INTRODUCTION

The problem of drug resistance has continued to plague efforts in malaria control and eradication since the development of resistance of both human and animal malaria parasites to antifolates was noted over thirty years ago, and resistance of *Plasmodium falciparum* to chloroquine was later observed (Doberstyn, 1984; Peters, 1985). Recently, the problem has been compounded by the rapid emergence of resistance of *P. falciparum* to pyrimethamine-sulphadoxine and the decreasing effectiveness of the conventional antimalarial quinine (Harinasuta et al., 1982). In order to deal effectively with this problem it is essential to know the biochemical mechanisms of resistance of various antimalarials. This in turn requires knowledge on mechanisms of action of these drugs. In this paper, attention will be selectively focused on biochemical aspects of resistance to pyrimethamine and chloroquine, as representatives of the antifolates and 4-aminoquinolines respectively.

Beale (1980) has considered a number of different mechanisms which can account for changes in drug sensitivity of various organisms: physiological adaptations, selection from mixed sensitive and resistant populations spontaneous nuclear mutation and subsequent selection of resistant mutants, induced mutation, mutation of extranuclear genes, changes in gene expression, and introduction of resistance transfer factors or other plasmids. Spontaneous gene mutation was considered as likely to be the most important mechanism for *Plasmodium* species, though others are also possible (Beale, 1980). Present evidence suggests that resistance to pyrimethamine or chloroquine is stably inherited, as a Mendelian character, and the presence of resistance-transfer factors has so far not been demonstrated. These mechanisms can lead to various means of biochemical expression of drug resistance. These include increase in target enzyme in the parasite so as to overcome inhibitory activity of the drug, alteration of the target enzyme leading to decreased affinity for the drug, decrease in drug uptake by the parasite, bypass of metabolic lesion by alternative metabolic pathway, and inactivation of the drug through parasite metabolism. Increase in specific activity of dihydrofolate reductase (EC. 1.5. 1.3), the target enzyme of pyrimethamine, was observed in pyrimethamine resistant *P. vinckei* and *P. berghei* (Perone, 1977) and also in resistant *P. falciparum* (Kan and Siddiqui, 1979). Alteration of dihydrofolate reductase, resulting in decreased sensitivity to inhibition by pyrimethamine, was shown to be the major basis for resistance in a *P. chabaudi* clone (Sirawaraporn and Yuthavong, 1984) and to accompany the enzyme specific activity increase in *P. vinckei* and *P. berghei* (Ferone, 1977). Decreased drug uptake is demonstrated by a number of chloroquine-resistant *Plasmodium* species (Macomber et al., 1966). A possible case of metabolic adaptation was shown in chloroquine-resistant *P. berghei*, in which haemoglobin catabolism was altered (Mahoney and Eaton, 1981), resulting in lack of accumulation of malaria pigment. Concrete examples of bypass of metabolic lesion are
missing, as also examples of drug inactivation by the malaria parasites as a means of expression of drug resistance.

ANTIFOLATES

Biochemical action of antifolates

Differences in folate metabolism between malarial parasites and their mammalian or avian hosts permit the use of antitumoroids as antimalarial agents. Although details remain to be investigated, it appears that, unlike the hosts, the parasites can synthesize folate cofactors de novo (Sherman, 1979). Recent studies, for example, indicated the presence of GTP cyclohydrolase (EC 3.5.4.16), the first enzyme in the folate pathway (Fig. 1) in *P. berghei*, *P. knowlesi* and *P. falciparum*, whereas this was absent in host erythrocytes (Krungkrai et al., 1985). Hence sulpha drugs are effective antimalarial agents since they block utilization of PABA by dihydropteroate synthetase (EC 2.5.1.15) resulting in depletion of dihydropteroate, an intermediate in the formation of folate cofactors. In addition to the de novo pathway, a folate salvage pathway may also exist in *Plasmodium* (Milhous et al., 1985).

The synthesis of tetrahydrofolate both in the parasites and the host must, however, proceed through the action of dihydrofolate reductase. The basis for chemotherapeutic effectiveness of antifolates such as pyrimethamine, trimethoprim and cycloguanil in malaria is the extremely tight binding of the drugs to the parasite enzyme as shown by correlation between the binding and in vivo activity (Ferone, 1977). The effect of these antifolates is potentiated when they are combined with PABA antagonists (Richards, 1966), probably largely due to simultaneous blockage of both utilization and synthesis of the folate cofactors respectively. Such combination also reduces the rate at which malarial parasites develop resistance to each component in the mixture, and when given together with mefloquine also delays development of resistance to any of the three (Peters, 1985). While the biochemical basis of linkage of resistance with polytherapy remains to be further investigated, the observation is in line with the location of resistance to these drugs on different genetic loci (Beale, 1980).

The biochemical action of antifolates may be attributed to their inhibitory effect on dihydrofolate reductase, which catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate. The latter serves as a cofactor for a number of one-carbon transfer reactions, particularly in the synthesis de novo of dTMP required for DNA synthesis (Ferone, 1977). Extensive comparisons have been made between dihydrofolate reductase from the parasites and the hosts. The enzyme from malarial parasites and other parasitic protozoa have molecular weights in the range of 100,000-200,000 while the mammalian and avian enzymes have molecular weight of approximately 20,000 (Ferone, 1977; Kan and Siddiqui, 1979; Sirawaraporn and Yutha-
Biochemical aspects of drug action in Malaria parasites

Vong, 1984). The enzyme from various malarial parasites and host erythrocytes have been shown to differ in molecular weights, $K_m$ values for dihydrofolate and NADPH, and other kinetic properties. The concentrations of pyrimethamine required for 50% inhibition of dihydrofolate reductase from $P. berghei$, $P. lophurae$ and $P. falciparum$ were less than those required for the enzyme from their corresponding hosts (erythrocytes from mice, ducklings and Aotus) by factors of 2000, 32 and 1200 respectively. These large differences in binding properties between the parasite and host enzyme probably account for the selective action of the drug on the parasites.

Further synthesis and utilization of tetrahydrofolate involves two other enzymes, serine hydroxymethyl transferase (EC 2.1.2.1) and thymidylate synthetase (EC 2.1.1.45) (Fig. 1). $^5$N, $^{10}$N-Methylene-tetrahydrofolate produced from tetrahydrofolate by the former enzyme is used in the methylation of deoxyuridylicate to form dTMP (deoxythymidylate) by the latter enzyme. Despite the importance of these two enzymes on folate metabolism, only a few studies on their characterization have been reported. Serine hydroxymethyltransferase has been studied only in $P. lophurae$ and $P. knowlesi$ (Platzer and Campion, 1976). The presence of thymidylate synthetase has been shown in various plasmodial species (Sherman, 1979). Detailed characterization of the enzyme from $P. berghei$ has been recently reported (Pattanakitsakul and Ruenwongsa, 1984; Pattanakitsakul et al., 1985). In this study, inhibition of thymidylate synthetase by FdUMP was antagonized by methotrexate, a potent inhibitor of dihydrofolate reductase.

A striking feature of enzymes of folate cofactors metabolism in malarial parasites and other protozoa is the association of dihydrofolate reductase with thymidylate synthetase, a phenomenon that has not been observed in mammalian or bacterial system.

Ferone and Roland (1980) reported the co-purification of both activities from $Crithidia fasciculata$ and $P. berghei$ on gel filtration and affinity chromatography. The bifunctional proteins have been further studied in $P. falciparum$, $P. lophurae$ and various species of flagellated protozoa, with the conclusion that this bifunctionality may be a common and unique feature of parasitic protozoa (Garrett et al., 1984). Association of the two enzymes from $P. berghei$ was also confirmed by Pattanakitsakul and Ruenwongsa (1984) through gel electrophoresis, although the two enzymes appear to have different stabilities on storage.

Synergism between antifolates and sulphur drugs

The synergistic action between antifolates and sulphur drugs has been of great practical use in malaria chemotherapy. However, little biochemical information, other than parallels drawn from studies on bacteria, is available on the mechanism of the synergistic action of these drugs in malaria. Since the sulphur drugs act on dihydropteroate synthetase, an enzyme located earlier in metabolic sequence to dihydrofolate reductase, the target enzyme for pyrimethamine and other antifolates, the sequential blockage by the drug combination is presumably the major basis for synergism observed. Theoretical studies (Harvey, 1982) have shown that synergism between inhibitors acting on different enzymes of a metabolic pathway can occur as a consequence of the non-linear nature of normal (Michaelis-Menten-Henri) enzyme kinetics, depending on the configuration of the pathway. The cyclic configuration of the folate pathway predictably leads to synergism between inhibitors of dihydropteroate synthetase and dihydrofolate reductase, and antagonism between inhibitors of dihydrofolate reductase and thymidylate synthetase (Harvey, 1982).
It is not known whether the degree of synergism, defined by the extent to which the observed inhibition of malarial parasite growth is greater than that expected from independent actions of, say, pyrimethamine and sulphadoxine, corresponds to that predicted from this theory of sequential blockage. An adequate explanation is, moreover, still missing for the effectiveness of the combination against parasites which are resistant to one or both of the components (Peters and Howells, 1978). In bacterial chemotherapy, the effectiveness of trimethoprim-sulphamethoxazole against sulpha-resistant organisms was noted by Poe (1976), who proposed that simultaneous binding of trimethoprim and sulpha by dihydrofolate reductase alone may form a molecular basis for the synergism. This proposal was disputed by a number of investigators. In particular, Baccanari and Joyner (1978) failed to observe any inhibition of the E. coli enzyme by sulphamethoxazole. Inspite of this controversy, simultaneous binding of two inhibitors on a single enzyme can in principle account for their synergistic action (Webb, 1965), and it may be worthwhile to examine this aspect in antimalarial synergism. We have recently found that pyrimethamine (0.03-1 nM) and sulphadoxine (0.1-2mM) exerted mutually potentiating inhibitory activity on partially purified dihydrofolate reductase from P. chabaudi (W. Sirawaraporn and Y. Yuthavong, unpublished observation). Although the sulphadoxine concentration required to observe this effect is rather high compared with the minimum effective concentration, it is within the range observed in malaria patients treated with this drug combination. However, pyrimethamine and sulphadiazine showed almost no mutually potentiating effect on inhibition of dihydrofolate reductase from P. chabaudi (W. Sirawaraporn and Y. Yuthavong, unpublished observation). In these limited examples, there is no obvious correlation between the in vivo effect of drug combination and the effect on dihydrofolate reductase activity.

**Resistance to pyrimethamine and its combination with sulpha drugs**

Ferone (1977) and other investigators found dihydrofolate reductase with increased specific activity in several pyrimethamine-resistant strains of P. vinckei and P. berghei. Increased enzyme activity was also found in pyrimethamine-resistant P. falciparum (Kan and Siddiqui, 1979). The specific activities for the rodent models are increased by 3-10 fold, while that for P. falciparum are increased by 30-80 fold. From active site titration with methotrexate, Ferone (1977) showed that the increase in specific activity of the enzyme from pyrimethamine-resistant P. berghei is due to increased enzyme catalytic sites, and not turnover number (activity of each site). It is unclear whether the increase in enzyme levels is due to gene amplification or altered regulation of gene expression. Coderre et al., (1983) showed that methotrexate-resistant Leishmania tropica, with 40-fold higher production of dihydrofolate reductase than the wild type, has amplification in specific regions of its DNA, and current evidence indicates that drug resistance in this case is a result of gene amplification. In view of some similarities in the structures and properties of the enzyme from various protozoa, i.e., Leishmania, Crithidia, Trypanosoma and Plasmodium, including its association with thymidylate synthetase (Garrett et al., 1984) it is possible that pyrimethamine resistance in Plasmodium is in some cases associated with dihydrofolate reductase gene amplification.

In addition to the increase in specific activity, dihydrofolate reductase from pyrimethamine-resistant P. vinckei and P. berghei also showed approximately 200 fold and 10-50 fold higher Kᵢ values (less affinity for binding) for pyrimethamine (Ferone, 1977). Moreover, while the inhibition is competitive...
with dihydrofolate for the enzyme from the sensitive strain, it is non-competitive for the enzyme from the resistant strain of *P. berghei*. Increase in the $K_i$ values were also found for cycloquanil and trimethoprim for the enzyme from resistant *P. berghei*. Although the degree of resistance observed *in vivo* to these drugs was greater than can be accounted for by the increase in $K_i$ values alone, this increase is probably an important factor contributing to the development of resistance. Additional features of the enzyme from resistant *P. vinckei* and *P. berghei* include approximately 10 fold increase in $K_m$ values for dihydrofolate, and decrease or lack of stimulation by potassium chloride. These changes in drug-binding and other kinetic properties of dihydrofolate reductase indicate structural alteration, possibly due to mutation in the structural gene for this enzyme. Both increase in the enzyme amount and change in enzyme properties imply that multiple genetic loci are involved in the development of resistance. The involvement of multiple genetic loci is possible considering the stepwise manner by which the resistance was achieved. However, the enzyme from resistant parasites obtained after a single course of pyrimethamine treatment also showed similar changes in both specific activity, and binding properties (Schoenfeld et al., 1974). Alternative explanations for these results are that these changes are linked and due to a single mutation, or that the single exposure resulted in selection of pre-existing resistant subpopulations. In view of the stability of pyrimethamine-resistant mutants and their neutral or slightly disadvantageous character comparing with sensitive parasites (Beale, 1980), it is possible that the sensitive and resistant subpopulations coexist in nature. Indeed, Thaithong et al. (1984) have shown that isolates of *P. falciparum* from Thailand consisted of heterogenous parasites with different sensitivities to chloroquine, pyrimethamine and pyrimethamine-sulphadoxine combinations. The increase in enzyme level and alterations in drug-binding affinity may indeed be separate characters of different resistant subpopulations, in which case the enzyme heterogeneity may be detectable through various methods of characterization and detailed kinetic analysis.

Some of the ambiguities discussed above should be removed through use of cloned parasites in the investigation. Sirawaraporn and Yuthavong (1984) have made a comparison between dihydrofolate reductase from a cloned strain of pyrimethamine-sensitive and a drug-resistant clone derived from it provided by the Edinburgh group. Both the specific activities and the turnover numbers of the enzyme from the two sources are comparable. There is therefore no significant difference in the amounts of the enzyme from both sources. The binding affinity of the enzyme from resistant parasites with pyrimethamine is, however, decreased by a factor of almost 200, and the inhibition is non-competitive with dihydrofolate as compared with competitive inhibition for the enzyme from sensitive parasites. The $K_m$ value for dihydrofolate of the enzyme from the drug-resistant parasites is increased by a factor of 4, and the enzymes from the two sources had different activity profiles with respect to pH and temperature, and different sensitivities to heat denaturation. The major basis for drug resistance therefore appears to be a structural change in the enzyme resulting in a large decrease in drug binding, and not an increase in enzyme content. It is also notable that the kinetic properties of thymidylate synthetase from the pyrimethamine-sensitive and resistant parasites were different in many respects (A. Ratanaphan and P. Ruenwongsa, unpublished observation), probably reflecting bifunctional nature of the thymidylate synthetase-dihydrofolate reductase.

In contrast, there is no essential difference between the kinetic properties of dihydrofolate reductase from pyrimethamine-sensitive
and resistant strains of *P. falciparum* (Kan and Siddiqui, 1979). An increase in enzyme content appears to be the only basis for drug resistance in this case. The various results obtained with studies on *P. falciparum* and rodent malaria models illustrate a range of biochemical mechanisms by which drug resistance may be achieved. As has been pointed out by Ferone (1977), other mechanisms are possible including increased substrate level, decreased folate cofactor requirement, utilization of folate end-products, decreased drug permeability and drug metabolism. These mechanisms still remain to be established in *Plasmodium* species.

There has been little biochemical information on the mechanisms of resistance of the malarial parasites to sulpha drugs or their combinations with folate antagonists. With *P. falciparum*, the problem of assessing sulpha resistance *in vitro* is complicated by the presence of high PABA content in the conventional medium used (Tan-Ariya and Brockelman, 1983; Milhous *et al*., 1985).

**4-AMINOQUINOLINES**

**Biochemical Action**

Chloroquine is the most well-known representative of the 4-aminoquinolines, which include drugs such as amodiaquine and quinacrine. The latter has an acridine ring system incorporating the quinoline ring structure. The quinolinemethanol drugs, quinine, quinidine and mefloquine, may share some aspects of their antimalarial action with the 4-aminoquinolines.

It has been proposed that quinoline antimalarials act by interacting with nucleic acids, DNA and RNA, primarily through intercalation (Hahn, 1974), thus disrupting the process of replication, transcription and subsequent steps in gene expression. These drugs, especially chloroquine, quinacrine, quinine and quinidine, were spectroscopically shown to bind DNA with apparent association constants of $10^5-10^6 \text{M}^{-1}$. These values are now considered too low to support an effective chemotherapeutic concentration of $10^{-7}-10^{-8} \text{M}$. Besides, mefloquine (Davidson, 1975) and some of the newer synthetic quinoline and phenanthrenemethanols prepared by the Walter Reed group give difference spectra that are extremely small compared with those obtained with the preceding drugs in the presence of DNA (B. Panijpan, unpublished results). It thus appears that drug action through DNA binding may not be tenable because mefloquine, which is effective against strongly chloroquine-resistant parasites, binds much less strongly and extensively. Other evidence against DNA as the primary target and site for these drugs' action is that the drugs do not seem to prefer the parasite DNA over that of the host enough for differential action. Yet another facet of antimalarial action of these drugs that is difficult to explain by DNA binding is the stage specificity of the drugs. All are blood schizontocides and kill the parasites only at the asexual blood stage. The liver-stage parasites, sporozoites and gametocytes are not killed by chloroquine at the normal therapeutic concentration. Other biochemical actions of chloroquine have been reported in relation to its possible mechanisms of action. Its inhibition of parasite-specific protease (Gyangeta *et al*., 1982), and of lysosomal phosphotipases (Matsuzawa and Hostetter, 1980) have been noted. Recently, inhibition of the digestion of endocytic vesicles containing host cell cytoplasm was suggested to be one of the first effects of chloroquine on intraerythrocytic (Yayon *et al*., 1984). How important these separate mechanisms are, remains to be determined further.

In the digestion of haemoglobin of the host cytoplasm ingested by the blood-stage parasite, the haem moiety, which is not covalent-bonded to the protein, eventually ends up in the food vacuole of the trophozoite as the malaria pigment, haemozoin. The haem
containing remnants of vacuolar haemoglobin digestion are expelled from the parasite at the mature schizont stage as part of the residual body.

The following phenomena have been observed when malaria-infected erythrocytes are treated with chloroquine. The drug accumulation capacity of *P. berghei*-infected mouse erythrocytes increases with the maturation of the parasites. At therapeutic drug concentration ($10^{-7} \text{M}$), chloroquine is principally accumulated in the parasites, mainly the food vacuole (Aikawa, 1972) while small but significant fractions are associated with the host membrane and host cytoplasm (Sirawarapornt *et al.*, 1982). The role of the host membrane is interesting since protease treatment of uninfected erythrocytes leads to a large increase in chloroquine accumulation capacity, leading Fitch *et al.*, (1978) to suggest that the binding site for chloroquine is intrinsic to the erythrocyte membrane. However, the distribution of chloroquine in the membrane and lysate fractions are different for protease-treated and malaria-infected erythrocytes, and become similar only when the latter are further treated with protease (Yuthavong, 1980). The membrane-binding site for the drug, exposed on protease treatment, was later shown by Chou *et al.*, (1980) to be ferriprotoporphyrin IX released from haemoglobin. Concurrently, Jearnpipatkul *et al.*, (1980) proposed from spectroscopic evidence that ferriprotoporphyrin IX of the haemozoin should be the site of binding, and perhaps action of chloroquine. Analysis for ferriprotoporphyrin IX present in the parasite gave an over 10:1 pigment to chloroquine molar ratio (Chou *et al.*, 1980).

Examples have been given of erythrocytes infected with drug resistant parasites being less capable of accumulating chloroquine than those infected with drug sensitive strains (Fitch, 1969), although this is not a universal phenomenon. Also, in some lines of *P. berghei*, there is a correlation between chloroquine sensitivity and the amount of haemozoin pigment present in the parasite (Ladda and Sprinz, 1969). The hemozoin pigment has been shown to contain mainly haemoprotein complex and ferriprotoporphyrin IX aggregates (Yamada and Sherman, 1979; Balasubramanian *et al.*, 1984). A recent study (Yayon *et al.*, 1984) using Mossbauer spectroscopy, however, shows that the iron in the malarial pigment of *P. berghei* is different from other known porphyrin iron compounds. An interesting phenomenon arising from chloroquine treatment is the light microscopic observation of the clumping of malaria pigment in the parasite's food vacuole (Warhurst and Thomas, 1975). This pigment clumping may be inhibited by quinolinemethanol drugs. It appears that pigment clumping may be inhibited by quinolinemethanol drugs. It appears that pigment clumping involves fusion of pigment-containing vacuoles and concentration and/or precipitation of pigmented materials. This occurrence is probably an energy-dependent step.

The more favoured binding site of chloroquine appears to be the ferriprotoporphyrin IX part of the haemozoin pigment (Balasubramanian *et al.*, 1984), although a different site, i.e., a parasite protein has been proposed (Yayon *et al.*, 1984). It has been shown that ferriprotoporphyrin IX and protoporphyrin IX (no Fe) bind chloroquine, quinine, quinacrine and mefloquine well (Jearnpipatkul and Panijpan, 1980). The binding constant of ferriprotoporphyrin IX chloroquine complex is in the order of $10^8 \text{M}^{-1}$ (Chou *et al.*, 1980). The major binding mode is most likely to be stacking of the drug and the aromatic rings (Jearnpipatkul and Panijpan, 1980; Panijpan *et al.*, 1983; Blauer and Ginsburg, 1982; Moreau *et al.*, 1982); chelation between side-chain nitrogen and Fe (III) of ferriprotoporphyrin IX (Warhurst, 1981) should not be the major mode of binding because chlo-
roquine ferriprotoporphyrin IX complex can exist at pH 5.9 when the drug is doubly protonated. A recent study in non-aqueous solution also demonstrated the unlikelihood of coordination of nitrogen residues of quinine, and raised the possibility of involvement of the alcohol group in the alkoxide form (Behere and Goff, 1984). The stoichiometry of drug: ferriprotoporphyrin IX is 1:2-3 (Panijpan et al., 1983). Smaller molecular aggregates of ferriprotoporphyrin IX can interact with membranes and lyse the parasite and the erythrocyte (Fitch et al., 1982; Panijpan and Kantakanit, 1982; Kirschner-Zilber et al., 1982). Known phospholipids and lipids extracted from erythrocytes have been shown to interact with ferriprotoporphyrin IX (Panijpan and Kantakanit, 1982). Erythrocyte lipids also bind well with chloroquine ferriprotoporphyrin IX complex. In addition, mitochondrial preparations also undergo lysis in the presence of ferriprotoporphyrin IX alone or plus chloroquine (B. Panijpan, unpublished results). Oxygen is important for the lysis in that its absence lowers haemolysis by about 50%. Ferriprotoporphyrin IX-induced erythrocyte lysis is enhanced in the presence of chloroquine, quinine, mefloquine and some membrane-binding agents (B. Panijpan, unpublished results). Quinoline, hydroxyquinolines and quinolines with anionic side-chains do not give any enhancement to the lysis. Perhaps ferriprotoporphyrin IX and ferriprotoporphyrin IX-drug complex form channels in the lipid bilayer and facilitate efflux and influx of ions and water (similar to an osmotic effect) to cause cell disruption. No resealing of erythrocyte ghosts has been observed with ferriprotoporphyrin IX-induced lysis, unlike the case of mild hypotonic lysis. The membrane damaging effect of ferriprotoporphyrin IX-drug complex has also been observed when it is inside the erythrocytes (Chou, 1980).

One may deduce from the above observation that ferriprotoporphyrin IX drug complex and ferriprotoporphyrin IX have a membrane damaging effect (Fig.2). Perhaps the first membrane of the sensitive parasite that is damaged in the presence of chloroquine is the autophagic vacuolar membrane. The mechanism probably involves protease digestion of methaemoglobin, releasing ferriprotoporphyrin IX which would be rapidly precipitated by the low pH, or sequestered by some molecules, probably soluble macromolecules. The presence of chloroquine would make newly released ferriprotoporphyrin IX more soluble at acid pH, less available for sequestration and shift the equilibrium of ferriprotoporphyrin IX from the sequestered state resulting in longer lasting ferriprotoporphyrin IX forms that can cause membrane damage. It is thought that sequestering agent is probably proteinaceous in nature because serum albumin and other proteins have been shown to bind ferriprotoporphyrin IX and ferriprotoporphyrin IX-chloroquine complex (Banyal and Fitch, 1982). This suggestion is reminiscent of the hypothesis by Laser et al., (1975), on the role of quinoline and acridine drugs in counteracting the
inhibition by protein of fatty acid induced lysis. The vacuolar membrane damage may lead to imbalance in pH and ions in the vacuole resulting in the starvation of the parasite because digestion of haemoglobin and metabolism of other substances may not take place normally. Based on the foregoing discussion, a model for chloroquine action can be proposed as in Fig. 2.

Resistance to 4-aminoquinolines and related drugs

It can be seen that, although the above mechanism of quinoline drug action offers new candidates for the primary binding site and target of drug action, some difficulties still remain to be explained. It is easy enough to imagine a simple factor that explains sensitivity and resistance to the same drug, e.g., parasite having more and less pigment, or variable amounts of sequestering agent. Cross resistance, e.g., resistance to chloroquine is usually associated with more resistance not only to amodiaquine, a 4-aminoquinoline, but quinine, a quinolinemethanol, as well. This can be explained simply on the basis of similarity in the drug structure and the binding site structure. But, what is the explanation for sensitivity to one drug and resistance to another in the same series, also frequently observed?

In the parasite vacuole, where the pH is presumed to be below 5, chloroquine is probably doubly protonated, but quinine is less protonated and mefloquine singly protonated because the latter’s ring nitrogen is not protonatable down to pH 2, probably due to steric hindrance of the two -CF3 groups and the electron withdrawing effects of these groups as well. If uptake of the drugs into the infected erythrocytes and the parasite and the subsequent protonation of the drug molecules needs energy, then it can be seen that quinolinemethanols, being less protonated and more hydrophobic, should be taken up more easily by the parasite. Washing out of radioactive chloroquine and mefloquine previously taken up by P. berghei in the presence of azide and 2,4-dinitrophenol was more easily done by buffer for the case of chloroquine than for mefloquine (W. Sirawaraporn, unpublished results).

Another difference in the properties of the 4-aminoquinolines such as chloroquine and quinolinemethanols such as mefloquine is the ability to bind to hydrophobic sites. Mefloquine by itself binds to lipids much better than chloroquine does (Chevli and Fitch, 1982; San George et al., 1984). As mentioned earlier, chloroquine can bind better to the membrane lipids in the presence of ferriproto­porphyrin IX. This differential lipophilicity of the drug types may partially explain differences in antimalarial action because mefloquine would be bound to the lipid membrane longer and take longer time to be cleared from the living system and may even penetrate the hydrophobic pockets of the haemozoin pigment better.

The release of at least some of the haem from haemoglobin and the conversion of this haem (Fe II) to ferriproto­porphyrin IX (Fe III) in the presence of oxygen would lead to reduction of O2 to superoxide, which can undergo reactions to give H2O2 and singlet oxygen. Two malaria parasites have been shown to have superoxide dismutase (EC 1.15.1.1) (Suthipark et al., 1982), whose catalytic activity is to dismute O2 to H2O2 and H2O and thus may minimize the deleterious effects of O2 and other active oxygen species. Some oxidant drugs have been shown to be effective antimalarials (Clark and Hunt, 1983; Pfaller and Krogstad, 1983). Perhaps the chloroquine-ferriproto­porphyrin IX complex may have enhanced oxidising potential also. It should be pointed out that micro­scopically visible pigment particles are large precipitates and pigment clumps may be larger ones still. Therefore, fine pigment, not easily visualized, may still exists as small particles
scattered out in numbers large enough for binding chloroquine and render drug sensitivity to the parasite. A quantitative estimation of by Mössbauer spectra, for example, showed that there was a similar amount of pigment iron in chloroquine-resistant as in chloroquine-sensitive *P. berghei* (Yayon et al., 1984). Difference in drug sensitivity therefore not simply related to either visible or total amounts of the haemoglobin pigment.

It should be remembered that membrane-directed drug action on the parasite can take place on the erythrocyte membrane, parasite membrane, parasitophorous membrane, the food vacuole membrane and other membranes. Differential resistance to 4-aminoquinolines and quinolinemethanols may reflect many possible differences in the properties of these drugs and their interaction with ferriprotoporphyrin IX in the haemoglobin. Drug uptake distribution, deposition and degradation, have all to be considered.

**CONCLUSION**

While there is little doubt that dihydrofolate reductase of the malarial parasite is the target enzyme for the action of pyrimethamine and other antifolates, other aspects of the folate metabolism of significance to chemotherapy still remains to be investigated. Outstanding among these are the mechanisms of drug synergism based on disturbance of this pathway, unique parasite enzymes in the early steps of the pathway, and the possibility of parasite utilization of the host folate cofactors. Molecular and genetic studies on the bifunctional dihydrofolate reductase-thymidylate synthetase also may yield unique insights for chemotherapy. The mechanism of resistance to antifolates have, like in other microorganisms, been shown in the malarial parasite to be of diverse types, each possibly requiring a different approach in surveillance and remedy. Work in the future should concentrate on resistance to both individual drugs and drug combinations like pyrimethamine-sulphadoxine. The linkage between emergence of resistance and use of drug combinations is also an aspect worthy of indepth studies at the molecular genetic level.

Unlike antifolates, the mechanism of action of chloroquine, other 4-aminoquinolines and related drugs is still a subject of considerable uncertainty, despite the fact that much progress has been made in the past few years. It is likely that the drug-ferriprotoporphyrin IX and drug-haemoglobin interaction is relevant to the drug action mechanism, possibly leading to subsequent damage to membranous structures. The sites of this damage, and their relative importance in the killing process, are still not known. Moreover, effects of chloroquine other than those arising from these interactions may contribute to the therapeutic effect. With these uncertainties still to be clarified, the mechanisms of resistance to chloroquine, not surprisingly, still remains to be elucidated. The stepwise nature of the increasing in resistance already suggests multiple mechanisms. The avoidance of lesions through altered metabolic pathway involving degradation of haemoglobin and formation of haemoglobin, or through prevention of complex formation with the drug, can conceivably arise through many mechanisms.

**SUMMARY**

Biochemical aspects of action of antifolates and 4-aminoquinolines and their resistance in the malaria parasites are reviewed, with emphasis on pyrimethamine and chloroquine respectively. Resistance to pyrimethamine has been shown to be associated with either an increase in the amount of parasite dihydrofolate reductase or a reduced affinity of the enzyme for drug binding, in line with the presence of a distinctive pathway for folate metabolism. The theories for drug synergism in the folate pathway are discussed with
respect to resistance to pyrimethamine and its combination with sulpha drugs. The biochemical basis for chloroquine resistance is still unclear, reflecting incomplete understanding of its mechanism of action. Data implicating the role of haemozoin and other components as a putative chloroquine receptor of the parasites are reviewed, and possible explanations for resistance are discussed.

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REFERENCES


FERONE, R. and ROLAND, S., (1980). Dihydrofolate reductase: thymidylate synthetase,


