pneumocystis carinii* to artemisinin (qinghaosu), a potent antimalarial agent, was determined in a short-term primary culture. In untreated cultures, trophozoites increased an average of 5-fold over 4 days. Inhibition of parasite growth was seen in cultures treated with arteminisin at concentrations as low as 0.5 μM. In contrast, artemisinin concentrations up to 100 μM had no effect on feeder layer cells. Further conformation of these results was obtained by utilizing the folate assay described above.

Deferoxamine, an iron chelator, was previously shown to have an anti-pneumocystis activity but it has a short half-life in vivo. Using the folate assay above, we examined the effect of a novel iron chelator with a long half life, desferoxamine hydroxyethyl starch (DFO-HES), on *Pneumocystis carinii*. It was determined that DFO-HES had an IC_{50} of 50 μM compared to a previously published value of 23 μM for DFO.

Malaria parasites, unlike their mammalian hosts, are dependent on *de novo* folate biosynthesis. The folate assay described above was utilized to investigate the importance of exogenous PABA. It was shown that the

parasite-infected red cells accumulate 5.4 ± 0.8 fmol radioactive folates per mg of protein from [^{3}H] PABA. In contrast, there was no detectable accumulation of radioactive folates in uninfected cells. Sulfadoxine, a DHPS inhibitor commonly used for treatment of malaria, was shown to have an IC_{50} of 10nM.

Para-aminosalicylic acid (PAS), an analog of PABA, has been widely used in the treatment of tuberculosis. Interestingly, PAS competitively inhibited DHPS and the uptake of PABA but was lacked *in vitro* antimalarial activity, suggesting a possibility that PABA is obtained from alternate sources , such as intraerythrocytic pools or biosynthesis of PABA. Using HPLC, it was determined that *Plasmodium falciparum* -infected red cells incubated in PABA- and folate-free media, contained 95.4 ± 17 pmol of PABA per mg protein (n=3) while the uninfected red cells had <1 pmol of PABA per mg of protein. On the other hand, in media containing PABA infected and uninfected red cells contained 153 ± 15 and 22 ± 10 pmol PABA/mg, respectively.These data suggest that *P. falciparum* may be capable of PABA biosynthesis.

Four *P. falciparum* mutants auxotrophic for PABA were analyzed for their intracellular PABA content. Although, all four strains were comparable to the parent clone in regards to PABA accumulation, only two strains were able to synthesize PABA *de novo*. This result substantiates that PABA biosynthetic pathway exists in *P. falciparum*.

Dedicated to my family for all their encouragement:

To Carmen, my wife,

to Gulamabbas and Mariam, my parents

and

Michael Rashad, my son,

for all the happiness he has brought us.

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I would like to express my deepest appreciation to Dr Steven Meshnick, for his guidance, help and friendship. I am also indebted to late Dr Donald Sloan, whose encouragement led me to join the biochemistry program. Furthermore, he was always kind, helpful and supportive. This effort would have not been possible without the help of my thesis committee. I thank especially Dr Allen Clarckson and other members for their time and support; Dr Gary Quigly, Dr Thomas Haines and Dr Horst Schultz.

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LIST OF ABBREVIATIONS

PABA: p-aminobenzoic acid

PAS: p-aminosalicylic acid

DHPS: dihydropteroate synthetase

PPPK: hydroxymethyldihydropterin pyrophosphokinase

DHFS: dihydrofolate synthetase

DHFR: dihydrofolate reductase

PCP: pneumocystis carinii pneumonia

AIDS: acquired immunodeficiency syndrome

HPLC: high pressure liquid chromatography

PHBA: para hydroxy benzoic acid

DFO-HES: deferoxamine conjugated to hydroxy ethyl starch

TMP/SMX: trimethoprim/sulfamethoxazole

IC_{50}: concentration at 50% inhibition

MDHP-PP: 6-hydroxymethyl dihydropterin pyrophosphate

PABG: para-aminobenzoyl glutamate

PPK: pyrophosphokinase

1. Introduction

1.1 The diseases

1.1.1 *Pneumocystis Carinii* Pneumonia

Pneumocystis carinii is an opportunistic pathogen which causes a potentially fatal pneumonia in patients with acquired immunodeficiency syndrome (AIDS) and other immunocompromized patients. It is an extracellular parasite which is ubiquitous in nature and only becomes dangerous when immune system fails, particularly, when the T cells are attacked by AIDS virus or are suppressed as a result of therapy for other diseases (Kovacs *et al.*, 1984)

The organism was first discovered in 1909 by Chagas who observed *P. carinii* in rats infected with *Trypanosoma cruzi*. He erroneously thought to be a part of trypanosome's life cycle. A year later, an Italian researcher Antonio Carini made same mistake when he found the microbe in lungs of *Trypanosoma-lewisi* infected rats. Two years later in 1912, Delonoe surprisingly found the same microbe in lungs of sewer rat and identified it as a distinct species, knowing of Carini's earlier description, named the novel microbe *Pneumocystis carinii* in honor of the Italian researcher (Reviewed by Frenkel, 1976).

P. carinii was not recognized as a medical problem in humans until after World War II when many malnourished infants in the overcrowded orphanages had a a pneumonia characterized by plasma-cell infiltrates

which was called, "plasma-cell interstial pneumonia". The disease was debilitating and had high mortality rate (Young, 1984).

The first breakthrough in treatment of PCP came in the 1960's, when Ivady and coworkers (Ivady *et al.*, 1967) found that pentamidine isethionate, a drug used to treat African trypanosomiasis, was effective in therapy for PCP. This led to decrease in fatality rate.

In the 1970's, the disease was considered as a sporadic phenomenon in immunocompromised hosts. PCP occured in children treated with chemotherapeutic agents for leukemia (Hughes *et al.*, 1973); in individuals with hypogammaglobulinaemia (Burke *et al.*, 1961); in patients treated with immunosuppressive drugs associated with renal transplantation; and in patients treated for various other inflammatory disorders (Hardy *et al.*, 1984; Walzer *et al.*, 1974)

PCP is the most common infectious disease occuring in AIDS patients. The number of PCP cases has increased as the number of AIDS cases have steadily increased to an estimated 1.5 million by the end of 1991 (Zackrison and Tsou,1991). As many as 80% of AIDS patients in United States develop PCP at some time during their sickness (Glatt *et al.*, 1988). PCP accounts for 43% of all infectious diseases in AIDS patients and is the major identifiable cause of death in 25% of AIDS patients (Leoung and Hopewell, 1990).

1.1.2 Malaria

Malaria, a disease caused by a protozoa of genus *Plasmodium*, is a cause of health problems in many third world countries. It is approximated

that 500 million cases of malaria occur in a year causing 2 million fatalities (Warhust, 1987).

In the early 1930's malaria was effectively controlled by using a combination of chloroquine and dichloro-diphenyl-trichloroethane (DDT) to curb anopheline mosquitoes. However, in the following decade chloroquine and DDT resistance strains started to appear which resulted in steady increase of malaria cases (Bruce-Chwatt *et al.*, 1981).

Currently there are four *Plasmodium* species which cause malaria in humans. These are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The mild cases of the disease caused by these parasites is characterized by fever, and anemia. If the disease is untreated life threatening complications can occur from extensive hemolysis and from diminished microcirculation (Wyler and Pasvol, 1986).

1.2 Biology of organisms

1.2.1 *Pneumocystis carinii*

1.2.1.1 Classification

The exact taxonomic classification of the organism still remains controversial despite many years of research. Until recently *P. carinii* was thought by most scientists and physicians to be a protozoa because the microbe was susceptible to anti-protozoa therapy and was also superficially similar to certain microspora and apicomplexa (Beaver *et al.* 1984). During the last two decades, however, several anomalous observations have challenged the taxonomic position of *P. carinii*. Electon microscopy studies have determined that the ultrastructure of *P. carinii* resembles a fungus of

yeast like characteristics rather than protozoan (Vavra and Kucera, 1970). The biochemical analysis of have shown that the cell wall of *P. carinii* cysts (a stage in *P. carinii* life cycle) consists of B-1, 3-glucan, a compound which is found in many yeast cell walls (Matsumoto *et al.*, 1988). Recently, more convincing evidence on taxonomic assignment has been obtained based on evaluation of rRNA. First, the comparison of nucleotide sequences present in 16S ribosomal RNA from different species suggests that *P. carinii* is more closely related to a *Saccharomyces cerevisae* (a yeast) than a protozoa (Edman *et al.*, 1988). Second, a comparison of 5S rRNA of *P. carinii* and other common fungi suggest that *P. carinii* is closely related to Rhizopoda, Myxomycota and Zygomycota (Watanabe *et al.*, 1989). The prevailing current opinion is that *P. carinii* is a fungus.

Another controversial issue has been whether there is one or several types of strains of *P.carinii*, each specific for a host species. When observed under light or electron microscope *P. carinii* isolated from humans or from rats do not exhibit any morphological difference. In contrast, the molecular biology tests using DNA hybridization technique have determined that the genomic DNA clones obtained from human *P. carinii* show variable hybridization to the ones from rat *P. carinii* (Tanabe *et al.*, 1988). Additional serological tests have shown a difference in surface antigen of the *P.carinii* isolated from rat and humans (Kim *et al.*, 1972). The existence of different *P. carinii* strains is not totally accepted yet, but seems probable. Some workers have used terminology for human isolates such as *P.carinii humanus* and *Pneumocystis jiroveci* (Frankel, 1972 and Hughes and Giglioti, 1988).

1.2.1.2 Life cycle

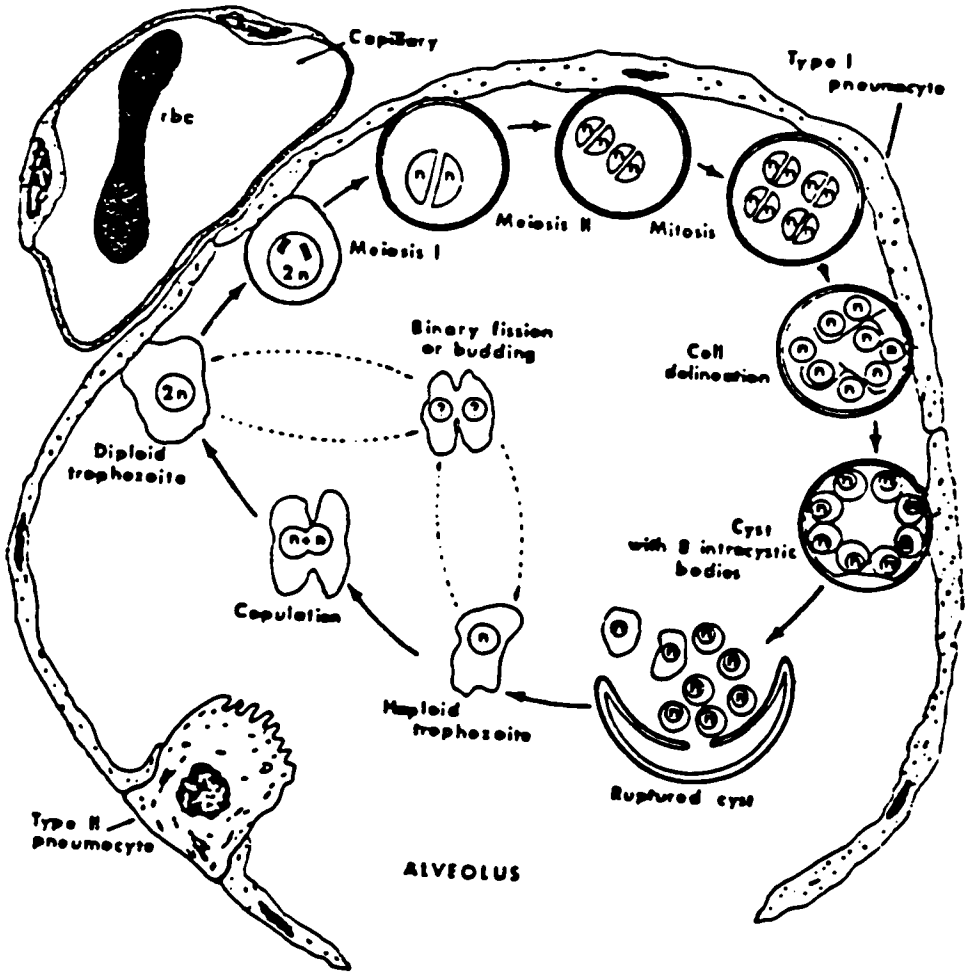
The life cycle and the mode of reproduction of *P. carinii* is poorly understood partially due to lack of long term cell culture. The information obtained about the life cycle is predominantly descriptive and imaginative and is based on transmission and scanning electron microscopy. So far only three developmental stages of *P. carinii* have been identified: cysts, sporozoites and trophozoites.

The cysts are thought to be a resistant stage of the parasite's life cycle (Campbell, 1972); they are thick walled and are mainly found attached to type I pneumocytes, alveolar spaces and other tissues. Cysts are oval or round shaped and are approximately 4 to 7 μM in diameter. Each cyst may contain upto 8 mononucleated intracystic bodies or sporozoites which are derived from individual trophozoites. Development of the sporozoites is thought to be an important step in replication of *P. carinii*. The sporozoites (1 to 2 μM in diameter) originate and develop within the cysts. Unknown factors cause the cyst to rupture, releasing the sporozoites which develops into mononucleated thin walled trophozoites (Figure 1).

The trophozoite measures approximately 2 to 8 μM in diameter and attaches to type I pneumocyte. Trophozoites develop into cysts; however, the process by which this development occurs is not clear. Freeze fracture and transmission electron microscopy studies have suggested that the development of the trophozoites into cysts involves conjugation, meiosis and mitosis (Itatani and Marshal, 1988; Matsumoto and Yoshida,1984; Yoshida,1989).

The following scheme, therefore, has been is proposed for life cycle of *P. carinii* : the mature cyst raptures releasing eight haploid sporozoites which subsequently become haploid trophozoites; two

Figure 1. Proposed life cycle of *P. carinii*. Obtained from Gutierrez(1989)



haploid trophozoites conjugate to form one diploid trophozoite which enters the cyst forming phase of life cycle by becoming a precyst which matures thus completing the cycle.

Electron microscopy studies have revealed that the precysts form a synaptolemal complex (indicative of meiosis). Therefore, the precysts nucleus divide twice by process of meiosis to form a cysts containing four haploid nuclei. As cysts mature, final mitosis produces eight nuclei. Each nucleus forms its own plasma membrane by partition of the cytoplasm thus becoming a sporozoite. It is also proposed that an asexual cycle occurs in which diploid or haploid trophozoites divide by process of binary fusion or budding (Yoshida, 1989).

1.2.1.3 Staining characteristics of *P.carinii*

Although the electron microscopy has played a major role in understanding the ultra-structure of *P. carinii*, its importance in diagnosis has not been utilized because the parasites can be easily detected by light microscope. Two basic forms of *P. carinii* stains are currently used; cyst walls stains and cytoplasmic stains that reveal trophozoites and sporozoites.

1.2.1.3.1 Cyst wall stains

As mentioned in section 1.2.1, cysts form of *P. carinii* contain large amounts B-1-3-glucan and weakly ionic sugars. The cyst wall stains require that the sugars and glucan first be chemically modified. In case of toluidine blue O, for example, the sugars are first esterified with a combination of sulfuric acid and acetic acid which then makes them susceptible to stain with toluidine blue O - a positively charged stain (Gossey *et al.* 1985). The

resulting stained cysts have a lavender appearance. In another cyst wall stain, Gamori's methamine silver, the sugars are first oxidized to aldehydes by chromic acid. The aldehyde sugars then react with exogenously added silver salts to form elemental silver which gives cyst wall a black appearance (Mahan and Sale, 1978).

Cyst wall stains are frequently used for diagnosis primarily due to their high contrast and their ability to distinguish the cysts from background tissue elements. The cyst wall stain are also reliable when used for identifying *P. carinii* in tissue sections but uptake of these stains by other fungi can cause difficulty in differentiating *P. carinii* from other fungi. In addition, distinction between empty cysts and the ones containing daughter cells is not possible when the cyst wall stains are used. Therefore cyst wall stains are unreliable for quantitating *P. carinii* in vitro.

1.2.1.3.2 Trophozoites stains

For evaluating parasites *in vitro*, many investigators prefer the trophozoites stains such as Giemsa (Kim and Hughes, 1973), and Diff Quick (Tollerud *et al.*,1989). Unlike cyst wall stains, trophozoites stains provide additional information about the life cycle of the organism *e. g.* number of sporozoites in a cyst. A major disadvantage of the stain is that it stains not only the organism but most of the background material and therefore it becomes very difficult to exactly quantitate *P. carinii.*

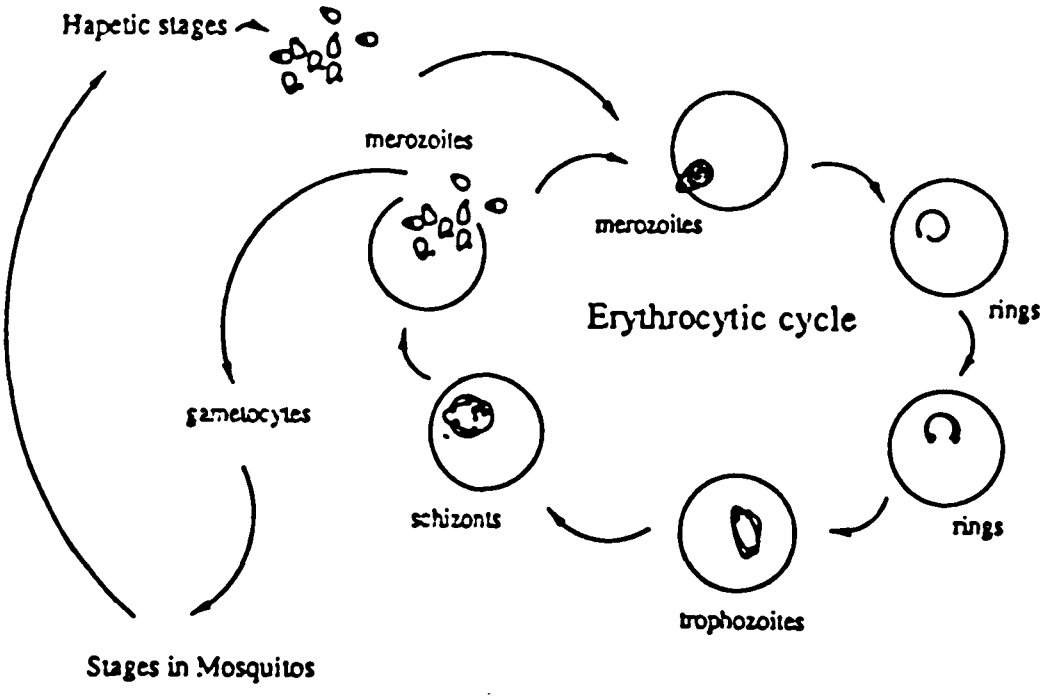
Giemsa stain is currently the most prefered of trophozoite stains. It consists of two dyes: Methylene blue and eosin and was originally developed for staining blood cells. Methylene blue is a positively charged thiazine dye, which is commercially available as an azure blend-an

oxidative derivative of the dye. Methylene blue apparently binds to RNA and causes the cytoplasm to be stained blue. Eosin is a negatively charged triarylmethane dye, which binds to positively charged histones, and stains the nuclei pink (Thompson and Hunt, 1966). Diff-Quick, a rapid stain commonly used for differential cell counts in bronchoalveolar lavage fluid and peripheral blood, is also effective and rapid in quantitating *P. carinii* (Smith and Bartlett, 1991). Diff-Quick is a modification of Giemsa stain and contains a Xanthene dye in addition to methylene blue and eosin. Xanthane dye enhances the function of eosin thus causing the organism to be stained rapidly.

1.2.2 Malaria

All *Plasmodium* species have a sexual life cycle in the invertebrate host, *i.e.* mosquito and asexual life cycles in vertebrate's erythrocytes and other tissues. The infection in vertebrates begins with a bite by a mosquito and injection of sporozoites into the blood stream. Once in the blood stream the sporozoites are carried to the liver. The sporozoites invade the hepatocytes and divide asexually. The sporozoites eventually matures into merozoites which are released into the blood stream to invade the red blood cells, where intraerythrocytic asexual life cycle begins by development of rings, trophozoites and schizonts. The mature schizonts produce a large number merozoites which lyse the erythrocyte. The merozoites are then released into the blood stream to invade more erythrocytes (Figure 2). The release of merozoites is associated with fevers and chills commonly found in malaria patients.

Figure 2. Life cycle of malarial parasites. Obtained with permission from Zhang (1992)



In addition to asexual life cycle in erythrocytes, some merozoites invade other red blood cells and differentiate sexually into gametocytes. The sexual cycle continues when the gametocytes are ingested by mosquitos. While in the stomach the gametocytes produce gametes, which fuse to form zygotes. The zygotes form an ookinete which penetrate the stomach wall. Once on the outside, the ookinete develops into a cyst-like structure called oocyte. In the oocyte thousands of sporozoites are produced by a asexual process called sporogony. When the oocyte matures, it bursts releasing many sporozoites which now enter salivary glands. A new life cycle is again initiated by infecting vertebrate host.

1.3 In vitro cultures

1.3.1 *Pneumocystis carinii.*

For many years investigators have been trying to culture *P. carinii* in vitro but with limited success. A continous long-term culture system has not been achieved but would be important for providing providing valuable information about the biology and biochemisty of the organism. A continous culture system could aid in the discovery of new anti-pneumocystis agents. Currently, evaluation of anti-*Pneumocystis* activity has relied on short-term culture system using feeder-layer cells and *P. carinii* from immunosuppressed rodents. In this section, a brief history of various culture system and details of current culture systems will be reviewed.

The first successful short term culture system of *P. carinii* was not reported until in the late 1970's by Pifer and her coworkers (Pifer et al, 1977). The culture system they used relied on feeder layer cells such as

primary embryonic chick epithelial lung cells (CEL) to support the *P. carinii* growth. *P. carinii* to seed the culture was obtained from lungs of immunosuppressed Sprague-Dawley rats or from human lung. The culture was initiated by inoculating CEL cells with *P. carinii* obtained from PBS washed lungs. The organism were then quantitated by counting cysts from toluidine blue stain. The organism were passed serially 4 times which resulted in 100 fold increase in number of cysts. However, attempts to reproduce these results have not been successful. For example, McMannigal (1979) reported that the cyst count only doubled when *P. carinii* were cultured with CEL as supporting cells (reviewed by Smith and Bartlett, 1984). Furthermore, the cysts counts are not reliable when used in culture (see section 1.2.3.1).

Various cell lines have been evaluated in order to determine which supporting cells would provide better yield and if the microbe could be grown in a continous culture. Latorre and his coworkers observed the growth of *P. carinii* in three different cell lines: MRC-5, Vero and Chang liver. Once again the organisms were obtained from immunosuppressed rodents. It was claimed that the organisms were serially passed 16 times that resulted in theoretical replication factor of 65,000 times (Latorre *et al.*, 1977). Although the yields are excellent, there are multiple problems with author's methodology. First, the inocula was not quantitated. Second, the organisms were not quantitated after each passage. A 16 times passage does not necessarily mean 2^{16} replication factor. Third, the attempts of Bartlett and coworkers (1979) to reproduce these results were not successful.

More promising results were obtained by Bartlett *et al.* using primary culture of human embryonic lung fibroblast cell line (WI-38) and MRC-5

(Bartlett *et al.*,1979). The organisms were obtained from homogenized lungs of immunosuppressed rats. The confluent cell lines were inoculated with supernatant containing *P. carinii* and the growth was assessed microscopically by counting number of trophozoites in Giemsa stained slide. The organism were subcultured twice with maximum yield of 10 folds when WI-38 cells were used as supporting cells. Furthermore, better yields were obtained when the cultures were maintained in 10% carbon dioxide and 5% oxygen.

Another cell line derived from human lung carcinoma, A549, reportedly was able to support *P. carinii* growth (Cushion and Walzer, 1984 and Cushion and Walzer, 1984). The organisms were obtained immunosuppressed rats or human lavage fluid. The homogenate from infected lungs were used to inoculate the cultures. The organism's nuclei were then quantitated by using Diff Quick stain, which stains both cysts and trophozoites. The *P. carinii* was subcultured three times and maximum of 10 fold increase in number of nuclei was observed. More importantly, when WI-38 was used as supporting cells, a maximum of 10 fold increase was observed, thus confirming the previous observations of Bartlett *et al.*

Many attempts has been made to culture *P. carinii* without using supporting cells with only limited success. In one such study, *P. carinii* isolated from the rat lung were cultured in a medium containing neopeptone and N-acetylglucosomine at pH 4. The *P. carinii* growth was quantitated by using Diff-Quick stain and counting the number of nuclei. It was observed that *P. carinii* growth increased upto 10 folds during the first 24 hrs and seized to increase after that (Cushion and Ebbets, 1990). This method might be useful to evaluate the efficacy of fast acting drugs such as pentamidine.

However, for slow acting drugs, the growth of *P. carinii* using supporting cells is still the best choice.

Most of culture system reported have yielded very low amounts of *P. carinii* with an average increase of 1 or 2 logs. Since it is not possible to subculture *P. carinii* more than 4 times, many investigators are now concentrating on obtaining large number of *P. carinii* in culture. In one such study, slow speed stirring vessels (spinner flask) containing Cytodex microcarrier beads were utilized to culture large quantities of trophozoites (Durkin et al, 1989). The monolayers of WI-38, Mv 1 Lu and A549 were first developed in spinner flasks for 3-5 days and the flasks were then inoculated with *P. carinii.* from homogenized lung. After 72 hrs of incubation, a maximum of 10 fold increase in number of trophozoites for all three cell lines was observed, which was similar to *P. carinii* grown in culture flasks. However, in using this method the authors were able culture large amounts *P. carinii.*

1.3.2 Malaria cultures

In early 1970's various methods were tried to culture *P. falciparum* but only with limited success. The big breakthrough came in 1976 when Trager and Jansen were able to successfully cultivate *P. falciparum* in a continuous culture. (Trager and Jensen, 1976). The parasites were cultured in human erythrocytes and in RPMI 1640 medium containing 10% human type A^+ serum. The cultures were maintained with 7 percent carbon dioxide and low oxygen at 38^0 C.

Later on, in mid 1980's, a complete in vitro hepatic system of *P. vivax* and *P. falciparum* was introduced by Mazier and his coworkers, who were

able to show that human hepatocytes inoculated with sporozoites were able to mature into merozoites. Furthermore, these merozoites were able to reinvade red cells and thus completing the hepatic cycle (Mazier *et al.*, 1984, and1985).

Recently, attempts have been made to culture *P. falciparum* in the absence of host cells (Trager, 1990). In this culture system, the merozoites were incubated in a medium containing 50% erythrocyte extract, ATP and pyruvate. After 16 hrs of incubation, the merozoites matured into rings.

1.4 Therapy and prophylaxis

1.4.1 *Pneumocystis carinii*

The various anti-Pneumocystis drugs can be classified into three categories: anti-trypanosomals, anti-folates and anti-malarials. Pentamidine isethionate was successfully employed to treat PCP when it was discovered in 1958. As described before the mortality rate in malnourished immunocompromised children and adults was 90% and with discovery of pentamidine isothionate, the mortality rate decreased to 20% (Hughes,1977). Pentamidine isethionate remained the drug of choice for many years until 1974, when Hughes and coworkers determined the efficacy of trimethoprim-sulfamethoxazole (TMP-SMX), an antifolate, against PCP in pediatric patients (Hughes *et al.*, 1974). Subsequent investigations suggested that TMP-SMX was as effective as pentamidine isothionate in treatment of PCP (Hughes, 1977). However, for AIDS related PCP the incidence of toxicities due to either drug is approximated to be over 60% (Davey and Masur, 1990). Of the two regimens, TMP-SMX is now prefered for treatment and prophylaxis of AIDS related PCP because TMP-SMX is

better tolerated (Hughes *et al.*, 1978). Many anti-malarials have been investigated for anti-*Pneumocystis* activity and drugs such as fansidar (also an anti-folate), 566C80 and combination of primaquine and clindamycin are currently on clinical trials. Only few promising regimens will be discussed here since the focus of this thesis is to understand therapeutic options targeting at folate metabolism.

1.4.1.1 Antifolates

The anti-folates specifically inhibit the biosynthesis of folates in the microbe and thus they impair the production of nucleotides, the building blocks of DNA. Human and mammalian cells do not possess the machinery for folate synthesis and therefore, they acquire the preformed folate as a vitamin. Details about mechanism of action of anti-folates and their relationship to folate synthesis will be described in the later sections.

1.4.1.1.1 Trimethoprim-Sulfamethoxazole

TMP-SMX is most often used drug for initial treatment of PCP. In mild cases of PCP, without obvious gastrointestial pathology (nausea, diarrhea and malabsorption), the drug is given orally at a total daily dose of 100 mg of sulfamethoxazole and 20 mg of trimethoprim per kg of body weight. In more severe cases the drug is administered intravenously (Lee *et al.*, 1989). The usual length of therapy is usually 21 days. For some unknown reasons the AIDS patients have higher incidence of adverse reaction than other immunocompromised patients (Gordin *et al.*, 1984). The adverse reactions that occurs range from minor headaches, fever, anemia, nausea, vomiting to major rashes and neutropenia. A change in therapy might be required if the

patients cannot tolerate the clinical and biochemical abnormalities. Infact, up to 50% of the patients require a change in therapy (Wofsy, 1987).

1.4.1.1.2 Dapsone or Trimethoprim-Dapsone.

Dapsone, an antifolate, is one of the investigational anti-*Pneumocystis* agents. Dapsone is only available as an oral agent for treatment of mild cases of PCP. Dapsone alone cannot be given to patients with glucose-6-phosphate dehydrogenase defiency because of risk of hemolysis. However, a combination of dapsone (20-50 mg) and trimethoprim (200 mg) in comparative trials appear to be as effective as trimethoprim-sulfamethaxazole in treatment of mild to moderate cases of PCP. In addition, trimethoprim-dapsone toxicities can be better tolerated and has fewer serious side effects than conventional agents (Leoung *et al.*, 1986; Hughes, 1988). Trimethoprim-dapsone seem more promising in treatment of mild cases of PCP than conventional regimens.

1.4.1.1.3 Other anti-folates

Various anti-folates have been evaluated for their activity against *Pneumocystis carinii.* The following anti-folates have shown some antipneumocystis activity: piritrexin (Fallon *et al.*, 1990), fansidar (a combination of pyrimethamine and sulfadoxine) (Fischl and Dickinson, 1986) and trimetrexate (Allegra *et al.*, 1987). Details of their mechanism of action is described in the later sections.

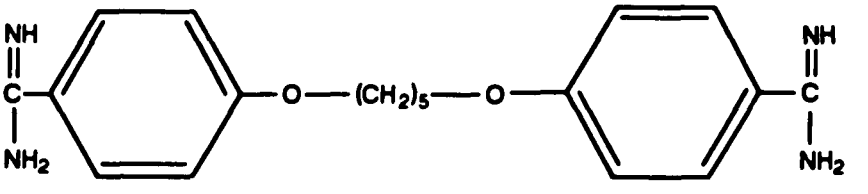
1.4.1.2 Pentamidine

Pentamidine, an aromatic diamidino compound (Fig. 3), is thought to prevent microbial DNA replication by binding to DNA and inhibiting its

topoisomerase (Tidwell *et. al.*, 1989) The exact mechanism of selective microbial toxicity is not yet well understood.

Pentamidine is the alternate drug most commonly used for treatment of mild to moderate cases of PCP in patients who fail to respond or have adverse reactions to TMP-SMX. But there are several drawbacks. First, Pentamidine (4 mg/kg/d) has to be administered intravenously once daily in order prevent sterile abscesses. Second, 45% of the patients on pentamidine require a change to other regimens due to adverse reactions. These reactions include anemia, creatinine elevation, hyponatremia and neotropenia. Third, pentamidine can also impair kidney function. Fourth, pentamidine can cause a profound drop in blood sugar (Sattler *et al.*, 1988).

Figure 3. Pentamidine Isethionate



1.4.1.2.1 Aerosolized Pentamidine

Because of high incidence and seriousness of toxicity associated with parenteral pentamidine, many researchers have focused on developing alternate methods for targeting the delivery of anti-pneumocystis drugs directly into the lungs. Aerosolized pentamidine is one of method of delivery that offers treatment at the site of infection and reduces systemic toxicity while maintaining therapeutic benefit.

In one study, 15 patients were given 600 mg of pentamidine through various nebulizers (aerosol devices). Thirteen of the 15 patients completed the 21 day therapy successfully. Two patients had to removed from medication due to other AIDS related problems. The only adverse effect was a cough and the patients responded to the cough medication (Montgomery *et al.*, 1987). However, in other studies there has been reports of more toxicity associated with earosolized pentamidine. The adverse reactions included cough, bad taste, salivation and bronchoconstriction (Smith *et al.*, 1988; O'Doherty *et al.*, 1988). The more serious adverse effects reported include bronchial bleeding (Miller *et al.*, 1989), dysglycaemia (Karboski and Godley,1988) and pancreatitis (Herer *et al.*, 1989).

1.4.1.3 Difloromethylornithine

Difloromethylornithine (DFMO), like pentamidine, is an anti-trypanosomal drug. DFMO inhibits first step in the synthesis of polyamines. Specifically, DFMO is an irreversible inhibitor of ornithine decarboxylase (Sjoerdsma, 1981).

In vitro studies (Cushion *et al.*, 1985) and experiments using rat models of PCP (Clackson *et al*, 1988) have determined that DFMO has an anti-pneumocystis activity. So far, DFMO has only been used as a salvage regimen for treatment of AIDS patients who do not respond to conventional treatments. Since DFMO is an alternate treatment, its efficacy has not clearly been defined and its treatment for AIDS related PCP remains unclear. Like other anti-pneumocystis drugs, DFMO related toxicities include gastrointestinal disturbances, high incidence of thombocytopenia (75%) and neutropenia in 65% of cases (Neibbart *et al.*, 1989).

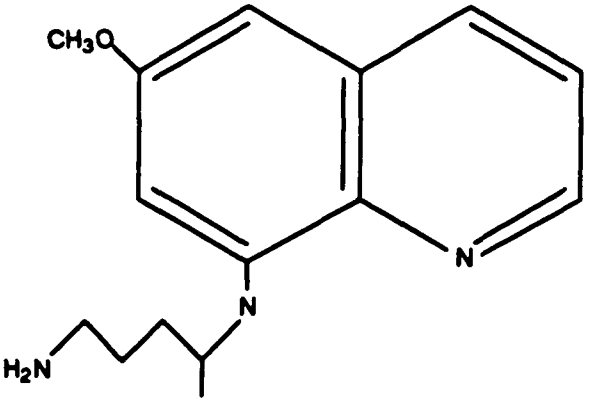
1.4.1.4 Anti-malarials with anti-pneumocystis activity

1.4.1.4.1 Primaquine and Clindamycin

Primaquine, or 8-aminoquinoline (Figure 4), has anti-malarial activity against all stages of the parasites life cycle (See section 1.4). Mirincamycin, a lincosomide, is an antibacterial agent which when used in combination with primaquine, enhances the antimalarial activity.

A combination of primaquine and Clindamycin, another lincosomide similar to mirincamycin, has now been reported to be efficacious against PCP in animal models (Queener *et al.*, 1988) and in humans (Ruf *et al.*, 1990). The mechanism of synergism by clindamycin on primaquine is unknown. However, primaquine has an effect on various steps in nucleic acid biosynthesis (Grewal, 1981); and clindamycin has a broad range of effects including penetration into the microbe and affecting its mitochondrial activity (Divo *et al.*,1985). It is possible that lower doses of both drugs is required because of their ability to concentrate in lung cells (Queener *et al.*, 1988).

Figure 4. Primaquine

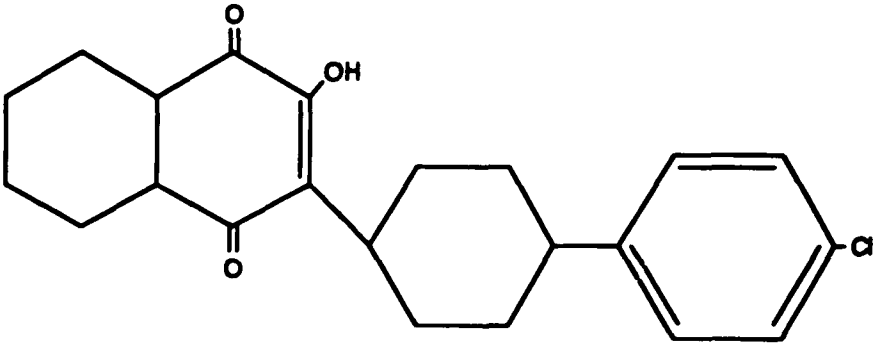


1.4.1.4.2 Compound Antavoquone (BW 566C80) or hydroxynaphthoquinone

Another anti-malarial, compound BW566C80 (Figure 5), was effective against *P. carinii* in animal models. In preliminary studies, the drug was associated with few adverse reaction and had long serum half-life (Hughes *et al.*, 1990). Compound 566C80, therefore, represents a new class of anti-*Pneumocystis* drug which act by inhibiting electon transport chain (Fry *et al.*, 1984).

Recently, the efficacy of compound 566C80 was determined in an open-label trial of 34 AIDS patients who had mild to moderately severe cases PCP. The results showed that 27 patients were treated successfully with the drug. However, four patients were removed from therapy due to adverse effects which included fever and rashes. Elevation of enzyme alanine aminotransferase was another side effect which was present in 9 of 34 patients (Falcon *et al.*, 1991). More clinical trials of 566C80 in comparison to SMX-TMP are currently in process and would give valuable information on the efficacy and toxicity of drug.

Figure 5. Formula for compound 566C80



1.4.2 Malaria Chemotherapy.

The numerous anti-malarial agents are classified according to their ability to act against different stages of parasite's life cycle (Table 2). The tissue schizontocides , such as proguanil, are commonly used as causal prophylactics and function to inhibit the growth of pre-erythrocytic life cycle of the parasite in the liver. Blood schizontocides act on erythrocytic stage of parasites life cycle, and these are generally used as curative agents. Inclusive in this group are drugs such as chloroquine and quinine. There are other agents which

Table 1. Classification of anti-malarial drugs

Class	Mode of action	Representative drugs
Tissue schizontocides	Inhibit growth in pre-erythrocytic stages.	Primaquine, proguanil, pyrimethamine
Hyponzoitocides	Kill the dormant liver stages (hypozoites)	Primaquine
Blood schizontocides	Act on erythrocytic and intra-erythrocytic stages	Chloroquine, quinine, quinidine, mefloquine artemisinin
Gametocytocides	Destroy sexual stage in the erythrocytes	Primaquine
Sporontocides	Inhibits development of oocysts in the mosquito	Pyrimethamine proguanil

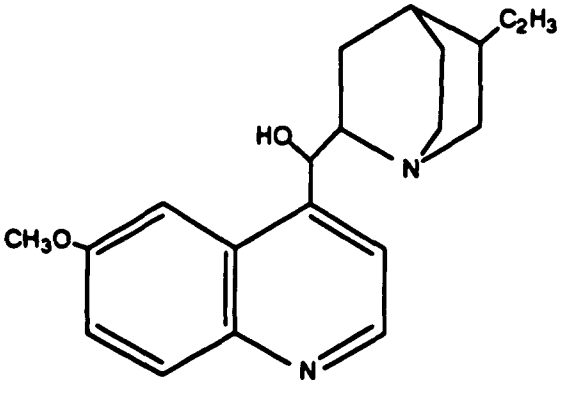
specifically act on parasite's developmental stage in the mosquitos and these are known as sporontocides. Since the main emphasis of this thesis is on *P. carinii*, the antimalarial will only be discussed briefly.

1.4.2.1 Qunolinemethanols

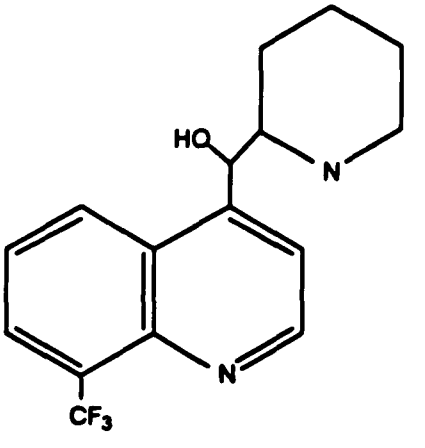
Quinine is one of the blood schizontocides which was first isolated from the bark of cinchota trees. The D-epimer of quinine, quinidine, and another quinoline methanol, mefloquine are also used for malarial therapy and prophylaxis. Although, the quinolinemethanols are effective anti-malarials, resistance towards mefloquine and quinine have been reported (Baudreau *et al.*, 1982; Marlin and Hall, 1990).

The mechanism of action of quinolines is unknown but may involve binding to hemin (Fitch, 1986; Warhust, 1981) or intercalation of quinine with DNA (Estensen *et al.* 1969; Davidson,1977).

Figure 6. Chemical structure of quinine and mefloquine



QUININE



MEFLOQUINE

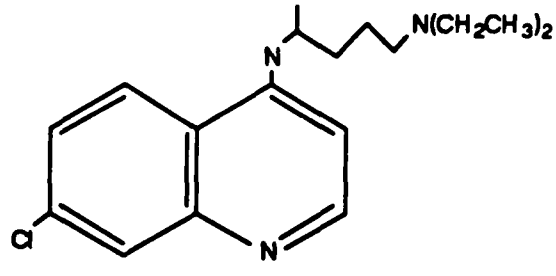
1.4.2.2 4-Aminoquinolines

Chloroquine , is another blood schizontocide, was the most effective antimalarial in the post war era. It is a chemical analog of quinine (Figure 7) and is administered orally as a prophylactic or therapeutic agent (Gustafsson *et al.*, 1983).

In malaria endemic areas, chloroquine resistant strains of *P. falciparum* are very common (Peters, 1987) . The chloroquine resistance strains were first reported in the 1950s and have continually increased thereafter. Furthermore, the *P. vivax* resistant strains have recently been reported (Whitby *et al.*, 1989).

Chloroquine has been investigated for almost five decades, and its mechanism of action is still controversial (Meshnick,1990). The first hypothesis

Figure 7. Chloroquine



proposed that the mechanism of selective toxicity was due to binding to DNA (Irvin *et al.*,1949). This hypothesis was accepted till early 1970s, when Homewood and his coworkers proposed that chloroquine impairs lysosomal function. It was suggested that chloroquine accumulates in the lysosomes and raises its pH (Homewood *et al.*, 1972). Few years later, in 1986, it was proposed that chloroquine binds to parasite's hemin and causes breakdown of the membranes (Fitch, 1986).

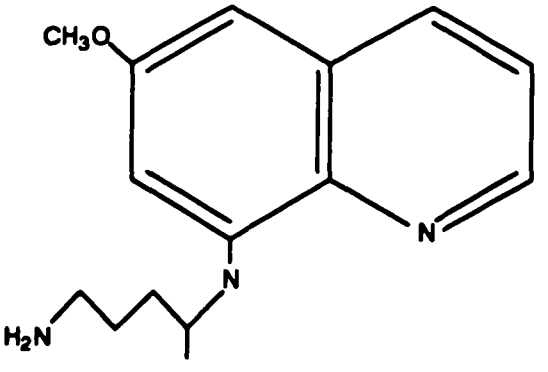
The hypothesis that chloroquine intercalates with DNA was recently reviewed by Kwakye-berko and Meshnick (1989 and 1990). These authors have suggested that the binding affinity is sequnce- and salt-dependent. However, the Kd was in millimolar range and could physiologically be too weak to be important. Despite the low affinity, it was suggested that the genome of the parasite contains 0.03-1% binding sites for the drug, which is enough to be toxic to the parasite.

1.4.2.3 Primaquine

Primarily used for preventing relapses, primaquine (Figure 8) is effective against tissue schizonts and gametocytes. Primaquine is metabolized by liver

into highly redox-active compounds. It was suggested that antimalarial activity of these compounds correlate to their ability to generate superoxide which subsequently cause oxidative damages (Bates *et al.* 1990).

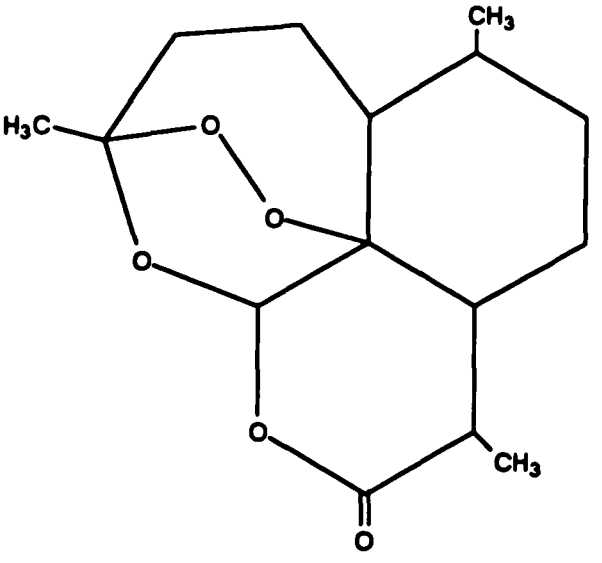
Figure 8. Primaquine



1.4.3.4 Artemisinin (qinghaosu)

Artemisinin (qinghaosu) is a promising new antimalarial agent derived from an ancient Chinese herbal remedy. Artemisinin (Fig. 9) and its derivatives, particularly artemether, have shown remarkable efficacy and are now undergoing clinical trials against drug-resistant malaria (Arnold *et al.*, 1990;China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalaria,1982; Luo and Shen,1987; Myint *et al.*, 1989; Wang and Xu, 1985).

Figure 9. Artemisinin



The antimalarial activity of artemisinin directly correlates with the breakdown of endoperoxide bridge to generate free radicals as evidenced by several studies. First, no antimalarial activity was observed when endoperoxide bridge was removed (Brossi *et al*, 1988). Second, artemisinin was determined to cause lipid peroxidation (Meshnick *et al.*, 1991) Lastly, ascorbic acid (a free radical scavenger) was shown to antagonize the effect of artemisinin (Meshnick *et al.*, 1989).Furthermore, the selective toxicity of

artemisinin is associated with binding to hemin, which is present in large amounts in the parasite (Meshnick *et al*, 1991).

1.4.3.5 Antifolates

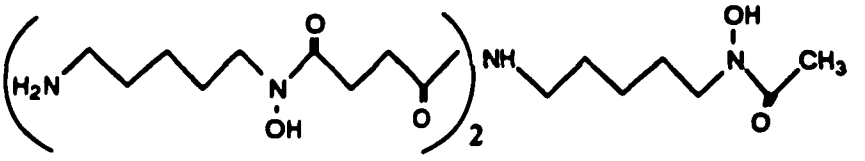
Antifolates are important in treating malaria were chloroquine resistant strains are prominent. The most commonly used anti-folates are: sulfa doxine in combination with pyrimethamine (Fansidar) and dapsone in combination with pyrimethamine (maloprim). Both of these drugs are only effective against *P. falciparum* malaria. Details of mechanism of action of sulfa drugs will be discussed in later chapters.

1.4.3.6 Chelators

According to Meshnick and Marr (1992), antimalarial chelators can be divided into two categories. The exogenous metal ion antagonists e.g. deferoxamine and exogenous metal ion synergist e.g. diethyldithiocabamate.

Deferoxamine (DFO) (Fig. 10) is an iron chelator, extensively used in treatment and prevention of iron-overload in chronically transfused patients (Propper *et al.*, 1976). The studies with DFO have indicated that the drug has an in vitro activity against *P. falciparum* (Raventos-suarez *et al.*, 1982). The mechanism of action of deferoxamine in *P. falciparum* does not involve exogenous iron chelation (Hershko and Peto, 1988), but depends on its ability to penetrate the infected red cell membrane (Fritsch and Jung, 1986). In addition, the derivatives of deferoxamine which cannot enter infected red cells do not have antimalarial activities (Scott *et al.*, 1990).

Figure 10. Deferoxamine



Deferoxamine is toxic when used to treat iron-overload patients. Some of the toxicities include ocular abnormality, hearing loss and infection complications (Reviewed by Weinberg, 1990). Therefore, it is conceivable that the adverse reactions observed will be predominant in clinical trials of malaria.

Diethyldithiocarbamate is a potent copper chelator which is used to treat alcoholism. The anti-malarial activity of diethylthiocarbamate is potentiated by intraerythrocytic and extracellular copper (Meshnick *et al.*, 1990)

1.5 Folate biosynthesis

1.5.1 *Para* Aminobenzoic Acid and folate biosynthesis in *Escherichia coli.*

The biosynthesis of para aminobenzoic acid (PABA) in *E. coli* is similar to synthesis of aromatic amino acids, *i.e.* tyrosine, tryptophan and phenyalanine, upto chorismic acid (Figure 11). The initial step of synthesis is the condensation of phosphoenol pyruvate (a glycolytic intermediate) and erythrose-4-phosphate (a pentose phosphate pathway intermediate) to form 3-deoxyarabinoheptulosonate 7-phosphate (DAHP). Dephosphorylation DAHP by 3-dehdroquinate synthase causes the cyclization of the ring to form 3-hydroquinic acid. Dehydration then results in formation of 3-

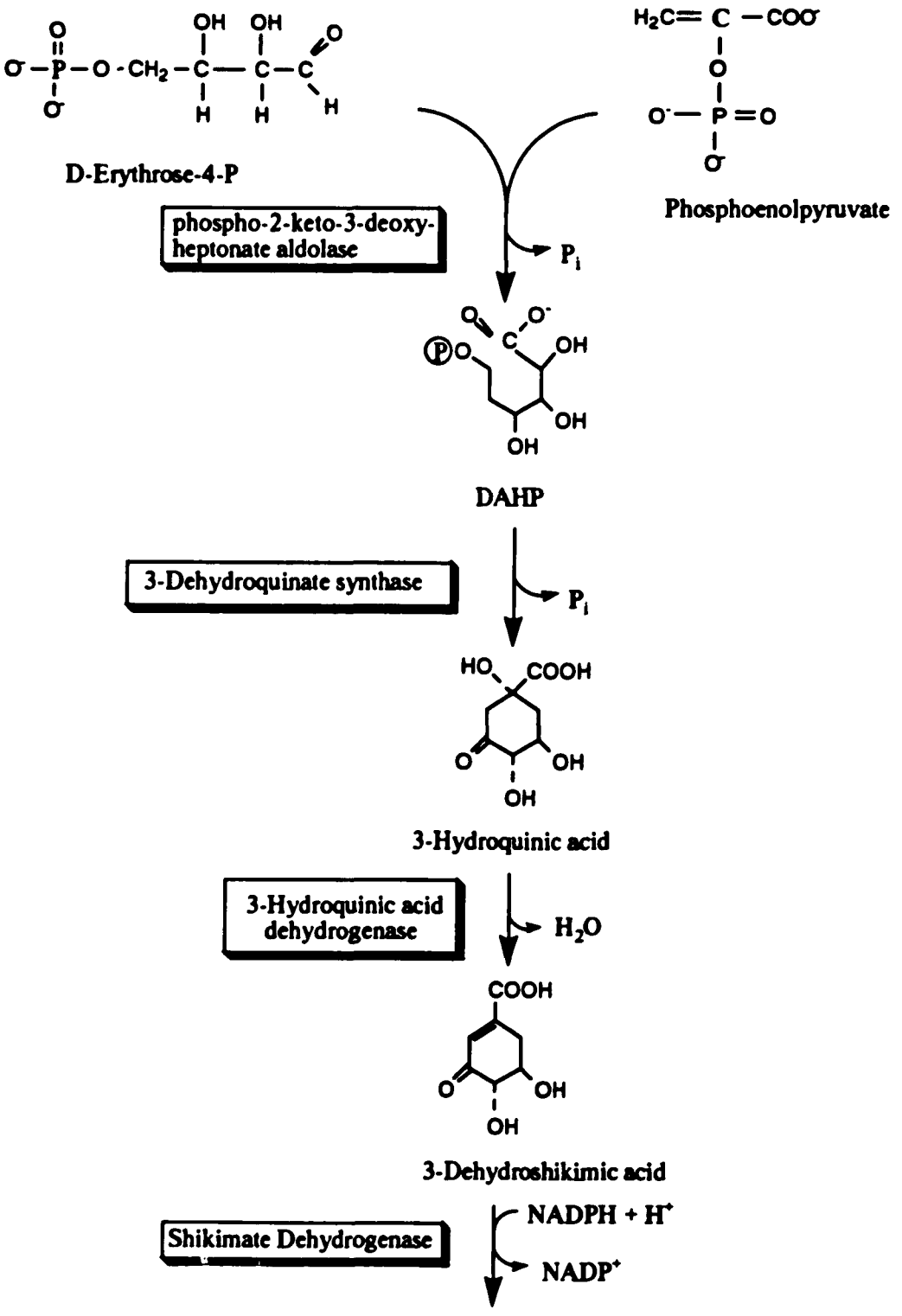
dehdroshikimic acid which is in turn reduced by NADPH to shikimic acid. Phosphorylation of shikimic acid yields shikimate 3-phosphate which condenses with another molecule of phosphoenol pyruvate to form an intermediate 5-enolpyruvoylshikimate 3-phosphate. Dephosphorylated of 5-enolpyruvoylshikimate 3-phosphate by chorismate synthase yields chorismate. The terminal step involves addition of amino group to chorismate from side chain of glutamine to form PABA.

The *de novo* folate synthesis in *E. coli* begins by phosphorylation of 6-hydroxymethyl pterin by enzyme 6-hydroxymethyl pterin pyrophosphokinase to form 6-hydroxymethyl pterin pyrophosphate, which then condenses with PABA to form pteroic acid. The later reaction is catalyzed by enzyme dihydropteroate synthase. The pteroic acid then reacts with glutamate and ATP, in a reaction catalyzed by dihydrofolate synthase, to form dihydrofolate (Richey and Brown, 1969). In the final step of folate synthesis, dihydrofolate is reduced by dihydrofolate reductase to form tetrahydrofolate (Figure 12). Tetrahydrofolate is an important molecule in one carbon metabolism (see section 1.5.2.1)

1.5.2 Folate metabolism in malarial parasites.

Folic acid was first isolated by Mitchell and his coworkers (Mitchell *et al.*, 1941) when they identified the haemopoietically active compound from spinach and other foliage. Folic acid is an essential vitamin which is required by man and other mammals but is synthesized *de novo* by malarial parasite and other microorganisms. Therefore, inhibition of folate biosynthesis is an important target for microbial chemotherapeutic agents.

Figure 10. A scheme for PABA synthesis in *E. coli*



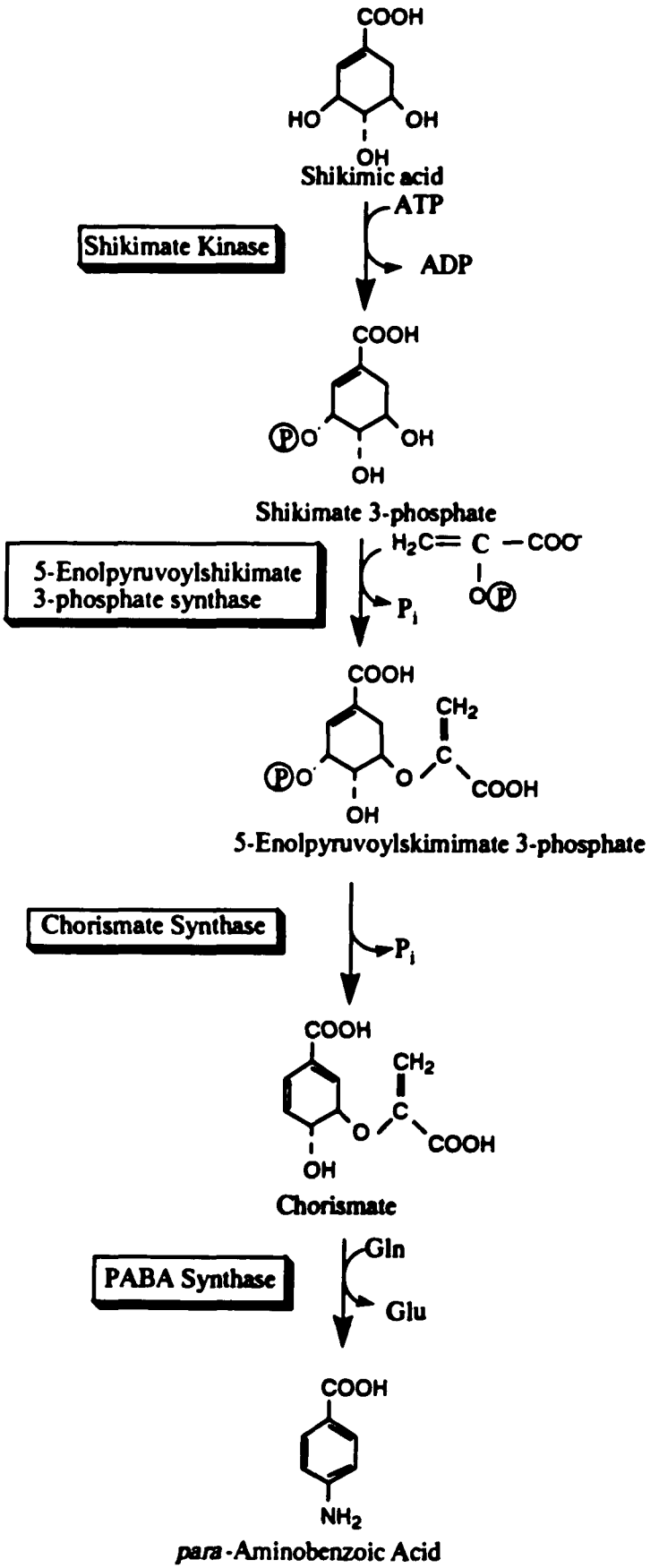
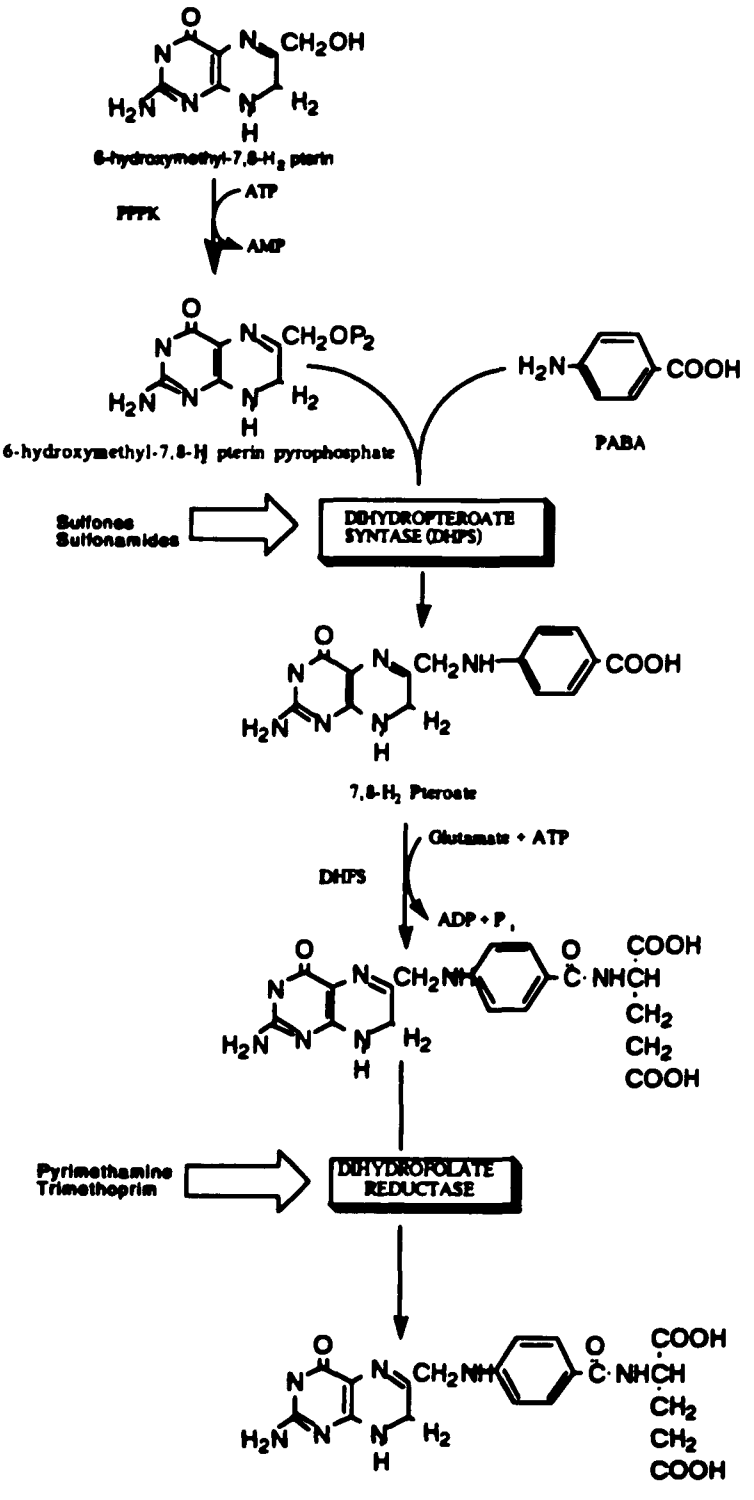

COOH
HO
OH
OH
Shikimic acid
Shikimate Kinase
ATP
ADP
COOH
OH
OH
Shikimate 3-phosphate
5-Enolpyruvoylshikimate
3-phosphate synthase
$H_2C=C-COO^-$
P_i
COOH
CH_2
C
O
COOH
OH
5-Enolpyruvoylskimimate 3-phosphate
Chorismate Synthase
P_i
COOH
CH_2
C
O
COOH
OH
Chorismate
PABA Synthase
Gln
Glu
COOH
NH_2
para-Aminobenzoic Acid

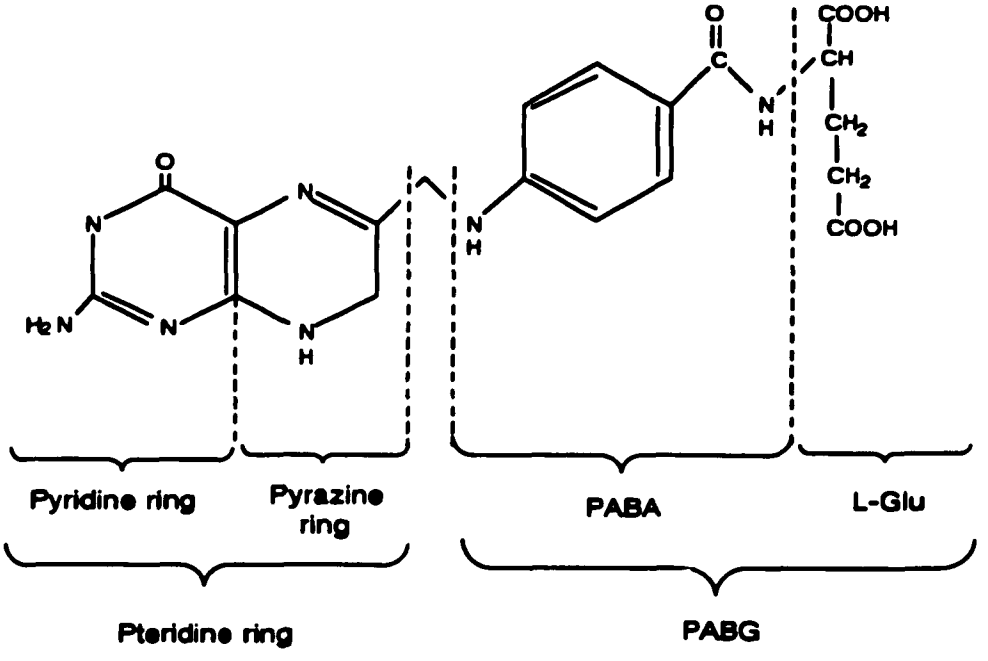
Figure 8. *De novo* folate biosynthesis in malarial parasites



1.5.2.1 Tetrahydrofolate metabolism.

Folate coenzymes serve as a one carbon donor or acceptor in variety of reactions. These reactions include one carbon transfer reaction of pyrimidines and purines, interconversion of amino acids, and initiation of protein synthesis (Brown and Williamson, 1982). The folic acid is comprised of three essential parts, a pteridine ring, PABA and glutamic acid (Fig. 13). It can undergo a reaction at any ring position, most notably addition of hydrogen atoms to 7,8-position leading to formation of dihydrofolate. Further reduction of dihydrofolate is driven by NADPH-dependent enzyme dihydrofolate reductase and leads to formation of tetrahydrofolate, a coenzyme which plays a crucial role in one carbon metabolism.

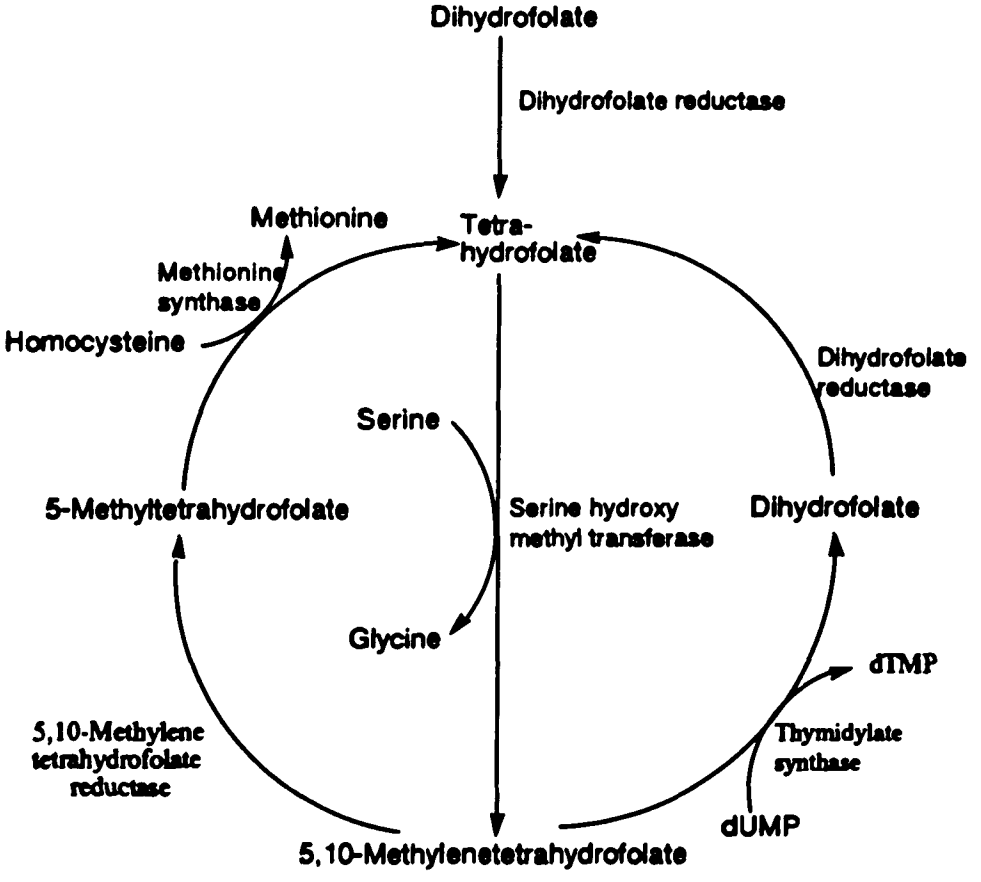
Figure 13. Chemical structure of dihydrofolic acid



The tetrahydro-derivatives exists as three different oxidation states depending on which one carbon unit is bonded to its N-5 or N-10 nitrogen

atom. The most oxidized form carry a formyl or formimino or methenyl group, while an intermediate group carries a methylene group. The most reduced form of tetrahydo-derivatives carries a methyl group.

Figure 9. Proposed pathway for one carbon interconversion in *Plasmodium falciparum*



In *de novo* synthesis of pyrimidines, for example, the one carbon unit of 5,10-methylenetetrahydrofolate is used in the synthesis of thymidylate from deoxyuridate in a reaction catalyzed by thymidylate synthase. The dihydrofolate generated from this reaction is then reduced to tetrahydrofolate, which in combination with serine is subsequently converted

by an enzyme, serine hydroxymethyl transferase, to 5,10-methylenetetrahydrofolate and glycine. In another reaction, 5,10-methylenetetrahydrofolate is reduced by a reductase to form 5-methyltetrahydrofolate, which reacts with homocysteine to form methionine and tetrahydrofolate (Figure 14).

1.5.2.2 Folate biosynthesis in malarial parasites

The existence of *de novo* folate synthesis in malarial parasites is partly presumed by presence of enzymes serine hydroxymethyltransferase and thymidylate synthase in *Plasmodium lophurae*. The parasite enzymes were distinct from that of the host in molecular weight, thermostability and pH optimum (Walsh and Sherman, 1968; Platzer,1977). More convinsing evidence comes from molecular biologist who have recently cloned and sequenced dihydrofolate reductase and thymidylate synthase bifunctional gene in *Plasmodium falciparum* (Bzik *et al.*, 1987). Recently, the enzyme serine hydroxymethyl transferase in *P. chabaudi* was purified and characterized (Ruenswongsa *et al.*, 1989) .

The evidence that malaria-infected cells utilize 5-methyltetrahydrofolate to synthesize methionine , also suggests the presence of methionine synthase (Smith *et al.*, 1976).

The *de novo* folate biosynthesis in malarial parasite was first demonstrated in *P. berghei* (Ferone, 1977). The activities of first two enzymes of folate biosynthesis i.e. the pyrophosphokinase and the dihydropteroate synthase were shown to coeluted when crude extract was run on a Sephadex G-200 column. However, the synthase and pyrophosphokinase activity could not be further separated . Recently, in

experiments using paper chromatography, Zhang and Meshnick (1990) were able to detect 10μUnits per mg of protein of dihydropteroate activity in P. *falciparum* extracts. The next enzyme in folate synthesis, the dihydrofolate synthetase, has yet to be identified in *Plasmodium* species. However, in experiments using metabolic labelling a complete pathway of folate biosynthesis in *P. falciparum* was discovered. Krungkrai and his coworkers were able to detect the synthesis of 5-methyl tetrahydrofolate from GTP, PABA and L-glutamate (Krungkrai *et al.*,1989), which possibly suggests that dihydrofolate synthase activity is present in *P. falciparum*.

What are the sources of 6-hydroxymethylpterin and PABA? In malarial parasites the pteridine ring is synthesized from guanosine 5'-triphosphate (GTP) by an enzyme GTP cyclohydrolase. This enzyme has been characterized and purified in *P. berghei* and *P. falciparum* and the inhibitors of GTP cyclohydrolase has been shown to inhibit the formation of 6-hydroxymethyl pterin (Krungkrai *et al.*, 1985). Furthermore, the synthesis of 6-hydroxymethyl pterin from labeled GTP has recently been reported (Krungkrai *et al.*, 1989).

The exact source PABA has yet to be determined, but there are several lines of evidence that show the source of PABA is at-least partly extracellular. The first evidence that exogenous PABA plays a role in growth of malarial parasites came from observation that sulfanilamides had anti-malarial activity in humans and this activity could be reversed by addition of PABA (reviewed by Sherman,1979). Further confirmation on importance of extracellular PABA was obtained from nutritional studies: In one study, it was shown that the parasitemias in *P. berghei* infected rats and *P. cynomolgi* infected monkeys was supressed as a result of milk diet containing low

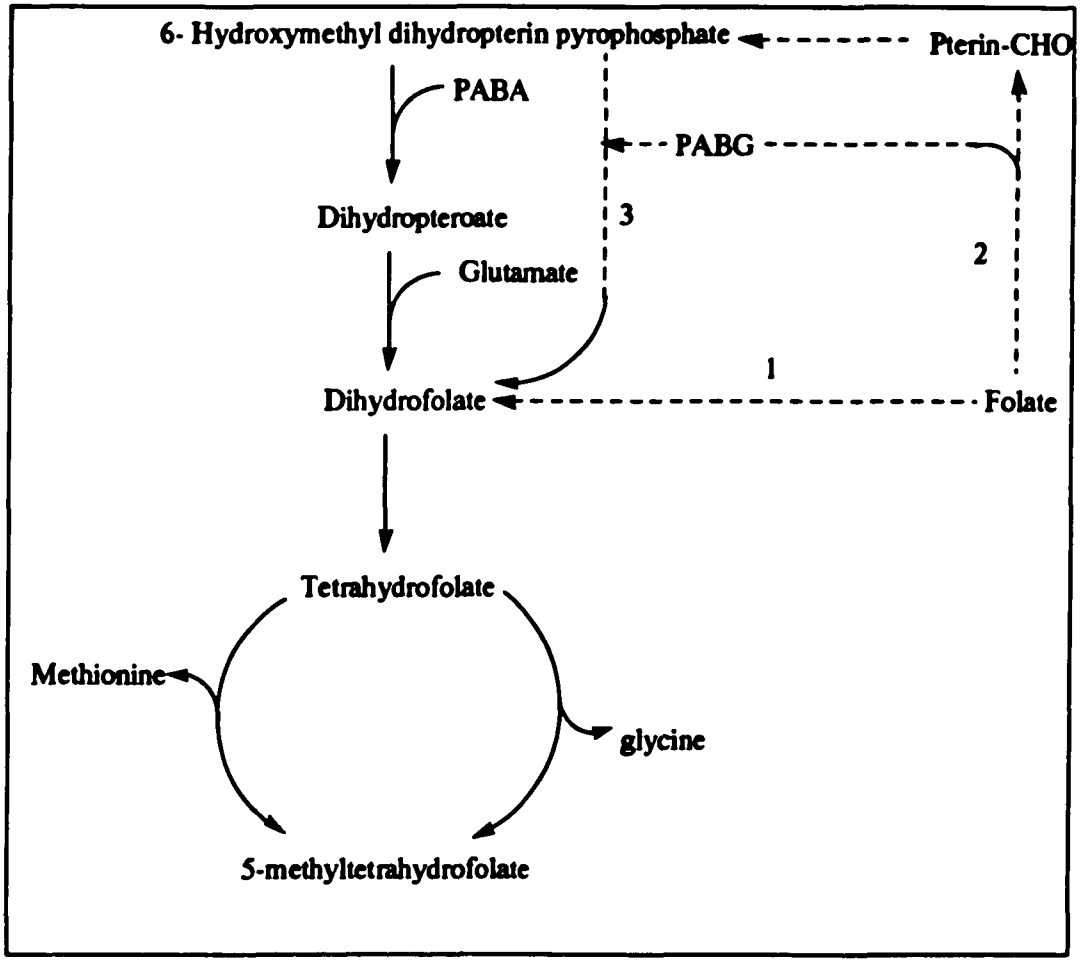
levels of PABA (Hawkins, 1954). In another study, it was suggested that the in vitro growth of *P. knowlesi* was enhanced by adding PABA to the culture medium (Anfinsen *et al.*, 1946).

It is also likely that the malarial parasites can synthesize PABA *de novo*. The experiments with sulfa resistance strains have determined that malarial parasites contain four of eight enzymes necessary for PABA synthesis (Dieckmann and Jung, 1986).

1.5.2.3 Folate Salvage Pathway

Folate salvage pathway in Plasmodium species was first observed by Milhous and collaborators (1985) when they suggested that folate antagonizes the inhibition of sulfa drugs more markedly than either PABA or PABG (Milhous, 1985). Further confirmation of this observation was obtained from metabolic labeling experiments which showed that radiolabelled folate was incorporated into 5-methyltetrahydrofolate and this incorporation was sensitive to sulfadoxine and pyrimethamine (Krungrai *et al.*, 1989). Therefore malarial parasites can utilize exogenous folate to convert into tetrahydrofolate, presumably by cleavage to form p-aminobenzoylglutamate (PABG) and pterin-6-aldehyde (pterin-CHO). PABG is an alternate substrate for DHPS and can then be converted to dihydrofolate in normal fashion. (Fig. 15).

Figure 15. Proposed *de novo* folate salvage pathway. (1) folate reductase (not present in malarial parasites), (2) pteridine-6-methylhydrolase, (3) DHPS (PABG = *p*-aminobenzoyl glutamate)



An alternate salvage folate pathway involves utilization of exogenous 5-methyltetrahydrofolate. In experiments using radiolabeled 5-methyltetrahydrofolate, it was suggested that the parasites utilize this metabolite to form methionine (Krungkrai *et al.*, 1990)

1.5.3 Folate biosynthesis in *P.carinii*

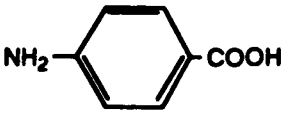
The *de novo* biosynthesis of folates in *P. carinii* was first demonstrated by Kovacs *et al.* (1989). Using an HPLC assay, these investigators showed that trimethoprim-sulfamethoxazole (Bactrim) and pentamidine inhibit the conversion of PABA to folates by *P. carinii* cultured in the presence of WI38 cells over 24 hours. Two years later, Comley *et al.* (1991) modified Kovacs *et al.* procedures and were able to measured PABA uptake and folate biosynthesis in freshly isolated *P. carinii* over 18 hours. Since, in these period of time, 90% of the incorporated PABA was converted into folates, PABA uptake was monitor to screen drug effects. However, since the organisms cease to synthesize folic acid after 24 hours, and since the organisms may not be actively growing, this method is only effective for drugs which are short acting, such as pentamidine, or which drugs which directly affect folate biosynthesis, such as sulfa drugs.

The enzymes involved in folate biosynthesis have yet to be identified and characterized. Recently, however, the multifuctional folic acid fas gene of *P. carinii* was cloned (Volpe *et al.*, 1992). It was demonstrated that fas gene of *P. carinii* has sequence homology to 6-hydroxymethyl pterin pyrophosphate, and dihydropteroate synthase.

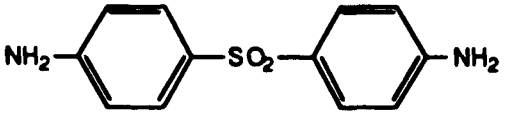
1.6 Inhibition of folate biosynthesis by sulfa drugs.

The sulfa drugs are categorized into two groups; the sulfonamides such as sulfadoxine and the sulfones such as dapsone (Figure 16).

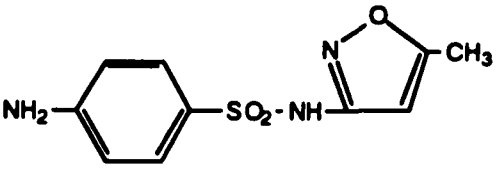
Figure 12. Structures of some sulfa drugs



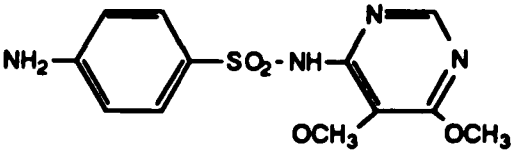
PABA



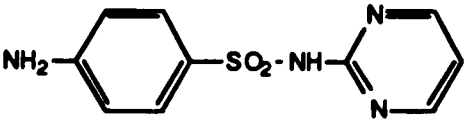
DAPSONE



SULFAMETHOXAZOLE



SULFADOXINE

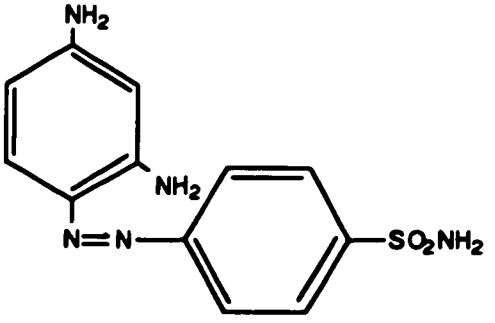


SULFADIAZINE

The development sulfonamides dates back to late 1910s when Heidelberger and Jacobs showed bacteriostatic properties of some azo dyes in vitro. However, these dyes lacked in vivo activity and were not useful as a chemotherapeutic agent. Few years later, Mietzch and Klarer (1935) synthesized new group of dyes which contained sulfonamide radical. One of these dyes, prontosil (Figure 17), had anti-streptococal activity in mice (reviewed by Anand,1980). In vivo studies of protonsil, lead to suggestion that the drug was metabolized by animals into sulfonamides and it was this metabolite that was responsible for anti-bacterial activity (Trefouel *et al.*, 1935).

The development of sulfonamides as a chemotherapeutic agent against bacterial infection, prompted many researchers to study efficacy of sulfonamides to treat other microbial diseases. One of the breakthrough came in 1938 when it was suggested that sulfonilamide and sulfachrysoidine were effective in treatment of human malarial infections (Hall, 1938), but nothing was made of it until later years.

Figure 17. Prontosil



Another group of sulfa drugs, the sulfones, were discovered in early 1940s. The sulfones such as dapsone are derivates of 4,4'-diaaminodiphenylsulfone (Figure 16). The importance of dapsone as a chemotherapeutic agent was immediately recognized when it was found to be efficacious in treatment of tuberculosis (Rist, 1940). In addition, dapsone was also effective in treatment of leprosy (Browne, 1969). The studies with leprosy subsequently lead to discovery of dapsone as an anti-malarial agent. It was realized that patients with leprosy had lower incidences of malaria and this was confirmed by microscopic analysis of their blood which showed the absence of malarial parasite in patients treated with dapsone compared to untreated (Archibald and Ross, 1960). Dapsone in combination with trimethoprim are still used for malaria prophylaxasis especially in chloroquine resistant areas. Malarial parasites show great variability in sensitivity towards sulfa drugs. For example, *P. falciparum* is very sensitive to sulfone and sulfonamides, while *P. vivax* is not.

1.6.1 Modes of Action

The observation that the anti-microbial activity of sulfanilamides can be reversed by PABA (Woods, 1940) was the first demonstration that PABA antagonism was the mode of action. Soon after that, Snell and Mitchell (1943) suggested that the mechanism of sulfonamide action was through competitive inhibition of PABA. In addition, noncompetitive inhibition by compounds not related to PABA e.g. serine was also observed.

The understanding of mechanism of sulfa drug inhibition at enzyme level was initiated by Shiota (1956) when he demonstrated the enzymatic synthesis of dihydropteroate and dihydrofolate in vitro. Additional in vitro

experiments using bacterial extracts confirmed that dihydropteroate was synthesized from PABA and hydroxymethyldihydropteridine. It was also suggested that formation of dihydroptreroate was inhibited by sulfonamides which were competing with PABA for active site of the enzyme (Brown, 1961).

The mechanism of inhibition by sulfones is similar to that of sulfonamides i.e. competitive inhibition of enzyme dihydropteroate synthase. Recently, detailed in vitro studies of DHPS from *P. falciparum* have suggested that sulfa drugs are actively concentrated by malarial parasites. This conclusion was based on observation that the Ki's for inhibition by sulfa drugs was much higher than required to inhibit parasite growth (Zhang and Meshnick, 1991)

1.6.2 Other targets of sulfa drugs.

Besides inhibition of DHPS, sulfa drugs have been implicated in inhibition of dihydrofolate reductase; a NADPH-dependent enzyme which catalyzes the formation of tetrahydrofolate from dihydrofolate. Experiments using partially purified dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistance *P. chabaudi*, have suggested that sulfadoxine inhibited former enzyme competitively and later enzyme noncompetitively. Furthermore, when pyrimethamine was used in combination with sulfadoxine, a potentiating effect on enzyme activity was observed. This result suggests that sulfa drugs have a serial effect on different enzymes (Sirawaraporn and Yuhavong, 1986).

In malarial parasites, targets other than folate biosynthesis pathway have been suggested for dapsone. Specifically, it was observed that

dapsone inhibited glucose utilization by intraerythrocytic parasites and this inhibition was reversed by increasing glucose concentration in the medium (Cenedella and Jarell, 1970).

1.7 Inhibition of folate biosynthesis: Dihydrofolate reductase inhibitors.

Unlike DHPS, dihydrofolate reductase is present in both the host and in the parasite. Fortunately, dihydrofolate reductase in malarial parasite differs greatly from mammalian enzyme in several ways. First, the two enzymes are structurally different and therefore, the affinity of inhibitors for the enzymes differ greatly. For example, pyrimethamine binds up to 1000 times more tightly to malarial dihydrofolate reductase than it does mammalian enzyme (Ferone, 1969). Second, the malarial enzyme cannot utilize folate, only dihydrofolate. Third, as mentioned before the malarial dihydrofolate reductase and thymidylate synthase exist as a single bifunctional protein while the mammalian enzymes are functionally separate (Bzik *et al.*, 1984).

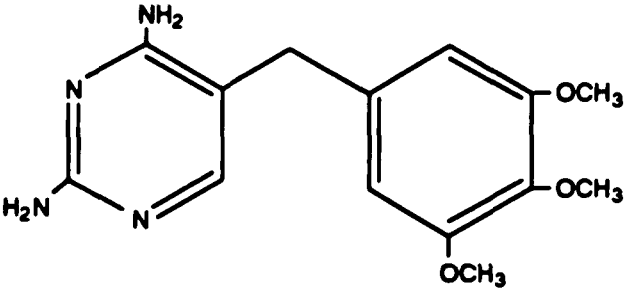
1.7.1 Mode of Action

Trimethoprim and primethamine (Figure 18) inhibits folate biosynthesis at much later stage than sulfa drugs. Specifically, they inhibits the enzyme dihydrofolate reductase which catalyzes the formation of dihydrofolate from dihydropteroate. Trimethoprim and other DHFR inhibitors are structural analogs of dihydrofolate and they inhibit DHFR competitively. As mentioned above the concentration of pyrimethamine required for inhibition of mammalian DHFR is much higher than that required to inhibit malarial parasite (Ferone *et al.* 1969).

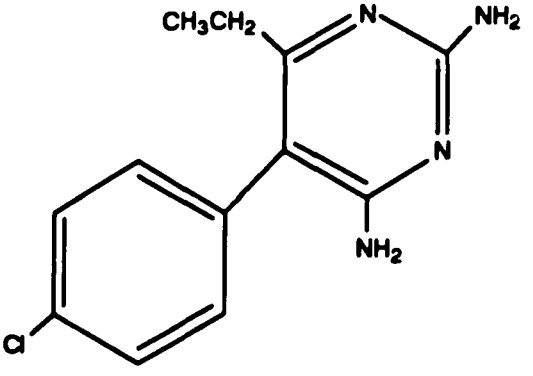
1.7.1.1 Synergism between sulfa drugs and DHFR inhibitors.

The antimalarial action of sulfa drugs and DHFR inhibitors was enhanced when a combination of both drugs was used (Hurley, 1959; Rollo, 1955). Therefore, the dosage of individual drugs for anti-malarial action was considerably reduced.

Figure 18. Inhibitors of dihydrofolate reductase



Trimethoprim



Pyrimethamine

However, the mechanism of potentiation is still a controversy. Originally, it was proposed that DHFR in *E. coli* was sequentially inhibited by sulfa drugs and then by DHFR inhibitors and that contributed to synergism observed (Black,1963). The fact that sulfonamides were also capable of inhibiting DHFR led to proposal of simultaneous inhibition hypothesis (Poe, M., 1976). Further support of simultaneous hypothesis was obtained from Sirawaraporn and Yuthavong (1986), when they observed potentiating effect of pyrimethamine and sulfadoxine for partially purified DHFR from *P. Chabaudi.*

1.8 Statement of the problems

1.8.1 *P. carinii*

1. Are other sulfa drugs less toxic more effective than sulfametaxazole ?

2. Can *Pneumocystis carinii* be cultured at least for a short time ?

3. Can this culture be used to screen new anti-*pneumosystis* drugs?

4. Can *P. carinii* in culture be quantitated accurately?

1.8.2 Malarial parasites.

1. Does malarial parasite utilize exogenous PABA to synthesize folate?

2. Can p-amino salicylate (an antifolate) inhibit folate biosynthesis and is it toxic to malarial parasites?

3. If PAS inhibits folate biosynthesis and does not kill, does it indicate a possible alternate action of sulfa drugs?

4. Is one of the possibilty that sulfa drugs inhibit PABA biosynthesis?

2. Methods

2.1 General Procedures

2.1.1 Chemicals and Drugs

The following chemicals and drugs were obtained from Sigma Chemical Company: cortisone acetate , Norit-A, Dowex-50, PBS buffer, 2-amino-4-hydroxy-6-hydroxymethylpterin, pyrophosphoric acid, dapsone, sulfadiazine, sulfaquinoxaline, and sulfamethaxazole. Sulfadoxine was received as a gift from Hoffman-La Roche (Nutley, NJ).

[^{3}H] PABA and [^{3}H] folate were from Moravek Biochemical Inc. (Brea, CA). 10% Deferoxamine-hydroxyethylstarchin 0.9% saline (DFO-HES), a high molecular weight complex of deferoxamine (Hallaway *et al.*, 1989), was a gift from Biomedical Frontiers, Minneapolis MN. This solution contained the equivalent of 17 mM deferoxamine. 10% Hydroxyethylstarch (HES) in 0.9% saline, also from Biomedical Frontiers, was used as a control. DFO-HES was iron saturated by mixing ferric chloride and DFO-HES for 30 min at room temperature. Sulfadoxine was a gift from Hoffman-LaRoche (Nutley, NJ). Biodegradable Counting Scintillant (BCS) was obtained from Amersham Inc. (Arlington Heights, IL). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO)

2.1.2 Infected Animals

Male Sprague-Dawley rats (150-200 g) were fed *ad libitum* and administered tetracycline in their drinking water (1 g/L). Subcutaneous injections of cortisone acetate (25 mg) were made twice a week. After 4 to 6

weeks of injections, those rats which showed signs of rapid breathing, blood in their noses, weight loss, and hair loss were used in the experiments.

2.1.3 Staining of *P. carinii*

The *P. carinii* were obtained from rats as described in section 2.2. Trophozoites were identified by Giemsa stain. Two µl of the lung supernatant were placed on a 1 cm^2 square etched on a glass slide with a diamond stylus. The slides were fixed for 2 min in methanol and stained for 40 min in Giemsa stain solution. The Giemsa stain was prepared by mixing 2 ml of Harleco Azure B type Giemsa stain (Harleco, Gibbstown, N.J), 2 ml of phosphate buffer (monosodium phosphate, 5.38 g/l; disodium phosphate 15.76 g/l; 2 drops of Triton X-100/l; pH adjusted to 7.2) and 46 ml of deionized water. After staining for 40 min, the slides were washed in a buffer containing 2 ml of phosphate buffer and 48 ml of deionized water. Trophozoites were counted double blind by light microscopy.

The cysts were counted by using modified toluidine blue O stain (Gossey *et al.*, 1985). Briefly, 10 µl of the lung supernatant were placed on a 1 cm^2 square etched on a glass slide with a diamond stylus and the slides were fixed in sulfation reagent (a mixture of 40 ml of glacial acetic acid and 15 ml of sulfuric acid) for 10 min. Sulfated slides were removed and rinsed in running tap water for 2 min. The slides were air dried and stained in toluidine blue O solution for 5 min. Toluidine blue O solution was prepared by dissolving 0.3 g of toluidine blue O in 60 ml of deionized water and 2.0 ml of concentrated hydrochloric acid, followed by 140 ml of absolute ethyl alcohol. Once the slides are stained, they are dipped five times each in 95 % ethyl alcohol, absolute alcohol, and xylenes.

2.2 Inhibition of *Pneumocystis carinii* Dihydropteroate synthetase by Sulfa drugs

Isolation of organism

P. carinii were isolated by a modification of the method of Gradus and Ivey (1986). Each rat was sacrificed by cervical dislocation. Their lungs were then quickly removed and kept at 4°C. Touch preps were made from the lungs and stained with Toluidine blue (Gossey *et al.*, 1985) and Gram stain. The experiment was continued if cysts were present, but no fungi or bacteria was evident. Subsequent inoculation of lung homogenate on bactarial plate confirmed the absence of contaminants. The lungs were homogenized by repeated passage through a 60-gauge wire screen. The resulting suspension was centrifuged at 2,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in PBS buffer. The suspension was carefully layered onto a preformed (40 min) 20% percoll gradient. After 40 min of centrifugation at 20,000 x g, the fluffy layer just above the lung tissue layer was removed. This layer was diluted with PBS and spun at 2,000 x g for 10 min. The supernatant was discarded and impression slides were made from the pellet. Usually, a pure preparation can be obtained with relatively few mammalian cells (<1%). When excessive mammalian cells or bacteria were observed, the organisms were discarded.

Synthesis of phosphorylated pterins.

Phosphorylated pterins were prepared by a modification of methods described by Shiota *et. al.* (1964), in which 50 mg of 2-amino-4-hydroxy-6-hydroxymethylpteridine and 5 g of pyrophosphoric acid were added to a 25-ml glass-stoppered Erlenmeyer flask. The mixture was protected from light

and stirred in a water bath at 60°C for 12 hours. The contents were transferred to a larger flask after addition of 30 ml of cold distilled water. A volume of 5 ml of 15% Norit A (acid washed) was added to the mixture and stirred for a few minutes and then filtered through a 0.45 μ Millipore filter (Millipore corporation). The filter was washed with 250 ml of distilled water, and thereafter adsorbed pteridines were eluted by adding 50 ml of an eluting reagent (equal volumes of 3N NH_4OH and absolute ethanol). The mixture was stirred for a few additional minutes. This elution step was repeated four times and the filtrates were pooled and evaporated to approximately 5 ml. The concentrated filtrate was applied to a Dowex-50 column, and the column was eluted with water until the effluent indicated no detectable fluorescence (monitored at 350 nm).

The eluate from the Dowex-50 column then was applied to a DEAE-cellulose column on-line in a Pharmacia FPLC unit. A gradient of 1L of 50mM LiCl in 50mM Tris-HCl (pH 7.2) and 150mM LiCl in 50mM Tris-HCl (pH 7.2) was used to develop the column. The column was allowed to flow at a rate of 8 ml/min. The elution of phosphorylated pteridines was monitored at 254 nm. Fractions from each peak were pooled and assayed for pyrophosphate (Grindey and Nichol, 1970). Fractions containing pyrophosphate were lyophilized (Labconco) and washed several times with absolute ethanol. The resulting pellet was dried using a Speed-Vacuum concentrator (Savant). The concentration of MDHP-PP was determined at 330 nm through use of a molar extinction coefficient equal to 6200 $M^{-1}cm^{-1}$ (Iwai and Okinaka, 1980).

Phosphate assay

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

Assays of pterin mono- pyro- and tri- phosphates

In order to distinguish 6-hydoxymethyl dihydropterin mono- pyro- and tri- phosphates, samples of pterin phosphates were first digested by alkaline phosphotase, and then assayed for both pterin and inorganic monophosphate concentrations. For the enzymetic digestion, a solution of 1 mg per ml alkaline phosphotase (5-10 units, Sigma) in 80 mM Tris-HCl, 2 mM $MgCL_2$, and 2 mM $ZnCl_2$ pH 8 was made freshly each time. The reaction solutions were mixed with samples in a 1:1 ratio, and incubated at 37°C water bath for 1 hour. At the end of the enzymatic digestion, an equal volume of ice cold 10 % TCA solution was added to precipitate protein and the reaction mixtures were centrifuged. The supernatants were then subject to pterin and phosphate assays. The standards of the phosphate assay, solutions of 0, 50, 100, 150, 200 µM Na_2HPO_4, were also treated in the same manner prior to the phosphate measurements.

Extraction of proteins from *P. carinii*

The organisms were suspended in 1ml of PBS buffer and were sonicated. The suspension was microfuged at 8,000 x g for 10 min. The supernatant was concentrated with a micro solute concentrator (Amicon). Protein concentrations were determined by method of Bradford *et. al.* (1976).

DHPS Assay Procedure

This procedure was adapted from methods described by Iwai and Okinaka (1980). The principle advantage of the adapted procedure is its enhanced sensitivity for DHPS activity.

The assay solution contained 100 mM Tris - HCl (pH 8.5), 5 mM NaF, 10 mM magnesium chloride, 2 mM ß-mercaptoethanol, 50 µM [^{14}C]-pAB (Amersham, Arlington Heights, Illinois specific activity 56.6 µCi/µM) and a final 100µM concentration of MDHP-PP. The reaction was initiated by addition of a cell extract (0.30 to 0.50 µgs) from either *E. coli* or *P. carinii* to the assay solution at room temperature. The reaction at zero time was represented by an aliquot immediately removed from the solution after the addition of the appropriate cell extract. The incubation of the remaining solution was allowed to proceed for 1 hr, and the reaction was terminated by placing both the zero and 1 hr-incubation samples in a boiling water bath for 2 min. These samples were clarified by centrifugation at 20,000 x g for 30 min, and then passed through a 0.45µm HA-type Millipore filter. DHPS was measured by two methods. Paper chromatography of the samples was performed according to method of Shiota *et. al.* (1964) and the HPLC method described below. Specific activities are presented as means $\pm$ the standard deviations. DHPS activity was observed to be stable, changing

less than 5 % when kept for twelve hours at room temperature or stored at -33° C for 1 month.

In order to determine the efficiency of conversion of PABA into folate, untreated cultures were exposed to [^{3}H] PABA for 48 hours, and then harvested, washed, pelleted and sonicated as described above. A 50 µl aliquot was removed, added to scintillation fluid and counted as above.

High performance liquid chromatography assay of DHPS.

A Waters Associates 6000 HPLC instrument equipped with a model 6000A solvent delivery system and a model U6K sample injector was employed in the analysis of the DHPS incubation solutions. A single µC_{18} column was first equilibrated with 50 mM ammonium phosphate buffer (pH 5.8). Sample volumes of 10 µl were injected onto the column using a Hamilton 801 microliter syringe. The assay solution was first eluted with the ammonium phosphate buffer (pH 5.8), then with 10% methanol balance water and finally with 50% methanol balance water. Samples (3 ml) from the elution profile were collected during the three-part elution and monitored through absorbance measurements at 254 nm. A 0.2-ml aliquot from each sample was added to 5 ml of aquasol and the samples were counted in a Rackbeta scintillation counter (LKB, Uppsala, Sweden).

Paper chromatography of DHPS

The [^{14}C]-PABA was separated from [^{14}C]-dihydropteroate by a paper chromatographic procedure. The reaction mixture (see section 2.3.1.4) was clarified by boiling and the resulting supernatant was spotted on 15 cm long 3 MM Whatman paper. The samples were eluted for one and half hours with

0.1 M potassium phosphate pH 7.0. The trails of samples were visualized under flourescent light and each trail, located between solvent front and base line, was cut into 10 equal parts. Each piece of filter paper was placed in a scintillation vial and 15 ml of Aquasol-2 was then added. The vials were counted in an LKB rackbeta liquid scintillation counter.

Inhibition and K_m Studies

K_m's were determined by measuring the activity of DHPS at substrate concentrations of 0 μM, 20 μM, 50 μM and 100 μM. IC_{50}'s and K_i 's were determined using data for which the drugs were present at concentrations of 0μM, 200μM, 400μM, and 500μM, except for dapsone in which the concentrations were 0μM, 200μM, 300μM, and 500μM. Plots of the log of DHPS activity versus drug concentration were made to determine IC_{50}'s. Dixon plots were constructed as described (Segal, 1976). All lines were drawn by linear regression analysis.

2.3 Folate biosynthesis by *Pneumocystis carinii in vitro* : sensitivity to sulfa and other drugs

Isolation and cultivation of organisms.

The *P. carinii* were obtained from the rats as described above. Rats were sacrificed and their lungs were then quickly removed and kept at 4°C. Touch preps were made from the lungs and stained with Toluidine blue (Gossey et al., 1985) and Gram stain. The experiment was continued if cysts were present, but no fungi or bacteria were evident. The lungs were homogenized by repeated passage through a 60-gauge wire screen. The

resulting suspension was centrifuged at 100 x g for 5 min. The supernatant was removed and used to inoculate the culture.

P. carinii was cultivated by a modification of previously published methods (Armstrong and Richards, 1989; Bartlett *et al.*, 1985). Twelve-well microtiter plates were seeded with 5 X 10^5 cells/well of epithelioid mink lung cell line Mv 1 Lu (American Type Culture Collection, Rockville, MD). Each well was incubated for 24 hrs or until the cells were >90% confluent in Eagle's Minimal Essential Medium (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 µg/ml). Each well was then inoculated with supernatants of infected lung homogenates which contained 100,000 cysts. The cultures were incubated in 5% O_2 and 10% CO_2 at 37 ^oC (Bartlett *et al.*, 1985).

In vitro folate assay

Folates were measured by taking advantage of their binding to mammalian folate binding protein (FBP) (Kamen and Caston, 1986). The assay was initiated by addition of desired drug to each well. For each experiment the cultures were set up in duplicates and 2 wells were used for each experimental point. After 24 hrs of incubation, 5 µCi of [^{3}H] PABA was added to each well and the cultures were incubated for additional 48 hrs. The organisms were removed by gentle agitation and washed three times with Dulbecco's phosphate buffer saline (PBS). Gram stain was again performed on an aliquot of each pellet to determine bacteria contamination. If no bacteria or fungi was evident, then each pellet was resuspended in 1 ml of PBS, sonicated at for 3 min each at 40 W using a microprobe (Heat

Systems Co., Melville, NY) , and then microfuged for 5 min at 14,000 rpm. The pellet was discarded and 50 µl of supernatant was saved for protein assay. The protein in the remaining supernatant was denatured by heating in boiling water for 2 minutes. The supernatants were then clarified by microfuging for 5 min at 14,000 rpm and divided into two equal portions. 50 µg of folate binding protein was added to one aliquot. Both fractions were then incubated for 20 min at room temperature. After 20 min, an equal volume of dextran-coated charcoal (5% activated charcoal [Sigma]:1% dextran [M.W. 220,000, Sigma]) was added to each fraction to absorb unbound PABA and folate precursors. The FBP-bound folates were separated from the dextran-coated charcoal by centrifugation for 10 min at 14,000 rpm. The supernatant was counted in biodegradable counting scintillant (Amersham Corp., Arlington Heights, IL.) on an LKB Rackbeta 1219 scintillation counter.

Validation of the assay.

To confirm that this procedure measures newly folates but not PABA, assays were performed using authentic [^{3}H] folate and [^{3}H] PABA as follows. Red blood cells were washed with PBS 3 times and resuspended in 1 ml PBS. The suspension was separated into two equal fractions. To one fraction 5nCi of [^{3}H] folate (specific activity, 50 mCi/µmol) was added and to the other fraction 5 nCi of PABA was added. Both fraction were sonicated as above. The folate assay on both fractions was performed as described previously (Zhang *et al.*,1992).

In order to determine the efficiency of conversion of PABA into folate, untreated cultures were exposed to [^{3}H] PABA for 48 hours, and then

harvested, washed, pelleted and sonicated as described above. A 50 µl aliquot was removed, added to scintillation fluid and counted as above.

Determination of IC_{50}.

At least 3 points were required for plots to determine the effects of drugs or PABA incorporation into folates. The plots were converted to straight lines by plotting percent inhibition versus log concentration. The data were fit by least square linear regression.

2.4 Susceptibility of *Pneumocystis carinii* to artemisinin *in vitro*

P. carinii was cultivated by a modification of previously described procedure (Section 2.2).Twenty-four well plates were seeded with 5 X 10^5 cells of epithelioid mink lung cell line Mv 1 Lu (American Type Culture Collection, Rockville, MD). Each well was incubated for 36 hrs or until the cells were >90% confluent in Eagle's Minimal Essential Medium (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 µg/ml). Each well was then inoculated with 2 X 10^5 trophozoites from supernatants of infected lung homogenates. The cultures were incubated in 5% O_2 and 10% CO_2 at 37 °C (Bartlett *et al.*, 1985). To count, the organisms were dislodged using a transfer pipette and then counted as described above. For each experiment, an entire well was harvested and counted each day for four days.

2.5 Folate metabolism in *Plasmodium falciparum*: studies of PABA biosynthesis and its importance as a target for sulfa drugs*In vitro* culture of *Plasmodium falciparum* and 3D7 mutants

Plamodium falciparum (FCR3) and the mutants were cultured in red blood cells by the method of Trager and Jensen (1976) in candle jars Culture media for the experiments consisted of PABA- and folate-free RPMI 1640 (GIBCO, Grand Island, NY), containing 0.2 % $NaHCO_3$, 40 mM Hepes, 20 mM dextrose, 40 mg/L gentamycin and 10 % (V/V) human type A^+ serum (Interstate Blood Bank, Memphis, TN) , at pH 7.1. Prior to use in experiments, red blood cells were washed three times in PABA- and folate-free RPMI 1640. Parasite culture was maintained at a hemotacrit value (percentage packed red cells in media) of 7 %. Blood smears were made daily and the parasitemia was determined with microscopy.

Folate Assay

Parasite-infected red cells of parasitemia 5-10% were incubated with [^{3}H]- PABA for 24 hrs and [^{3}H] folates synthesized were measured by modification of the methods of Kamen and Caston (1986). Folte synthesis in uninfected red cells was measured as control. Parasite-infected and unifected red cells were washed 3 times with PABA- and folate-free RPMI-1640 medium (PFF RPMI), Preincubated in PFF RPMI containing 10% serum for 48 hrs, and then incubated in the same medium containing 7 μM [^{3}H] PABA for 24 hrs. Cells were then pelleted and washed 3 times with PBS, resuspended in 1 ml of PBS and lysed by sonicating for 1min at 40 W using a microprobe (Heat systems Co., Melville, N.Y). The lysates were microfuged

for 5 min at 14,000 rpm (Brinkmann Instruments Co., Westbury, NY), and 50 µl of the supernatant was saved for protein determination. The remaining supernatant was treated in a manner similar to section 2.2

Preparation and concentration of red cells infected with parasites for PABA analysis.

Infected and uninfected red cells were incubated with PABA- and folate- free media with or without added PABA (6.3 µM) for 48 hrs. After incubation, the infected red cells were centrifuged at 500 X g for 5 min and concentrated by the gelatin flotation method (Fairfield *et al.*, 1983) to a parasitemia of 40-50%. These parasite-infected red cells were washed in phosphate buffered saline (PBS) three times before they were used in experiments.

The uninfected and the concentrated infected red cells were sonicated with a Sonifier Cell Distributor (Ultasonics Inc., L.I., NY) using a microprobe at 40 watts for 45 sec. A volume of 10 µl of the sonicate was removed for protein determination and to the rest of the homogenates an equal volume of 2 M perchloric acid was added. The denatured proteins were removed by centrifuging at 16,000 X g for 5 min and the supernatant was filtered through a 0.45 µM Acrodisc filter. (By adding known amounts of ^{3}H-PABA to the homogenate prior to addition of perchloric acid, it was found that supernatant contained 43% ± 1%of total radioactivity). To the supernatant obtained, 5 nmol of para-hydroxy benzoic acid was added as an internal standard.

HPLC analysis of PABA

PABA content was measured by adaptation of method of Lindsay *et al.* (1988). HPLC analysis was performed using a Gilson liquid chromatograph (Middleton, WI) fitted with Dual Rainin Rabbit HP pumps (Rainin, Woburn, MA). The samples was then injected through a Rheodyne injector (Rheodyne, Cotati, CA) containing 100 µl sample loop. PABA was separated by reverse phase HPLC using 4.6 X 250 mm C18 Hypersil reverse phase column (Phenomex, Torrence, CA) packed with 5 µm Spherisorb. The eluate was monitored using a Gilson HM Holocrome ultraviolet/visible detector (Gilson, Middleton, WI) at a fixed wavelength of 266 nm in a flow cell volume of 11 µl . The mobile phases consisted of (A) 0.1 M sodium citrate buffer at pH 3.5 adjusted with HCl. (B) 10% of acetonitrile (90% double distilled water, v/v). A linear gradient (2 ml/min) startining at 100% A leading to 20% B was run for 30 min. Thereafter the column was washed for 20 min at 50% B before regenerating the starting conditions.

A standard curve was constructed by adding various amounts (1-50 nmol) of PABA to red cell extracts and by determining the ratio of the areas of the PABA peak to the internal standard peak.

Determination of IC_{50}

At least 3 points were required for plots to determine the effects of drugs or PABA incorporation into folates. The plots were converted to straight lines by plotting percent inhibition versus log concentration. The data were fit by least square linear regression.

3. Dihydropteroate Synthetase of *Pneumocystis carinii* : Studies of Its Inhibition by Sulfa-Reagents.

As discussed before, two enzymes involved in the biosynthesis of tetrahydrofolate that are important as targets of antifolates are the DHPS and DHFR. DHPS catalyzes the formation of dihydropteroate from PABA and 6-hydroxymethyldihydropterin pyrophosphate (MDHP-PP). DHFR catalyzes the formation of tetrahydrofolate from dihydrofolate. Sulfones and sulfonamides act as competitive inhibitors of DHPS substrates while drugs such as trimethoprim and pyrimethamine act as inhibitors of DHFR substrates. In this report we have measured the inhibitory action of several sulfonamides and sulfones on the parasite associated with DHPS using a new high pressure liquid chromatography (HPLC) enzyme assay.

3.1 Results

3.1.1 *P. carinii* DHPS

Because the enzymatic reaction is terminated in a boiling water bath, we examined the stability of our substrates and products under these conditions. When 0.5M solutions of PABA MDHP-PP and DHP were boiled for 2 minutes, corresponding HPLC peak areas (2 µl injections) were changed by less than 10%. Figure 19 illustrates the HPLC elution profiles from incubation mixtures containing *E. coli* and *P. carinii*. As shown in Figure 1, for both organisms, [^{14}C]-pAB repeatedly eluted at 5 min with 50 mM ammonium phosphate, whereas the product (pteroic acid) eluted repeatedly in 50% methanol at 28 min. Standard samples of PABA and pteroic acid eluted in a similar manner. Using the HPLC to isolate the

product and measure DHPS activity, the *E. coli* homogenate was found to contain 451 ± 46.5 (n=4) μunits/mg (A unit of DHPS activity is defined as one μmole dihydropteroate synthesized per hour). This is similar to the specific activity determined using the standard assay (320 ± 30 (n=3) μunits/mg). In contrast, *P. carinii* homogenates had too little enzyme activity to be measured by the paper chromatography method . Using the HPLC method, with which we were able to detect as little as 1 μunit of activity, *P. carinii* homogenates were found to have 13.50 ± 3.92 (n=4) μunits/mg. On the other hand, uninfected lung homogenate had no detectable activity (< 0.002 μunits/mg).

3.1.2 The inhibition of *P. carinii* DHPS

Having designed this repeatable and quick assay procedure, we then determined the K_m's for both substrates and the effects of five sulfa-drugs on the *P. carinii* DHPS activity. The K_m for pAB was 71 μM (Figure 22). Table 2 lists the calculated IC_{50} and K_i values of these sulfones and sulfonamides.IC_{50} and K_i values were calculated by extrapolation from the graph (Figure 20 and 21). In all cases $R^2 > 0.92$. As defined in Table 2, the drug which most effectively inhibited this DHPS activity was dapsone. IC_{50}'s of greater than 500 μM were observed with sulfadoxine (42 % inhibition at 500 μM) and sulfadiazine (10% inhibition at 500 μM).

3.2 Discussion

In this report, we have demonstrated that *P. carinii* contains DHPS activity. Initially, we attempted to measure *P. carinii* DHPS by published assays (Iwai and Okinaka, 1980) but found that the organism's activity

was below detectable levels. Accordingly, we developed a new HPLC-based assay with enhanced sensitivity and specificity. Using this new assay we have compared the inhibitory actions of several sulfones and sulfonamides in *P. carinii* (Table 2). Of the drugs tested, dapsone (IC_{50} = 215μM, K_i=9 μM) was the most potent inhibitor, whereas sulfadiazine was the weakest. Sulfaquinoxaline, sulfamethoxazole, and sulfadoxine all had intermediate inhibition with IC_{50}'s of 400-600 μM and K_i's of 60-150 μM.

Although there is a great need for new treatments for PCP, the development of such agents has encountered several severe obstacles. First, it has been exceedingly difficult, if not impossible, to date to obtain pure preparations of organisms which are not contaminated by mammalian host material. Second, an easy and reliable long-term *in vitro* cultivation method for these organisms has not yet been developed. Third, the animal model for PCP is difficult to quantitate and to adapt for the testing of drugs.

Measurements of the inhibitory effect of sulfa drugs against the *P. carinii* DHPS offers promise as a means of developing new chemotherapeutic agents. The HPLC assay is simple and reproducible and, since mammalian tissue does not contain DHPS activity, it is possible to measure the relative inhibitory effects of drugs even in the presence of the host cell contamination.

For the drugs tested, IC_{50} values were up to 30 fold higher than K_i values. For dapsone, the K_i (9μM) was closer than the IC_{50} (250 μM) to the serum concentration of dapsone in patients with AIDS (4 μM - 16 μM)

(Lee *et al.*, 1989) and the *in vitro* inhibitory concentration of the drug reported by Kovacs *et al.* (1989). In addition, this value was similar to the K_i for dapsone in *E. coli*, 5.9 μM (McCullough and Maren, 1973; Shiota, 1984). These data suggest that the K_i may be a more useful indicator than IC_{50}.

The effects of various sulfa drugs were quite different on the *P. carinii* DHPS than on the *E. coli* DHPS. For example, the K_i for sulfamethaxazole in *E.coli* is 0.13μM (McCullough and Maren, 1973; Shiota, 1984), whereas in *P. carinii* the K_i is >400-fold greater (59 μM). Furthermore, in *E. coli* sulfadiazine (K_i= 2.5 μM) is a more effective inhibitor of DHPS than dapsone, whereas in *P. carinii* the opposite is true. These differences may reflect fundamental differences in enzyme active site structure between the two species.

In summary, the data presented here indicate that various sulfonamides and sulfones have inhibitory effects on the *P. carinii* DHPS. Further studies of inhibitors of the *P. carinii* DHPS may uncover further leads for the development of new therapeutic regimens.

4. A new drug screening procedure for *Pneumocystis carinii* based on *in vitro* folate biosynthesis

Typically, potential antimicrobial agents are first identified by *in vitro* screening. Unfortunately, it has been exceedingly difficult to screen drugs *in vitro* against *P. carinii*. One reason is that *P. carinii* does not grow well in culture. Although several groups have established short-term primary culture systems, the organisms must be grown in the presence of feeder-layer cells and cannot be successfully passaged (Bartlett *et al.*, 1979). Furthermore, there are many differences between various culture methods. For example, different types of feeder-layer cells are used from one lab to another. Also, methods for quantitating organisms differ in that some investigators prefer cyst counts (Pifer *et al.*, 1977), other prefer trophozoites (Bartlett *et al.*, 1979) while others prefer to count nuclei (Cushion and Walzer, 1984). Perhaps as a result of these problems, widely divergent differences in *in vitro* activities of drugs have been reported (Cushion, 1989). Clearly, there is a need to develop a new *in vitro* assay for screening anti-*Pneumocystis* agents.

We now report a new method for quantitating and screening drugs for anti-pneumocystis activity against actively growing organisms. This method takes advantage of the fact that *P. carinii* synthesizes folic acid *de novo* (Kovacs *et al.*, 1989), while mammalian cells do not. Thus, by measuring the conversion of radiolabelled exogenous p-aminobenzoic acid (PABA) into folic acid, parasite metabolic activity can be measured without fear of interference from mammalian feeder layer cells. The parasite-derived folate pool is then easily separated using mammalian folate binding protein (FBP),

which has been shown to bind various methylated and polyglutamated folate derivatives, but not folate precursors such as dihydropteroate (Kamen and Caston, 1986).

4.1 Results

4.1.1 *In vitro* folate biosynthesis by *P. carinii*

P. carinii utilize exogenous PABA to synthesize folates. After incubating for 48 hrs with [^{3}H] PABA, the organism have synthesized 9.95 ± 0.75 fmol radioactive folate per mg total protein (data obtained from 4 independent experiments, each of which was carried out in duplicate). In wells containing feeder layer cells but no parasites, less than 0.1 fmol per mg total protein can be detected. The cultures used in this study are viable; during the 72 hours used for this assay, trophozoite numbers increase 4-5 fold. There was a substantial amount of non-specific uptake of radioactivity as well. Total parasite-associated radioactivity was greater than twice the radioactivty in parasite-associated folate.

The assay procedure used is specific for folates and was linear for 48 hrs (Table 4). In red cell homogenates, >85% of exogenously added [^{3}H] folic acid was bound to folate binding protein while <1% of exogenously added [^{3}H] PABA was bound.

4.1.2 Screening of anti-folates

Having designed a quick and reliable folate assay method, various drugs were screened for potential antipneumocystis activity. Three sulfa drugs, dapsone, sulfamethoxasole, and sulfadoxine, were first evaluated for antipneumocystis activity (Table 3). Dapsone was the most potent inhibitor of

folate biosynthesis with IC_{50} < 1μM Sulfamethoxazole, a component of Bactrim, had an intermediate effect with an IC_{50} of 3.5 μM. Sulfadoxine, a component of Fansidar, was least effective in inhibiting folate biosynthesis, with an IC_{50} of 12 μM. In addition, trophozoites counts were taken each day. Figure 23 shows the average growth curve of controls and sulfa-treated organims.

4.1.3 Screening of other anti-Pneumocystis agents

In order to determine if the folate assay can be used to screen other anti-pneumocystis agents which are not anti-folates, we first determined the inhibitory effect of pentamidine (Table 3). Pentamidine was shown to inhibit PABA incorporation with an IC50 of 5μM.

Iron chelators have previously been shown to have antipneumocystis activity. We measured the antipneumocystis activity of a novel iron chelator, DFO-HES, which is a high molecular weight conjuage of desferoxamine and hydoxyethyl starch. DFO-HES had an IC_{50} of 42 μM (the concentration represents deferoxamine equivalents) (Fig. 23). This is similar to IC_{50} previously found for deferoxamine alone (Weinberg and Shaw, 1991) and confirmed here (Table 3). The inhibitory effect was likely to be due to iron sequestration, since iron saturated DFO-HES had no activity. HES alone at an equivalent concentration (2 mg/ml) did not have any detectable inhibitory effect (data not shown).

4.2 DISCUSSION

The inhibition of the conversion of [^{3}H] PABA into folates can be used to assess the efficacy of drugs against *P.carinii in vitro*. As expected,

dihydropteroate synthesis inhibitors, dapsone, sulfadoxine and sulfamethoxasole, inhibit *P. carinii* folate biosynthesis. However, other types of drugs, such as iron chelators, artemisinin and pentamidine, also block the observed conversion of PABA to folic acid. This presumably occurs because these drugs kill or cause severely injure the organisms.

The *de novo* biosynthesis of folates in *P. carinii* was first demonstrated by Kovacs *et al* (1989). Using an HPLC assay, these investigators showed that bactrim and pentamidine inhibit the conversion of PABA to folates by *P. carinii* cultured in the presence of WI-38 cells over 18-24 hours. Comley *et al.* measured PABA uptake and folate biosynthesis in freshly isolated *P. carinii* over 18 hours. Since, in these period of time, 90% of the incorporated PABA was converted into folates, PABA uptake was monitor to screen drug effects. However, since the organisms cease to synthesize folic acid after 24 hours, and since the organisms may not be activly growing, this method is only effective for drugs which are short acting, such as pentamidine, or which drugs which directly affect folate biosynthesis, such as sulfa drugs. The assay reported here differs from the two previous reports in several important ways. First, the method described here, using folate binding protein, is a much easier way to measure incorporation into folates than HPLC. Second, unlike the Comley assay, this assay measures folate biosynthesis in actively growing organisms exposed to drug for 72 hours. Thus, slower acting drugs can be detected. Interestingly, for incubations of this length of time, PABA uptake cannot be substituted for measurement of conversion of PABA into folates. Less than half of the parasite-associated counts were present in folic acid.

Of the three antifolates used, dapsone was most potent inhibitor of folate biosynthesis, sulfamethoxasole was intermediate, and sulfadoxine was the least potent. This order of activity is the same as the order of activity against the *P.carinii* dihydropteroate synthetase (Merali *et al.*, 1990). It is also the same as the order of activity against *P. falciparum in vitro* (Zhang and Meshnick, 1990). Suprisingly, the concentrations which inhibit *pneumocystis* folate biosynthesis are approximately 10x lower than those which inhibit dihydropteroate synthetase (Merali *et al.*, 1990). There are several possble explanations for this difference. First, the parasites might concentrate sulfa drugs as has been observed in malarial parasites, where the concentrations which inhibit growth are 100-1000x lower than the concentrations which inhibit dihydropteroate synthetase (Zhang *et al.*, 1992). Second, sulfa drug might have an additional target other than dihydropteroate synthetase (Sirawaraporn and Yuthavong, 1986).

Deferoxamine, an iron chelators, has been shown to have antipneumocystis activity both *in vivo* and *in vitro*. But does it kill parasites by depriving them of iron or must it be taken up by the parasites? In the case of malaria, when the antimalarial activity of deferoxamine was first discovered, it was believed to be due to its ability to sequester iron and prevent its uptake. However, later investigations revealed that the antimalarial activity of this compound was dependent on its ability to penetrate the parasite. Accordingly, a high molecular weight agregate of deferoxamine, deferoxamine-dextran, was found to lack antimalarial activity *in vitro*. *P. carinii* appears to be different, however. DFO-HES, was shown to inhibit parasite growth i*n vitro* (Figure 24). The mechanism of *P. carinii* growth inhibition by DFO-HES presumably involves the chelation of

extracellular iron, since iron saturated DFO-HES was inactive. Unfortunately, DFO-HES was found to lack *in vivo* antipneumocystis activity in rats (R. Tidwell, personal communication).

In summary, the data presented here indicate that the folate assay can be used to screen not only anti-folates but various other classes of drugs. Using this assay we were able to quantitate accurately actively growing parasites for 48 hrs. Further studies of drugs by using in vitro folate assay would lead to development of novel anti-*Pneumocystis* agents.

5. Susceptibility of *Pneumocystis carinii* to artemisinin *in vitro*

Artemisinin (qinghaosu) is a promising new antimalarial agent derived from an ancient Chinese herbal remedy. Artemisinin and its derivatives, particularly artemether, have shown remarkable efficacy and are now undergoing clinical trials against drug-resistant malaria (Arnold *et al.*,1990; China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalaria, 1982; Luo and Shen, 1987; Myint *et al.*, 1989; Song and Zhao. 1989). In addition, artemisinin and artemether have recently been shown to be active against other parasitic organisms, including *Toxoplasma gondii* and *Schistosoma mansoni* (Yang *et al.*,1990; Shuhua and Catto, 1989). We report here that artemisinin is also active against *P. carinii in vitro.*

5.1 Results

5.1.1 The effect of artemisinin on *P. carinii* and feeder layer cells in culture.

Artemisinin was obtained from the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland, and stored as an ethanolic solution at -70° C. Artemisinin was added at the following concentrations to cultures immediately after inoculation: 100 µM, 50 µM, 5 µM, 0.5 µM and 0.1 µM. Final ethanol concentration was never greater than 0.3%. A pentamidine concentration of 15 µM was used as a positive control.

P. carinii trophozoites increased in number for four days by an average of 5.3-fold (Figure 25). The inhibitory effect of pentamidine was not seen on day 1 of culture (<26%) but was evident on days 2 and 3 of culture, when inhibitions by 63% and 79%, respectively, were seen. In contrast, the effect of 100 μM artemisinin was evident after 1 day (Figure 26), inhibiting growth by 63%, 76%, and 78% on days 1,2, and 3, respectively.

Artemisinin at concentrations of 50 and 5 μM also completely inhibited the growth of *P. carinii* for four days, without affecting the feeder layer cells (Figure 27). No effect was observed at an artemisinin concentration of 0.1 μM, while artemisinin at 0.5 μM had an intermediate effect.

As controls, feeder layer cells were incubated for 4 days in the presence of 100 μM, 50 μM, 10 μM and 1 μM artemisinin, but in the absence of parasites, under conditions identical to those used for parasite culture. No effect was observable under phase contrast microscopy. Feeder layer cells from the 100 μM artemisinin control were also dislodged from the wells by trypsinization and found viable by trypan blue exclusion (>95%). Furthermore, 0.5% ethanol had no effect on either the feeder layer cells or on parasite growth over 4 days (data not shown).

The *in vitro* folate assay previously described in section 4.1 also shows that artemesinin inhibit PABA incorporation at concentrations similar to that which inhibited growth (IC50=0.8μM).

5.1.2 Discusssion

Artemisinin, a potent antimalarial agent, inhibits the growth of *P. carinii* in culture at concentrations as low as 0.5 μM. In contrast, artemisinin has no effect on Mv 1 Lu feeder layer cells at a concentration of 100 μM, indicating that the drug is selectively toxic to the parasite. An artemisinin concentration of 0.5 μM caused a 55% inhibition of growth. This results is similar to the concentration which inhibits *Toxoplasma gondii*, 0.3 μM (Yang *et al.*,1990), but is considerably larger then the concentrations which inhibits malaria growth, 20 nM (Meshnick *et al.*, 1989).

In this study, we used a short-term *in vitro* culture system to assess the effects of artemisinin on *P. carinii*. Others have reported increases in the number of trophozoites for as long as 10 days, accompanied by a 2 to 10 fold increase in cell number (Cushion, 1989). Short-term cultures have also been used to demonstrate the antipneumocystis activities of trimethoprim-sulfamethaxazole, pentamidine, dapsone, primaquine, trimetrexate, and others (Cushion, 1989)

Artemisinin and its derivatives represent a promising new class of antiparasitic drugs which act as free radical generators (Meshnick *et al.*, 1989). They are currently being used in the treatment of malaria in China (China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalaria, 1982; Luo and Shen, 1987; Wang and Xu, 1985), Burma (Myint *et al.*, 1989), and Vietnam (Arnold *et al.*,1990). The results presented here suggest that they are potentially useful in the treatment of PCP.

6. Folate metabolism in *Plasmodium falciparum*: studies of PABA biosynthesis and its importance as a target for sulfa drugs

As described in the introduction, sulfa drugs such as sulfadoxine are analogs of PABA. The role of PABA in *P. falciparum* is not clear. Extracellular PABA has been reported to be an essential nutrient in growth of *P. berghei* (Hawkins, 1954) and *P. falciparum* (Kretschmar and Voller, 1973). In contrast, there is evidence that de novo PABA synthesis occurs in *P. falciparum* and activity of four enzymes in PABA biosynthethic pathway has been dectected (Dieckmann and Jung, 1986). We now report that *P. falciparum* is capable of *de novo* PABA synthesis when extracellular PABA is depleated. Furthermore, studies with sulfadoxine and para-aminosalicylate (PAS) have shown to inhibit folate biosynthesis and cause intracellular PABA build-up.

6.1Results

6.1.1 Incorporation of exogenous [^{3}H-] pABA into folates

To confirm that exogenous [^{3}H]-PABA can be used by parasites, the [^{3}H]-folate synthesis in parasite-infected red cells was measured. After incubating with 7 μM [^{3}H]-PABA for 24 hours, parasite-infected red cells contained 5.4 ± 0.8 fmole folates per mg total protein (adjusted to 100% parasitemia; data obtained from 5 independent experiments each with triplicate measurements). In contrast, there was no detectable level of [^{3}H]-folates in normal red cells (< 0.05 nmole folates per mg protein).

6.1.2 Inhibition of folate biosynthesis by p-aminosalicylate and sulfadoxine.

Sulfadoxine, a component of fansidar, was first evaluated for its ability to inhibit incorporation of [^{3}H]-PABA into [^{3}H]-folates (Figure 28). Sulfadoxine was shown to inhibit PABA incorporation with an IC_{50} of 7 nM (Table 5). p-Aminosalicylate (PAS),0 an antifolate primarily used to treat tuberculosis (Mandell and Sande, 1985), was also evaluated for anti-malarial activity (Figure 29). PAS was shown to inhibit folate biosynthesis at higher concentration than sulfadoxine with an IC_{50} of 300 nM (Table 5). Incorporation of exogenous PABA is inhibited by sulfadoxine at concentration 5000 times lower than it inhibited DHPS activity. PAS also inhibited incorporation of exogenous PABA at concentration 150 times lower than it inhibited DHPS activity. One possible explanation of lower IC_{50} for incorporation of PABA in both PAS and sulfadoxine treated cells is that both of these drugs are concentrated by the parasites (Zhang *et al.*, 1991).

Sulfadoxine concentration of 357nM was toxic to parasites but PAS concentration of up to 1 mM was not (unpublished data, Zhang and Meshnick). This suggests that sulfadoxine does not kill by inhibiting DHPS alone, since PAS also inhibits but does not kill. One potential explanation is that the sulfa drugs inhibit PABA synthesis*in vivo* where as PAS does not. Another possible explanation is that sulfadoxine inhibits the uptake of PABA by infected red cells whereas PAS does not. Both of these possibilities are investigated here.

6.1.3 De novo PABA biosynthesis by *Plasmodium falciparum*

Figure 30 shows HPLC profiles from *Plasmodium falciparum* infected red cells and uninfected red cells incubated in PABA- and folate-free media. PABA was only detected in infected red cells. In PABA- and folate-

free media, *Plasmodium falciparum*-infected red cells contain 95.4 ± 17 pmol/mg (N=3) while the uninfected red cells have <1 pmol/mg of protein (Figure 31). In media containing PABA, on the other hand, infected and uninfected red cells incubated contain 153 ± 15 and 22 ± 10 pmol PABA/mg, respectively (Figure 30). Therefore, PABA biosynthesis occurs in infected red cells and does not occur in uninfected red cells.

6.1.4 Analysis of *de novo* PABA biosynthesis by *P. falciparum* mutants auxotrophic for exogenous PABA

The PABA contents of *P. falciparum* wild type strain 3D7 and its mutant clones D11, E10, B5 and E6, obtained from Drs Glen MacCorkey and Thomas McCutcheon of National Institute of Health (NIH, Bethesda, Maryland), were analyzed fo

r their PABA contents. These mutants were selected to PABA auxotrophs The wild type and the clones were incubated in PABA-free media prior to HPLC analysis. The results show that 3D7 has a PABA content of 22 pmol/mg while the mutants D11 and E10 have PABA contents of <1pmol/mg. In contrast, B5 and E6 contain 38.5 and 2 pmol/mg of PABA, respectively (Table 6).

6.1.5 The effect of sulfadoxine and PAS on *de novo* folate biosynthesis

To determine if the anti-folates inhibit PABA biosynthesis or cause PABA build-up, the effect of sulfadoxine and PAS on PABA content of *P. falciparum* infected-red cells was analyzed. Table 7 shows that parasite infected-red cells contain 206 pmol/mg protein of PABA (results obtained

from 3 independent experiments). In contrast, sulfadoxine and PAS cause the parasite infected-red cells to an accumulate 350 and 305 pmol/mg protein of PABA, respectively.

6.6 Discussion

Malarial parasites utilize exogenous PABA to synthesize folates. There are several pieces of evidence for this. Firstly, PABA stimulates parasite growth both in vivo and in vitro (Ferone, 1977). Secondly, PABA antagonizes the effects of antifolates (Watkins et al., 1985; Brockelman and Tan-Ariya, 1982). Thirdly, as we have shown here, parasite-infected red cells convert exogenous [^{3}H] PABA into [^{3}H] folates.

Incorporation of exogenous [^{3}H]PABA was inhibited by sulfadoxine and PAS at IC_{50} of 7 nM and 300 nM, respectively. Furthermore, sulfadoxine inhibited the PABA incorporation at concentration 5000 times lower than that inhibit parasite DHPS. A similar situation was observed with PAS, with inhibition of PABA incorporation 150 times lower than that inhibit parasite DHPS.

Why are the IC_{50} so different from K_i? The parasites can concentrate sulfa drugs as previously determined by Zhang and Meshnick (1991). These investigators analyzed the uptake of [^{3}H] sulfamethoxazole by parasite infected-red cells and showed that intraparasitic concentration was 20 times higher than the extracellular concentration . But the difference was not suffecint to explain the 200 fold difference between LD_{50} and K_i previously observed. Therefore, the concentration of sulfa drugs by the parasites is not the only explanation for the observed difference in IC_{50} and

K_I. Both PAS and sulfadoxine inhibit DHPS but only sulfadoxine kills, suggesting a possibility that sulfadoxine have an alternative mechanism.

One of the alternatives is that sulfadoxine might inhibit PABA uptake by the parasites. Previous studies with sulfadoxine have shown that it does not inhibit PABA uptake by the parasite (Zhang's thesis, 1991). Additional experiments here have shown that PAS concentration of upto 20 μM does not significantly alter PABA uptake.

Another possible alternate would be that the sulfa drugs could inhibit *de novo* PABA biosynthesis *i.e.* by feedback inhibition. Previous reports have shown that PABA is not an essential nutrient for sulfa-resistant plasmodia and instead they might be capable of *de novo* PABA biosynthesis (Dieckmann and Jung, 1986). These investigators have demonstrated the presence of four enzymes in PABA biosynthetic pathway namely: shikimate dehdrogenase, shikimate kinase, PABA synthase and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase. But the mere presence of these four enzymes does not prove their activity in vivo. We assayed and detected that *P. falciparum* (FCR 3)-infected red cells contain 7x as much PABA as uninfected red cells, when both are incubated in the presence of PABA. When incubated in PABA-free media, *P.falciparum*-infected red cells contain 1/3 less PABA while uninfected red cells contain no measurable PABA (Fig. 2). These data are consistent with the observations of Dieckmann and Jung and suggest that PABA biosynthesis occurs in infected but not uninfected red cells.

Further confirmation of *de novo* PABA biosynthesis by malarial parasites was obtained by testing PABA content of the mutant clones of

3D7 wild type. From the results obtained, two types of mutants (B5, E6 and D11, E10) are evident. The mutants B5 and E6 are capable of synthesizing PABA endogenously when extracellular PABA is depleated, while D11 and E10 do not. The reason PABA requirement is not clear. But could represent the parasite with reduced or altered capacity to produce PABA endogenously.

Having determined the PABA content of the parasites, we analyzed the effect of sulfadoxine and PAS on *de novo* PABA synthesis. At high concentration both drugs cause build-up of PABA with sulfadoxine more than PAS, suggesting that DHPS inhibition occurs *in situ* and that neither drug inhibits PABA uptake or biosynthesis significantly.

Why doesn't 1 mM PAS inhibit the parasites growth while sulfadoxine inhibits growth at concentration of < 1μM? Since sulfa drugs have been implicated as inhibitions of DHFR (Sirawaraporn and Yuhavong, 1986); It is a possibility that sulfadoxine will inhibit DHFR and PAS might not. This possibility has to be further investigated.

In summary data presented here that *P. falciparum* incorporates exogenous [^{3}H] PABA into [^{3}H] folates and this incorporation can be inhibited by sulfadoxine and PAS. However, the concentration required for inhibition of PABA incorporation is much lower than the reported concentration for inhibition of DHPS. Further investigation of *P. falciparum* showed that the parasite is capable of *de novo* folate biosynthesis and at high concentration both drugs cause build-up of PABA, suggesting that both drugs inhibit DHPS in vivo, but do not inhibit PABA uptake or biosynthesis.

Figure 19. HPLC elution profiles of the DHPS assay components from extracts of *E. coli* (A and B) and *P. carinii* (C and D). Column flow is from the right to the left. A) *E. coli* extract, t = 0. The region where PABA eluted contained a total of 3,470 cpm, whereas the DHP region contained 434 cpm. B) *E. coli* extract, t = 1 hr. The region where PABA eluted contained a total of 1,849 cpm, whereas the DHP region contained 1,498 cpm. C) *P. carinii* extract, t = 0. The region where PABA eluted contained a total of 5,038 cpm, whereas the DHP region contained 122 cpm. D) *P. carinii* extract, t = 1 hr. The region where PABA eluted contained a total of 1,942 cpm, whereas the DHP region contained 430 cpm.

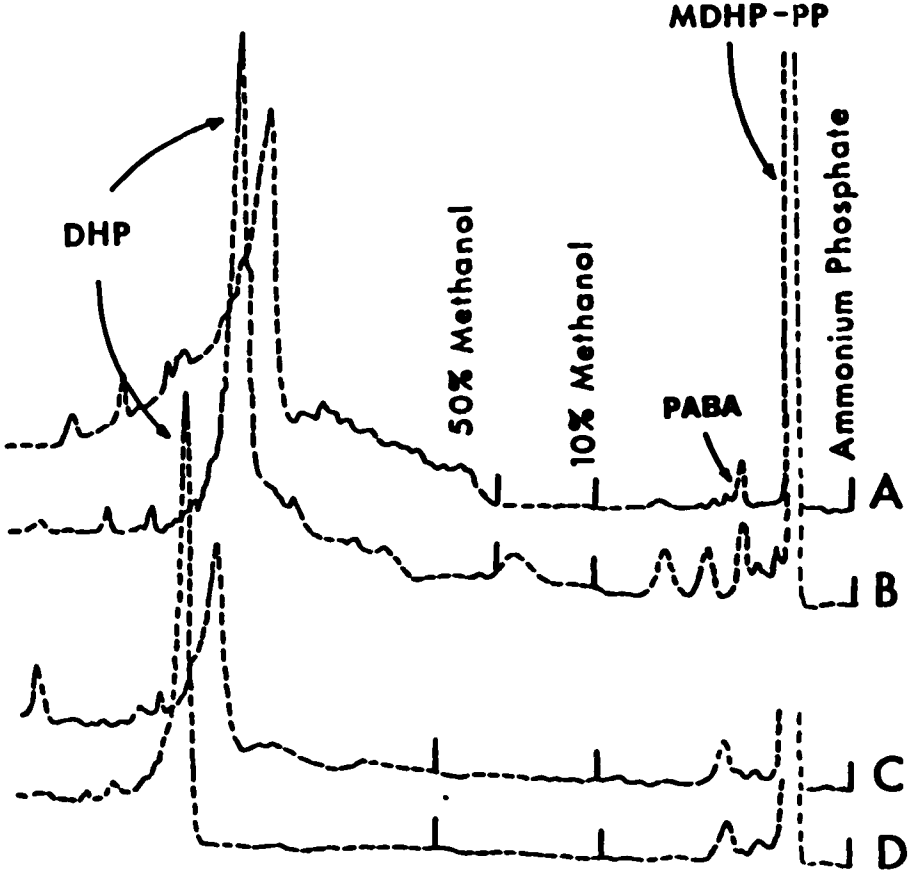


Figure 20. Dixon plot for dapsone (A) and Sulfamethoxazole (B)

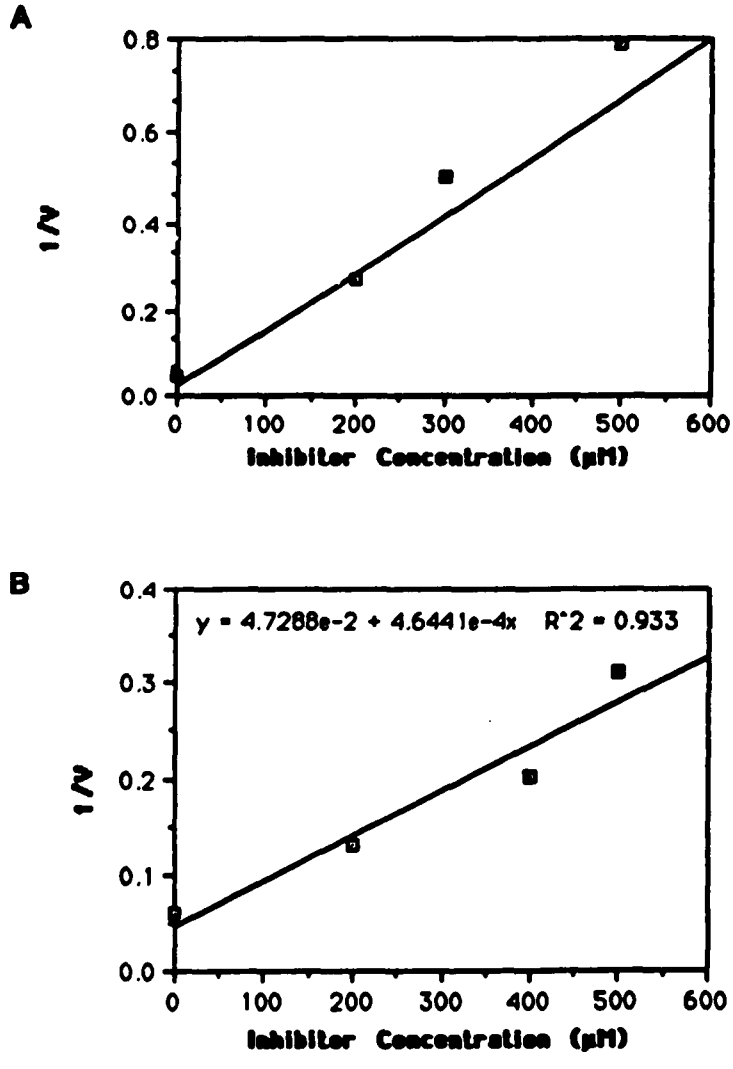
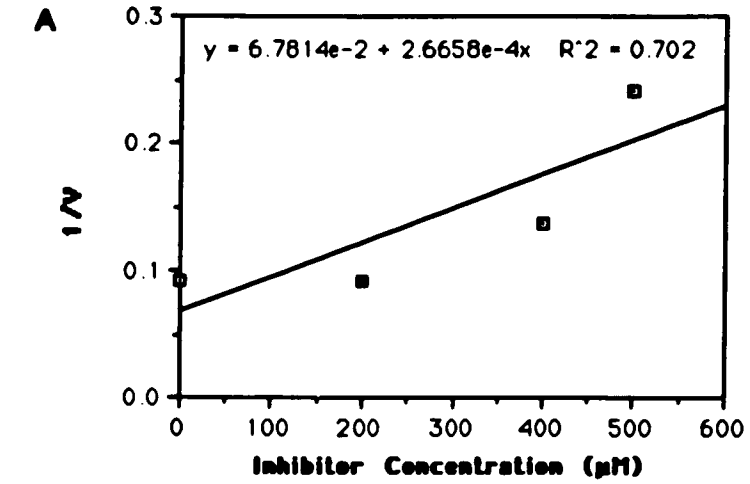
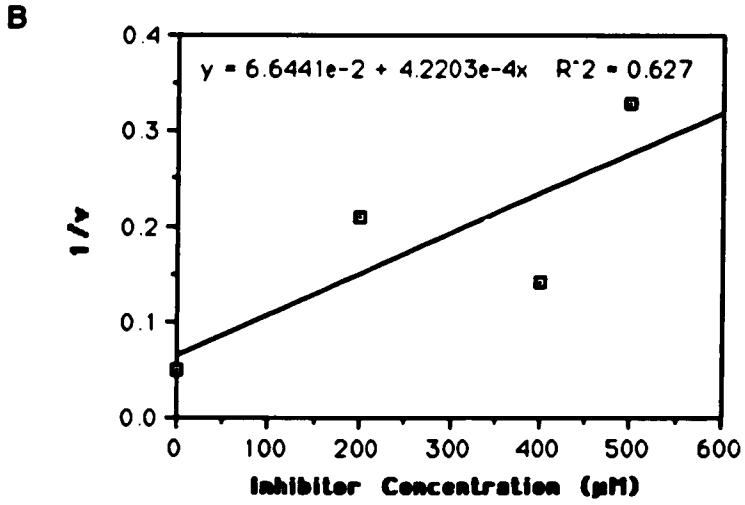


Figure 21. Dixon plots for sulfadoxine (A) and sulfaquinoxaline (B)



Ki = 148.5



Ki = 92

Figure 22. Linewever Burke plot for dihydropteroate synthase using PABA as a substrate

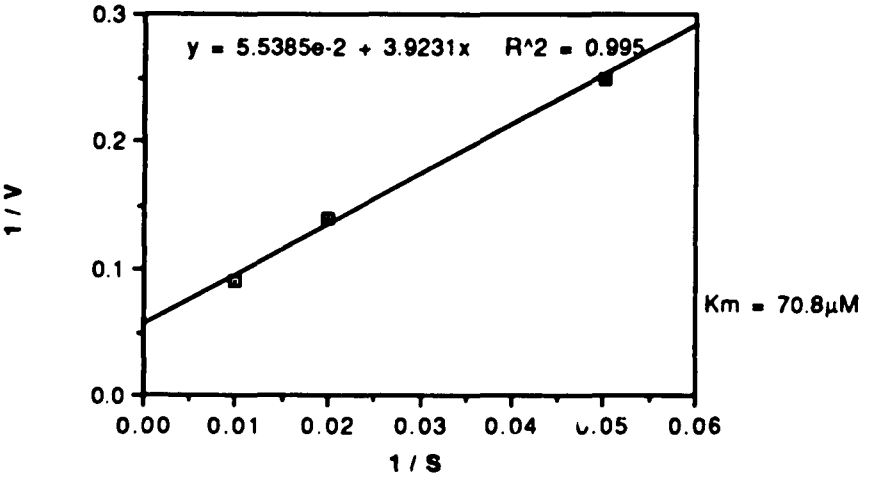


Figure 23. The growth curve of *P.carinii* showing the effect of 50 μM dapsone, sulfamethaxazole and sulfadoxine. The trophozoites were counted using Giemsa stain as detailed in the methods.

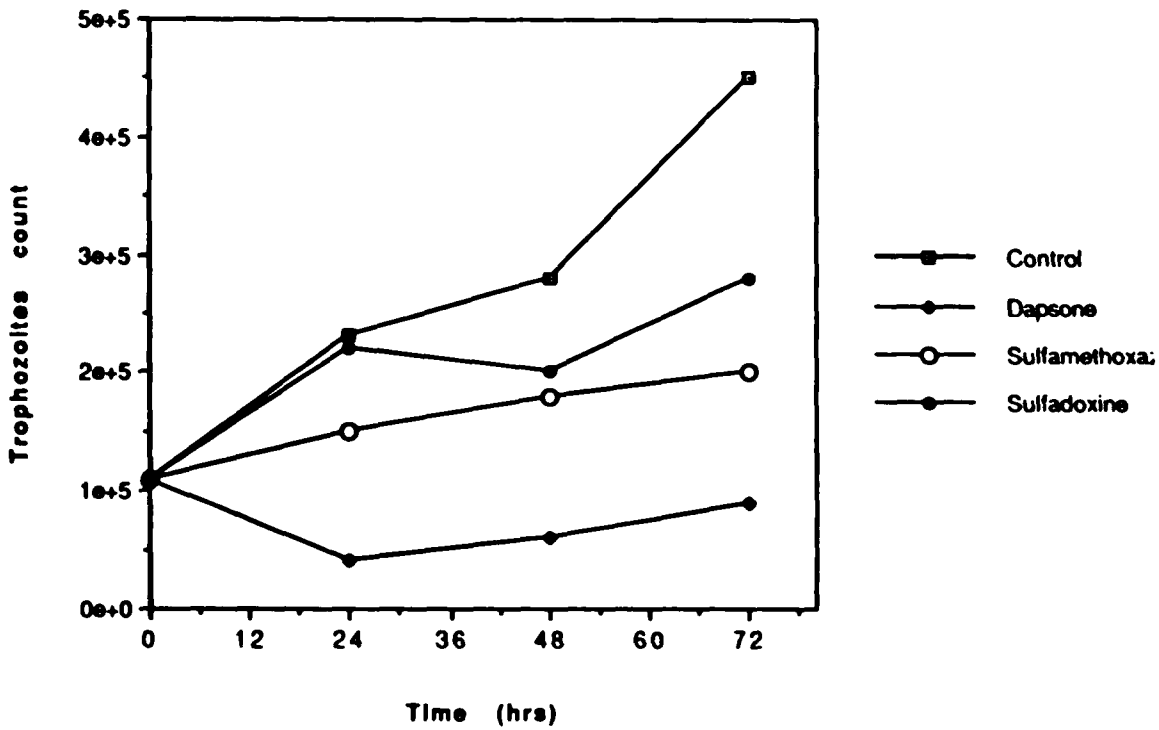


Figure 24. The effect of deferoxamine conjugated to hydroxyethyl starch (DFO-HES) on *P. carinii in vitro*. Presaturation with $FeSO_4$ reversed the inhibitory effect of DFO-HES. Percent inhibition expressed as a reduction in number of trophs in control well.

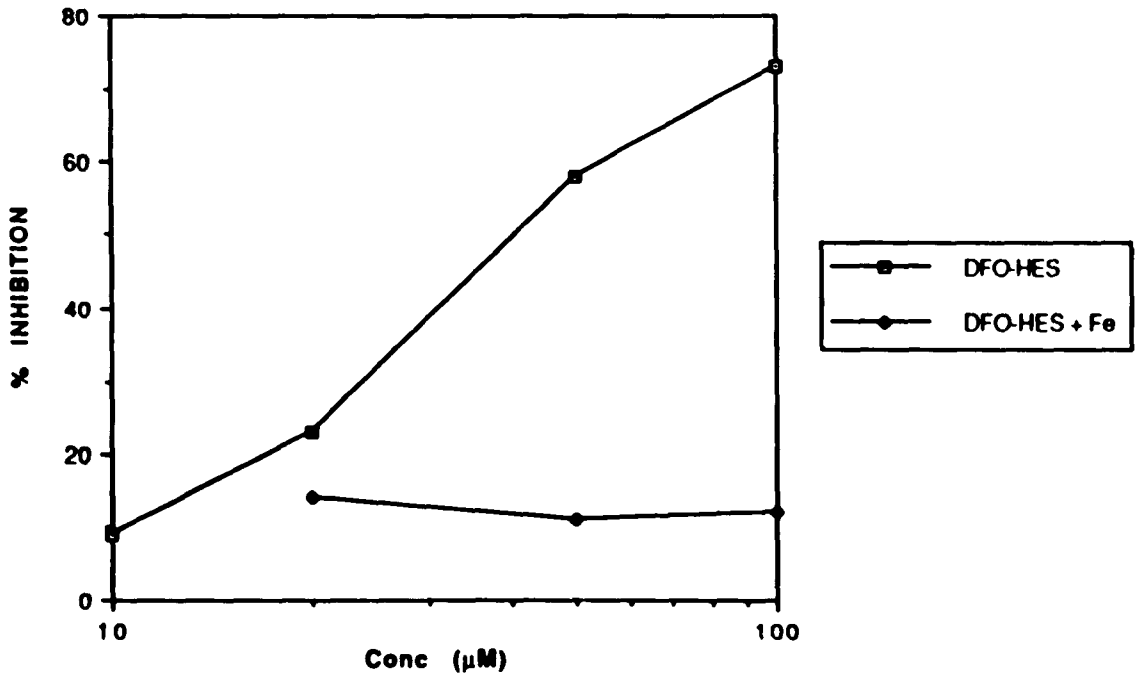


Figure 25. Growth of P. carinii in culture. Each point is the average of four experiment ± standard deviation.

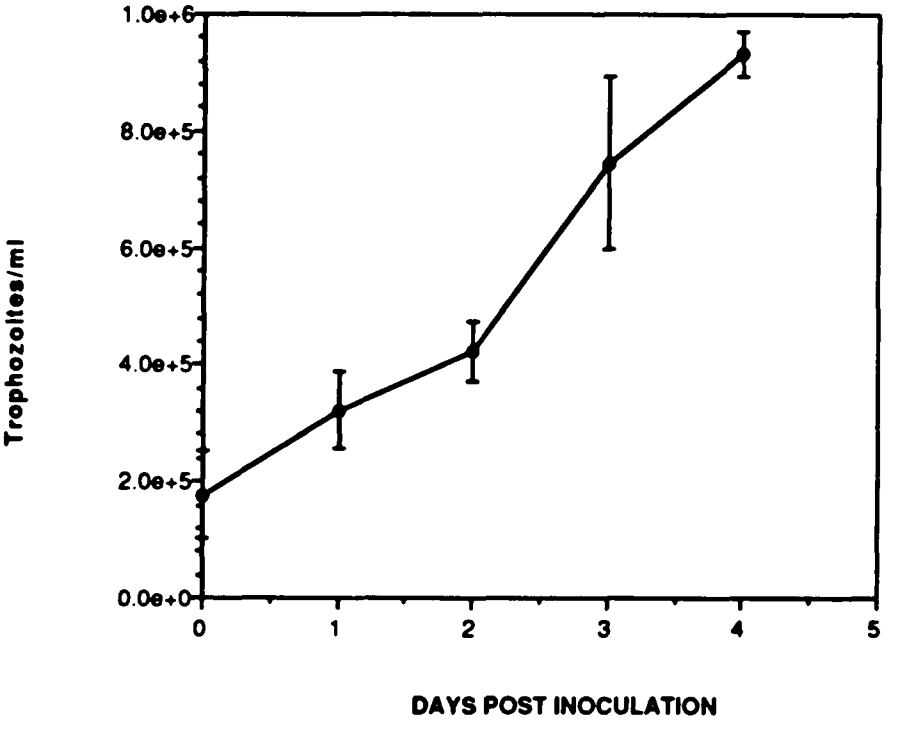


Figure 26. Growth of *P. carinii* in culture in the presence of 100 μM artemisinin, 15 μM pentamidine or no addition.

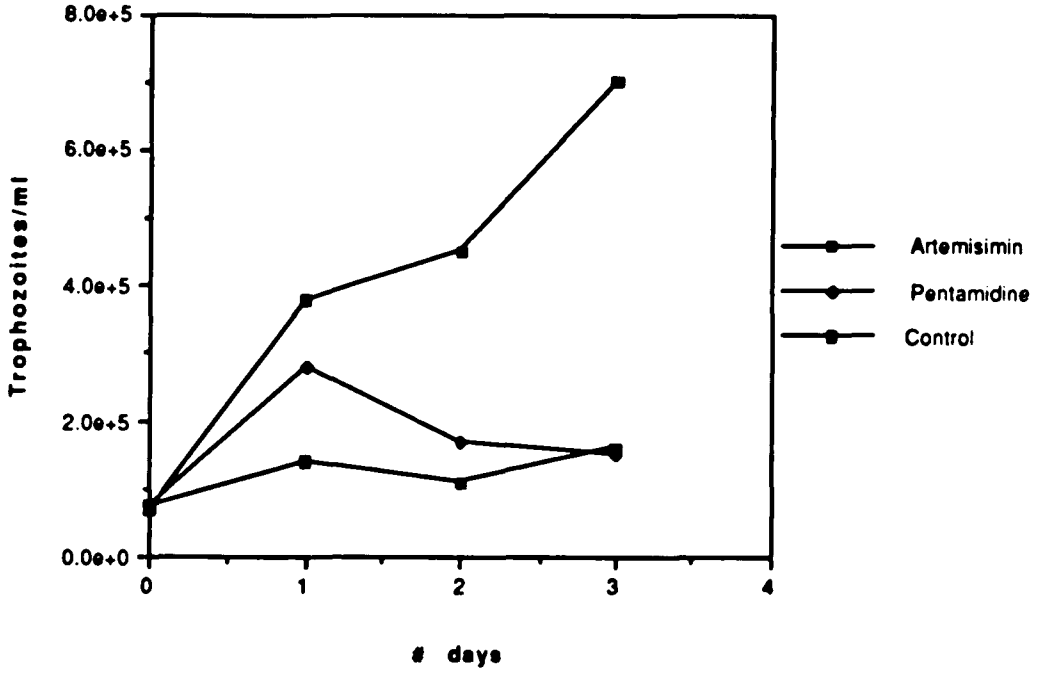


Figure 27. Inhibition of *P. carinii* growth in culture by various concentrations of artemisinin.

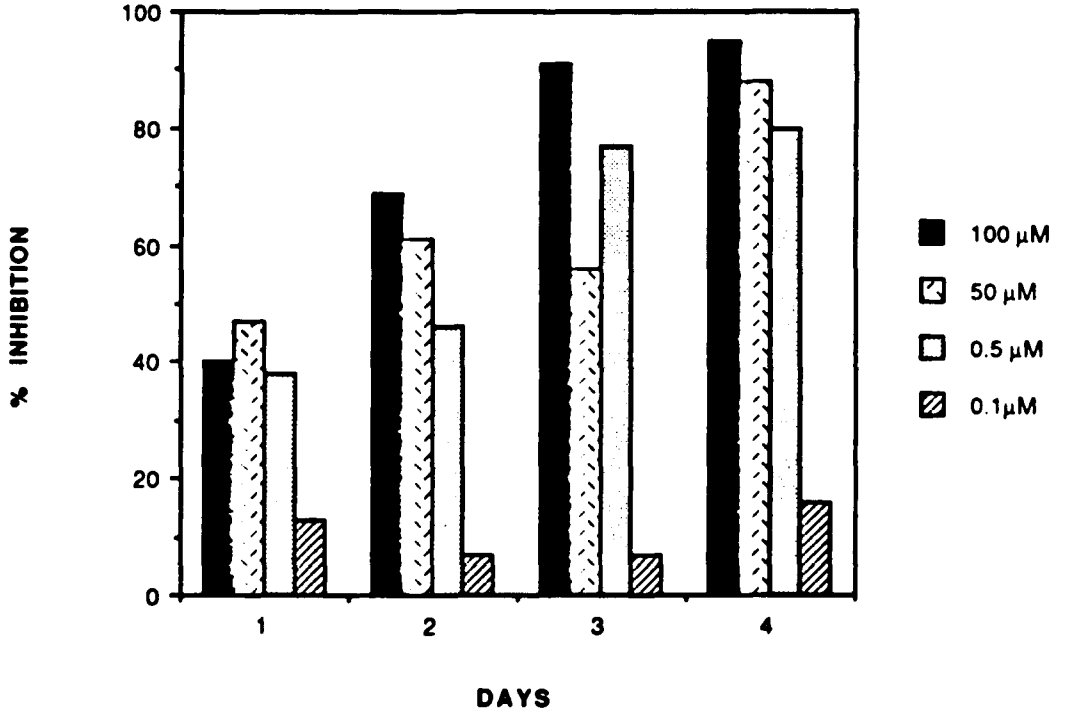


Figure 28. Inhibition of *P. falciparum* folate biosynthesis by various concentrations of sulfadoxine.

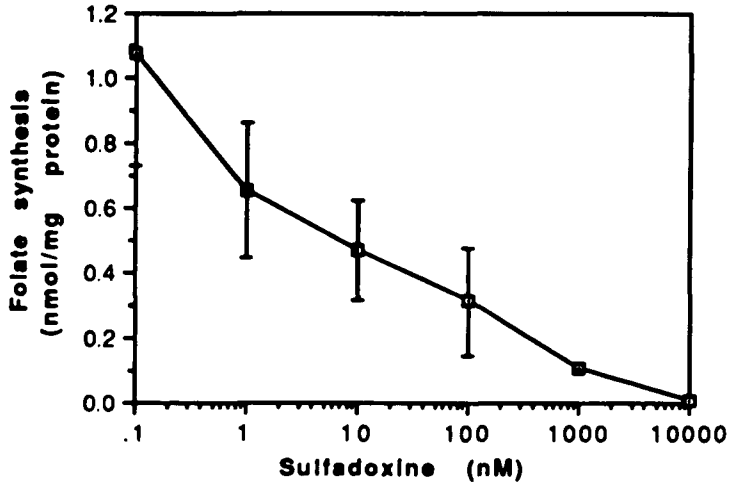


Figure 29. Inhibition of *P. falciparum* folate biosynthesis by various concentrations of PAS.

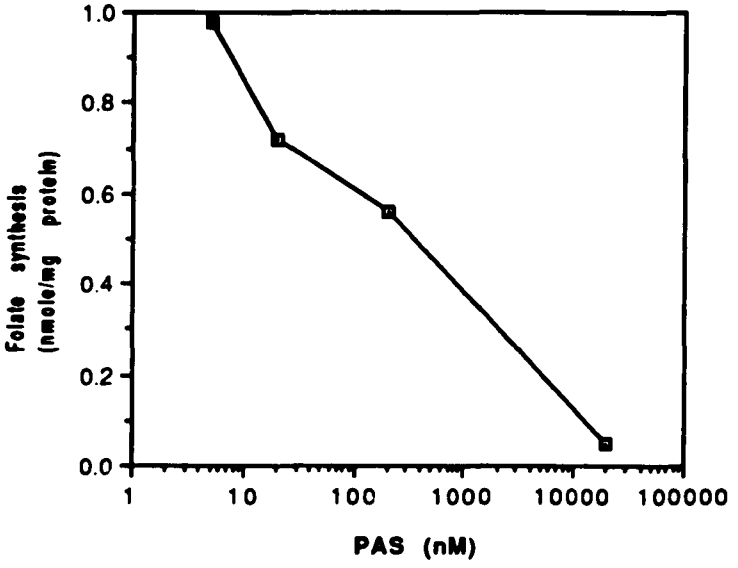


Figure 30. HPLC profiles of A. *Plasmodium falciparum* infected red cells B. Uninfected red cells. Both type of cells were incubated in PABA- and folate-free media for 48 hrs. The identified peaks are =PABA and 2=PHBA (intrenal standard)

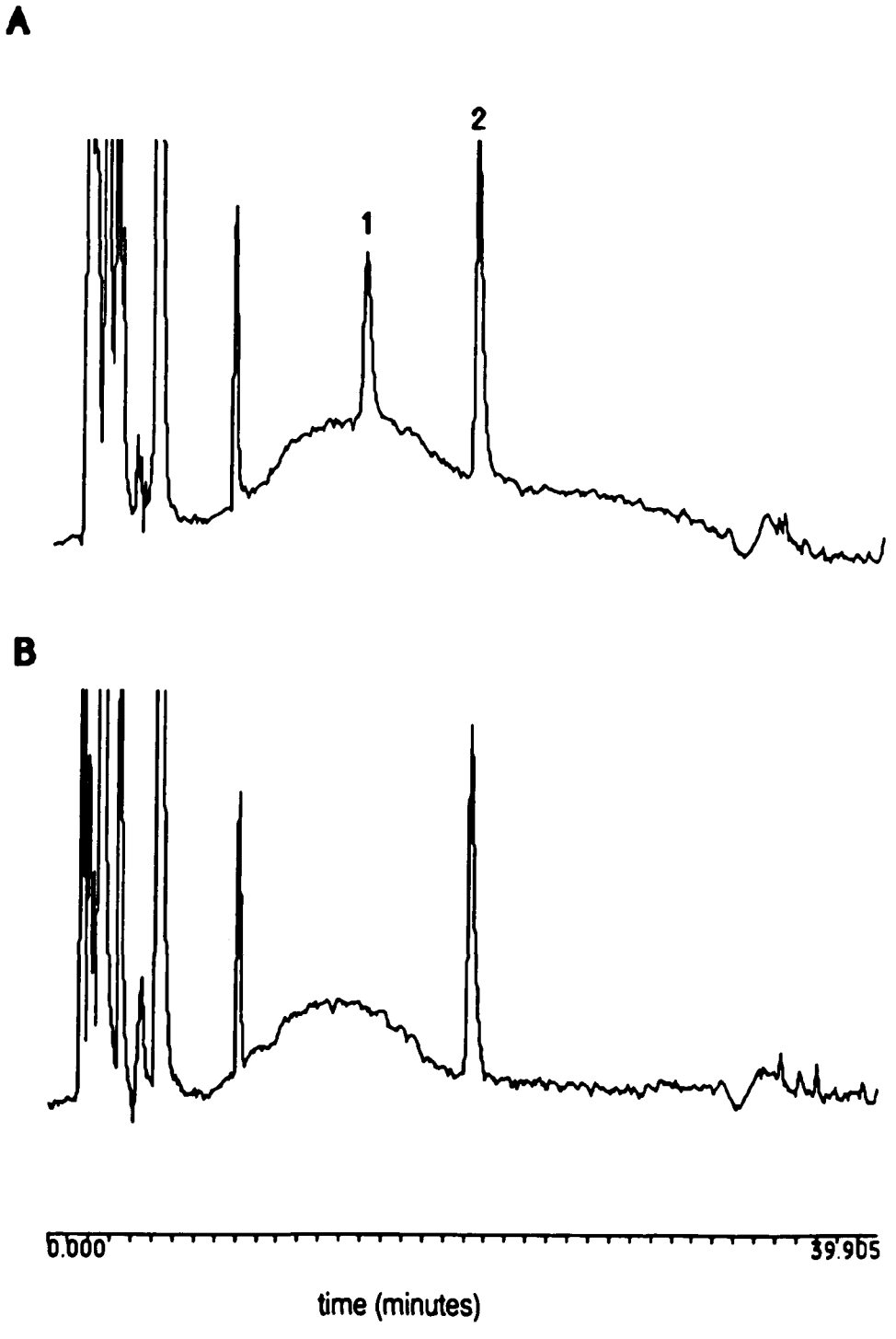


Figure 30. PABA contents of *Plasmodium falciparum* infected red cells (FCR 3) and uninfected red cells (RBC) in the presence and absence of PABA containing media. Bars represent means of 3 measurements ± standard deviations.

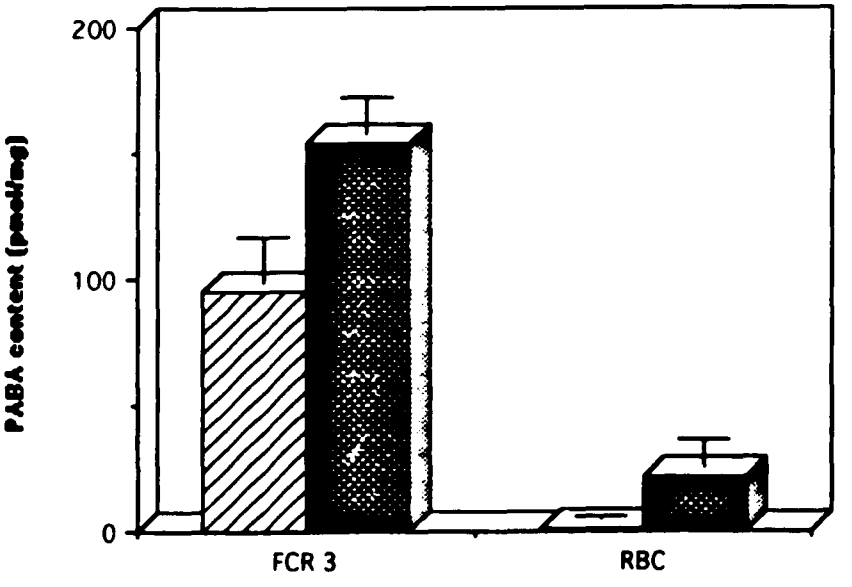


Table 2. Effectiveness of sulfones and sulfonamides as inhibitors of dihydropteroate synthetase activity.

Compound	IC_{50} (μM)	K_i (μM)
Dapsone	215	9
Sulfaquinoxaline	425	92
Sulfamethaxazole	440	59
Sulfadoxine	>500[a]	149
Sulfadiazine	>500[b]	---

[a] 42% inhibition at 500 μM
[b] 10% inhibition at 500 μM

Table 3. Effects of various drugs on [^{3}H] PABA incorporation by *P. carinii*

Drug	Conc(μM)	% Inhibition Trial 1	Trial 2	Average
Sulfamethaxazole	100	103	99	100
	10	92	90	91
	1	26	21	24
Sulfadoxine	100	94	94	94
	10	70	65	68
	1	18	12	15
Dapsone	100	100	100	100
	10	97	97	97
	1	78	70	74
Artemisinin	100	98	94	96
	10	101	99	100
	1	57	54	55
Pentamidine	100	98	94	96
	10	59	70	64
	1	27	8	17
Desferoxamine	100	100	75	87
	23	53	59	46

Table 4. Time course experiment for determination of linearlity of folate assay

Time (min)	PABA incorporation (fmol/mg proten)	# Cysts
0	0	50,000
24	1.25	57,175
36	1.17	69,250
48	1.97	97,100

Table 5. Comparison of the effects of sulfadoxine and PAS on PABA and hypoxanthine incorporation by *P. falciparum*

Drug	IC50 for PABA incorp.	IC50 for hypo-xanthine incorp*
Sulfadoxine	7 nM	357 ± 33 nM
PAS	300 nM	>1000 μM

* Results from Zhang (1992)

Table 6. PABA contents in wild type 3D7 and its mutant clones (D1, E10, B5, E6). The organisms were incubated in PABA- and folate-free media for 48 hrs. Average of duplicate experiment are shown.

Strain	PABA content (pmol/mg)		
	Exp 1	Exp 2	Average
3D7	25	19	22
D11	<1	<1	<1
E10	<1	<1	<1
B5	31	48	39.5
E6	2	2	2

Table 7. The effect of sulfadoxine and PAS on the PABA content of *P. falciparum*

	PABA content (pmol/mg protein)
Control	206 ± 28*
Sulfadoxine (100 μM)	304 ± 40
PAS (100 μM)	350 ± 53

* means ± S.D. (n=3)

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