THE EFFECTS OF CHLOROQUINE-RESISTANT AND CHLOROQUINE-SENSITIVE STRAINS OF <u>PLASMODIUM BERGHEL</u> ON RODENT HEPATIC DRUG-METABOLIZING ENZYMES

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ABSTRACT

Title of Dissertation: Effect of chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium berghei* on rodent hepatic drug-metabolizing enzymes Guan-Hong Song, Doctor of Philosophy, 1993 Dissertation directed by: R. Andre, Professor, Department of Preventive Medicine and Biometrics

Malaria is one of the most important health problems in the world. The emergence and rapid spread of chloroquine resistance in malaria parasites is a major contributing factor to the failure of malaria control programs and to the disease's recent worldwide resurgence. The mechanisms for chloroquine resistance have not been fully elucidated, although several hypotheses have been proposed in the last 30 years.

The present study determined the effect of infection with chloroquine-resistant (RC strain) or chloroquinesensitive (N strain) *P. berghei* on cytochrome P450-dependent enzyme activities, growth rate, and virulence. Mean patent period of infection with the N strain was 7.28 days, and with the RC strain was 18.67 days. Mortality caused by the N strain was 100% and that by the RC strain only 2.50%. The chloroquine-resistant parasites grew more slowly and were less virulent than the chloroquine-sensitive parasites. Infection with the N strain decreased total hepatic cytochrome P450 content and benzo(a)pyrene hydroxylase activity of the uninfected controls by 37% and 40%,

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respectively (P<0.01). There were no significant differences in these measures between mice infected with the RC strain and uninfected mice. In contrast, infection with the RC strain increased total activity of benzphetamine Ndemethylase by 83% over infection with the N strain and by 68% over uninfected controls (P<0.01). However, there was no significant difference in the N-demethylase activity between livers of mice infected with the N strain and uninfected mice.

In summary, infection with chloroquine-resistant *P. berghei* enhanced the N-demethylase activity, but did not alter cytochrome P450 content. Chloroquine is primarily metabolized by cytochrome P450-dependent N-dealkylase in humans and other mammals; therefore, the enhanced cytochrome P450-dependent N-dealkylation, in hosts infected with drug-resistant malaria parasites, may enhance the rate of metabolism of chloroquine, and thus reduce the drug pressure faced by chloroquine-resistant malaria parasites. This may be a mechanism of chloroquine resistance in malaria parasites.

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THE EFFECT OF CHLOROQUINE-RESISTANT AND CHLOROQUINE-SENSITIVE STRAINS OF *Plasmodium berghei* ON RODENT HEPATIC DRUG-METABOLIZING ENZYMES

By

Guan-Hong Song

Dissertation submitted to the Faculty of the Department of Preventive Medicine and Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1993

DEDICATION

To my wife, Miao-Fang Zhang, who joined me and gave me much physical and moral support as a virtuous assistant during my course of study toward my Ph.D. degree. She even abandoned her own academic pursuits and career to support my study.

To my two daughters, Wei Song and Juan Song, who were forced to lived on their own when they were teenagers while their parents completed their education.

I would not have been able to finish my study and be awarded the Ph.D. degree without the whole-hearted support of my wife and my daughters. The dissertation and Ph.D. degree are due in large part to them.

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ABBREVIATIONS

The following abbreviations will be used in the text:

BPD - benzphetamine N-demethylase

- BPH benzo(a)pyrene hydroxylase
- CaM calmodulin
- CoA coenzyme A
- FP ferriprotoporphyrin IX
- IE irradiated erythrocytes
- IR irradiated chloroquine-resistant strain of
 P. berghei
- IS irradiated chloroquine-sensitive strain of
 P. berghei
- 3-MC 3-methylcholanthrene

MDR - multidrug resistance phenotype

MFOS - microsomal mixed function oxidase system

N strain (SS) - chloroquine-sensitive strain of

P. berghei

NADPH - reduced form of nicotinamide adenine dinucleotide-phosphate

PB - phenobarbital

PBS - phosphate buffer saline solution

pfmdr - multidrug resistance-like genes contained in
 P. falciparum

Pgh - the pfmdr protein product

RC strain (RS) - chloroquine-resistant strain of *P. berghei*

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SE - standard error

SGOT - serum glutamic-oxalacetic transaminase

SGPT - serum glutamic-pyruvic transaminase

UC - uninfected mice (control)

INTRODUCTION

A. Human Malaria and Its Importance in Human Life:

1. Impact of Malaria on Human Life: Malaria is caused by mosquito-borne parasites. The parasites causing human malaria are Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale. In general, P. falciparum is most prevalent in the tropics and subtropics. It causes the most dangerous form of malaria, and remains one of the greatest killers of the human race when not treated promptly. In a non-immune population, the death rate during an epidemic may exceed 10% or be as high as 50% in limited areas. In Africa, more than 25% of the children who contract falciparum malaria die (U. S. Agency for International Development, 1985). Plasmodium vivax is a less pathogenic species and has nearly worldwide distribution between summer isotherms of 16-20 °C in the northern hemisphere and 20 °C in the southern hemisphere. Vivax infections predominate in temperate regions.

The importance of malaria as a cause of human morbidity and mortality exceeds that of any other parasitic disease. Over 40% of the world population, or 2.17 billion people, remain exposed to varying degrees of malaria risk in 99 countries. The global incidence of malaria is estimated to be nearly 120 million clinical cases each year, with nearly 300 million people carrying the parasites. The World Health Organization reported in 1991 an annual death rate of 800,000 for children in the African region (WHO, 1992).

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Through its direct impact on health and its indirect effects on economic development, migration and military conflict, malaria has played and continues to play an important role in human life. It not only makes a notable impact on the resident population in endemic areas but also impacts on the non-immune vistor (e.g., military troops and travellers). In World War I, more than 100,000 British and French troops on the Macedonian front were sidelined due to malaria. The U.S. Army recorded over 500,000 malaria cases, and the Navy and Marine Corps recorded another 90,000 cases during World War II.

Based on the latest statistics of the World Tourism Organization, 30 million people visit malaria-endemic countries from nontropical countries each year (Steffen and Behrens, 1992). In the past decades malaria imported into the developed world has been on the increase. In Ghana between 1986 and 1989, the malaria attack rate in travellers from the UK increased four-fold overall but eight-fold in business travellers, resulting in an incidence rate of over 2% monthly (Phillips-Howard et al., 1990). Mortality for imported falciparum malaria varies from 3.8% in both the USA and Germany to 8.7% in Japan and 0.4% in both Switzerland and the UK (Steffen and Behrens, 1992).

Due to high attack rates, malaria imposes a heavy burden on activities and is probably one of the principal obstacles to economic development in some regions. According to the Minister of Health of Indonesia, malaria caused at least 30 million cases annually, directly affecting more than 2

30% of the population of that country. Among these cases were 12 million wage earners who were unable to work an average of five days/case because of malaria, representing an annual loss of more than 60 million US dollars. In the Philippines, prior to large-scale control, the economic loss caused by mortality from malaria was estimated at a total of 15 million US dollars per year (10,000 deaths; actuarial value of one life, US \$1500) (Wernsdorfer & Wernsdorfer, 1988).

2. Failure of Malaria Eradication in the World and the Future of Malaria Control: In the 1940s, DDT was developed as one of the four great weapons used against malaria; the others were larvicides (e.g., larviciding oils and Paris Green), drainage, and administration of quinine. Antimalarial strategy changed drastically due to the persistence, low cost, economy of application and safety of DDT. The philosophy of malaria control switched from larviciding to residual insecticidal spraying of houses, a strategy that made large-scale and area-wide control feasible (Service, 1992).

The worldwide program of malaria eradication was formally endorsed by the Eighth World Health Assembly in 1955 (WHO, 1957). Within 15 years of the start of this remarkable international endeavour, malaria had been eliminated from the whole of Europe, the Asian part of the former USSR, several countries of the Middle East, most of North America including the whole of the USA, most of the Caribbean, large areas of the northern and southern portions of South America, Australia, Japan, Singapore, Korea and Taiwan. By 1970, the eradication program had freed 727 million people from the risk of malaria; this represented 53% of the population of the originally malarious areas (Bruce-Chwatt, 1985).

From 1973 to 1977, the number of reported cases of malaria throughout the world had increased by 2.5 times, due to the reduction of malaria control activities in many countries (WHO, 1982). In sub-Saharan Africa there has been virtually no reduction in malaria prevalence except in a few southern countries, while in other regions, such as South America and Asia, there have been dramatic resurgences (Service, 1992). In the face of these setbacks, WHO was forced to abandon its goal of eradication and declare malaria control a more practical target. Reasons include technical obstacles (e.g., the exophilic habits of some anopheline species, resistance of malaria vectors to insecticides, resistance of plasmodia to antimalaria drugs) and administrative, socio-economic, financial and political problems (e.g., increasing cost of insecticides and wages, poor surveillance and case detection, political and social instability, agricultural development, and shortage of trained personnel).

Valid strategies of malaria control should integrate a number of methodologies, including education, mosquito control, vaccine research and antimalarial drugs. At present there is a focus on two approaches. One uses low technology and community participation, and is exemplified by the use of insecticide-treated mosquito nets. The other is a proposed high technology strategy, the production of transgenic

mosquitoes. In the mainland of China, some of the low technology net trials have been accompanied by a dramatic decline in malaria (Li et al., 1989). Alonse et al. (1991) showed that, in the Gambia, insecticide-treated nets reduced malaria-specific mortality in children aged 1-4 years by 70%. However, there can be problems with nets. For example they afford little protection in regions where vectors bite early at night before people, especially children, are safely in bed. Peoples' acceptance of sleeping under nets in hot climates and in very crowded houses can vary considerably. The possibility of Anopheles developing pyrethroid resistance or changing their biting habits to feed earlier and/or outof-doors remains a threat. Oaks et al. (1991) argue that because of the tremendous variation in malaria epidemiology in the study areas, it is difficult to evaluate precisely the impact of insecticide-treated mosquito nets on malaria.

The future control strategy of using transgenic mosquitoes refractory to *Plasmodium* development is very ambitious. There will undoubtedly be numerous technical and ethical problems associated with the development and release of transgenic refractory *Anopheles* into nature. A project of this magnitude, requiring a vast amount of basic research in *Plasmodium/Anopheles* interaction, *Anopheles* transgenic technology and *Anopheles* ecology, can by no means be envisioned on a short-term basis. Its implementation will necessitate a major scientific and generous financial investment (Brey, 1991).

The prospect for the development of effective malaria

vaccines is reasonably good, but the realization of this goal will require some time. Parasite antigenic diversity and genetic restrictions of immunological responses to these antigens in the human population pose great problems to the effectiveness of subunit malaria vaccines now being developed. The necessity for safe and effective adjuvants and for incorporation of T-cell epitopes in the vaccine molecule are additional major constraints in subunit malaria vaccine development (Siddiqui, 1991). It was anticipated by some that by now we would have several good vaccines for field evaluation, but apart from the controversial "Patarroyo vaccine" (Patarroyo et al., 1988), we are still waiting for a practical malaria vaccine (Target, 1991). Now many believe that at best, vaccines of the immediate future will give only partial protection (Playfair et al. 1990), ameliorating morbidity and mortality.

While antimalarial drugs can prevent death, they are not normally deployed with this sole purpose in mind. They are more frequently used to protect from malaria infection and to cure the disease. Often, drugs account for only a fraction of the economic costs of malaria and its management. In fact the costs of repeated visits to the doctor and time lost from work because of a recrudescence of disease may far outweigh the higher cost of a more effective and more expensive drug (Schapira et al., 1993). Since experience suggests that drug resistance cannot be easily contained, and that development of resistance to newer drugs is probable, scientific effort must focus more on maximizing the safe and effective use of current operational drugs (Bjorkman and Phillips-Howard, 1990b). Chloroquine may continue to provide adequate clinical efficacy even in areas with reported resistance (Breman and Campbell, 1988). Despite the fact that nearly all countries in sub-Saharan Africa now have evidence of chloroquineresistant *P. falciparum*, chloroquine remains the most widely-used antimalarial on the continent. It is still a most valuable drug, although as a treatment of falciparum malaria its days may be numbered (White, 1992).

3. Emergence and Spread of Chloroquine-Resistant Malaria 1920s, antimalarial drugs have Parasites: Since the constituted a fundamental component of malaria control. In tropical Africa there was increasing drug pressure from the mid-1970s, reaching a peak in 1988 with the use of an estimated 191 tons of chloroquine base (WHO, 1990). The drug was widely used at subtherapeutic doses, especially in children; this produced a relatively fast selection for and spread of chloroquine-resistant parasites, manifested by both an increasing frequency and degree of resistance. As a result of this massive use of antimalarial drugs for prophylaxis and treatment with subcurative doses (Wernsdorfer and Payne, 1991) and the use of medicated salt (Payne, 1988), drug resistance in the malaria parasites has rapidly spread. Anecdotal reports suggest that malaria resistant to chloroquine emerged as early as 1957, both along the Cambodia-Thailand border areas and in Colombia. Thereafter, the chloroquine-resistant P. falciparum dramatically spread in South America and expanded westward, northward, and

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towards the Pacific Islands from South-East Asia. A new focus had appeared in Africa by 1977, and then all of East and Southern Africa and the offshore islands became involved (Table 1).

By 1990, the distribution of chloroquine-resistant *P.* falciparum had extended to such a degree that it became almost identical to that of the species as a whole (Bjorkman and Phillips-Howard, 1990a). Chloroquine resistance has been reported in 73 of 95 countries and territories afflicted by autochthonous *P. falciparum* (Wernsdorfer, 1991). The continued increase in geographic distribution of chloroquineresistant *P. falciparum* is a major contributing factor to failure of malaria control programs and to the worldwide resurgence of the disease during recent years. In addition, there are reports of chloroquine resistance in *P. vivax* from Papua New Guinea and Brazil(Schurkamp et al., 1992; Garavelli and Corti, 1992). These foci of chloroquine-resistant *P. vivax* will further complicate malaria control; however, vivax malaria fortunately is usually not fatal.

B. <u>Summation of Past Research Hypotheses about</u> <u>Chloroquine Resistance</u>: Chloroquine is still the mainstay of antimalarial chemotherapy due to its rapid action, low toxicity and low cost, although the rapid spread of chloroquine-resistant plasmodia has now compromised its efficacy in malaria control and treatment. In order to devise strategies to avoid, override or circumvent parasite resistance to this important drug, it is essential to understand its mechanism of action and the potential

American, Asian and African countries with Table.1 chloroquine-resistant P. falciparum a _____ Year of first America Asia Africa report _____ Colombia; Venezuela. Cambodia. 1957-1960 1961-1965 Brazil; Guyana. Thailand; Vietnam. 1966-1970 Panama. Malaysia; Burma; Bangladesh; Lao PDR; Philippines. India; Indonesia; 1971-1975 Surinam. China; Papua New Guinea. 1976-1980 Bolivia; Peru. Solomon Islands; Kenya. Vanuatu. Tanzania; Burundi; Iran; Pakistan; 1981-1985 Fr. Guyana. Sri. Lanka; Bhutan. Sudan; Zaire; Burkina Faso; Madagascar;Malawi; Mozambique;Uganda; Zambia; Angola; Central African Republic; Gabon; Namiibia; Senegal; South Africa; Zimbabwe; Benin; Cameroon; Congo; Ethiopia; Rwanda; Somalia. 1986-1989 Afghanistan; Nepal; Comores; Eq. Guinea; Oman; Yemen. The Gambia; Guinea; Nigeria; Mali; Cote D'Ivoire; Niger; Swaziland Togo; Botswana; Chad; Djibouti; Ghana; Liberia Guinea Bissau; Sierra Leone. _____ ^a Data taken from Wernsdorfer W H and D Payne, 1991., and Payne

D., 1987.

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mechanism by which resistance could develop. Several hypotheses about the mode of action of chloroquine and mechanisms of chloroquine resistance have been investigated: ferriprotoporphyrin IX complex formation; lysosomotropic; Pglycoprotein; DNA intercalation; calmodulin-mediated effector proteins; and parasite detoxification hypotheses. These involve repression and depression of genes, modifications in drug absorption and binding, metabolic changes, and changes in virulence and host-parasite relationships. However the mode of action of chloroquine, as well as the mechanisms responsible for the drug resistance, have not yet been fully elucidated.

1. <u>Ferriprotoporphyrin IX Complex Formation</u> <u>Hypothesis</u>: This hypothesis is based on the ability of ferriprotoporphyrin IX (FP), a product of hemoglobin digestion, to form a tight complex with chloroquine.

Malaria parasites, during their asexual development, rapidly increase in both size and number, and the rate of plasmodial protein synthesis must, of necessity, be commensurate with such prodigious growth. The principal protein of the erythrocyte, hemoglobin, remains the most abundant reservoir of amino acids available to the growing parasite (Sherman, 1977). Hemoglobin consists of FP, a prosthetic group heme which is bound to its apoprotein, globin. Brown (1911) suggested that the malaria parasite contained proteolytic enzymes capable of splitting hemoglobin into globin and hematin (ferriheme hydroxide), and that the globin was further metabolized and used as a source of amino acids or oligopeptides for the parasites, with hematin accumulating as malaria pigment during parasite development.

A century ago, Marchiafava and Bignami (1894) realized that quinine exerts its effect only when malaria parasites actively digest hemoglobin to produce hemozoin (malaria pigment). Plasmodium falciparum gametocytes which survive quinine or mepacrine treatment produce little or no pigment after drug exposure (Mackerras and Ercole, 1949). Studies of chloroquine resistance in rodent malaria showed that hemoglobin digestion apparently is an absolute requirement chloroquine susceptibility of malaria parasites. for Chloroquine-resistant rodent malaria parasites either produce no pigment or cease producing it during exposure to chloroquine(Peters, 1964; Peters et al., 1965; Howells et al., 1968). When the parasites revert to chloroquine sensitivity, they also invariably revert to pigment production (McChesney and Fitch, 1984; Jacobs, 1965; Thompson et al., 1967; Peters, 1968c). These findings may be germane only to rodent malaria, however, since this phenomenon has not been observed in human malaria parasites.

Controversy still exists regarding hemozoin. Sherman et al. (1968) surmised that it is simply a partially degraded hemoglobin. Homewood et al. (1972b) refuted their hypothesis and proposed that malaria pigment and schistosomal pigment may not be simple breakdown products of hemoglobin, but may be synthesized by the parasites from hemin (derived from hemoglobin) and other as yet unidentified constituents.

Subsequently, Yamada and Sherman (1979) suggested that P. lophurae hemozoin is composed of FP, insoluble monomers of globin (molecular mass of 15 KDa), and a protein of plasmodial origin (molecular mass of 21 KDa). Formation of hemozoin plays a role in protection of the parasite against molecular oxygen and compartmentalization of the iron porphyrin, a product of hemoglobin digestion by the plasmodia. Ashong et al. (1989) obtained pure preparations of hemozoin from chloroquine-sensitive (NF54) and chloroquineresistant (K1) strains of P. falciparum and proposed that the preparations contained 41-45% FP and a glycine-rich polypeptide (approximately 14 KDa molecular mass) synthesized by the parasite. The iron porphyrin residuum from hemoglobin digestion could be detoxified by crystallization as a complex with a parasite-derived protein. However, Goldie et al. (1990) concluded through analyses of P. falciparum hemozoin that almost all of the protein component of hemozoin comes from hemoglobin.

The specific role of FP in hemozoin production also remains a subject of ongoing study. FP is highly hemolytic to mouse erythrocytes (Chou and Fitch, 1980, 1981) and also lyses the membranes of *P. berghei* isolates from mouse erythrocytes (Orjih et al., 1981). FP causes nearly total loss of potassium from normal erythrocytes and swelling of the erythrocytes. It results in hypotonic lysis and kills malaria parasites in the erythrocytes (McChesney and Fitch, 1984). FP released from hemoglobin would be expected to bind to nearly all available protein (Banyal and Fitch, 1982;
Lovstad, 1986; Zhang and Hempelmann, 1987) and cellular membranes (Dutta and Fitch, 1983) with possible diminution of its toxicity. The toxic form of FP evidently exists only transiently, as it is rapidly processed to a form that does not bind chloroquine in subcellular preparations of infected erythrocytes (Fitch and Chevli, 1981). Heme was said to be oxidized first to hematinic acid and later to hematin, a toxic substance which was rendered both safe and insoluble by conjugation with a nitrogenous moiety to form the typical malaria pigment (Peters, 1964; Peters et al., 1965). A recent report from Slater and Cerami (1992) suggested that the parasite has a heme polymerase activity that converts the parasite-toxic free heme to relatively nontoxic malaria pigment. The activity of FP as it relates to the mechanism of chloroquine resistance is only partially understood. FP has high affinity and significant specificity for chloroquine. When released in erythrocytes, FP binds chloroquine with an apparent dissociation constant of approximately 10^{-6} M, and if FP is placed in the erythrocyte membrane from the outside, it has an apparent dissociation constant for chloroquine of approximately 10^{-7} M (Fitch, 1986). Chloroquine competes with the soluble heme binder of parasite origin that normally sequesters FP to form a chloroquine-FP complex. It is believed that this chloroquine-FP complex then causes abnormal membrane permeability and kills the parasite (McChesney and Fitch, 1984). The complex formed between chloroquine and FP lyses uninfected mouse

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erythrocytes and the membranes of *P. berghei* and *P. falciparum.* Lysis of the parasites is enhanced by the chloroquine-FP complex in comparison with FP alone (Chou and Fitch, 1980, Orjih et al., 1981, Fitch et al., 1982, Chou and Fitch, 1981, Dutta and Fitch, 1983). Chloroquine alone was said to have no action on either red cell or parasite membranes, but this statement must be related to the concentration of chloroquine (Peters, 1987).

Fitch (1983) assumed that chloroquine acts by delaying the sequestration of FP into malaria pigment, thereby allowing FP to exert its intrinsic cellular toxicity. Accelerated processing of toxic FP into malaria pigment would cause chloroquine resistance. Orjih et al. (1988) modified this hypothesis and proposed that malaria parasites can detoxify FP both by sequestering it and by decomposing it. In the latter case, chloroquine would cause the accumulation of a toxic chloroquine-FP complex by delaying the decomposition of FP by hydrogen peroxide. Thus increased availability of hydrogen peroxide could cause chloroquine resistance in malaria.

Some clues to the mechanism of chloroquine resistance were also obtained from observations on chloroquine-resistant *P. berghei* and *P. falciparum*. In their studies, Macomber et al. (1966) and Fitch (1969) found red blood cells infected with chloroquine-resistant *P.berghei* accumulated only onehalf of the amount of chloroquine that was found in cells infected with chloroquine-sensitive *P. berghei*. Fitch (1970) demonstrated that chloroquine-resistant *P. falciparum* accumulated less chloroquine by high affinity binding than do comparable chloroquine-sensitive strains. These researchers postulated that the antimalarial activity may result from the concentrating mechanism within the parasitized cells, and that resistance may result from the impairment of this mechanism.

There is a parallel between the progressive hemozoin aggregation in digestive vesicles and the rise of chloroquine concentration in the parasitized red cells (Macomber and Sprinz, 1967). FP was identified as the receptor responsible for accumulation of chloroquine and a mediator of its antimalarial activity (Chou et al., 1980, Fitch, 1989). McChesney and Fitch (1984) described the topography of the receptor as follows: (1) a flat surface large enough to accommodate planar ring systems of 30-40 Å²; (2) a chemical group in the flat surface that favors interaction with compounds having a nitrogen in their ring system; (3) an anionic site located in the proper geometric relation to the flat surface to attract the protonated terminal nitrogen atom in the side chain. FP possesses these topographical features (Fitch, 1972, Fitch et al., 1974, Chou et al., 1980).

Since high affinity accumulation is due to binding of chloroquine to FP (Chou et al., 1980), less accumulation represents inaccessibility or absence of FP. Therefore, chloroquine resistance in malaria may be due to inaccessibility or absence of FP in the parasitized erythrocyte. Wood et al. (1984) reported that chloroquinesensitive *P. berghei* infected erythrocytes retain two- to four-times more FP than comparably infected chloroquineresistant *P. berghei*. The chloroquine-resistant strain proteolyzes hemoglobin more rapidly or efficiently than the chloroquine-sensitive strain, and the chloroquine-sensitive strain preferentially accumulates FP. The explanation of the apparent difference in the proteolytic activity could be due to a chloroquine-resistant protease, like trypsin, cleaving only a few specific peptide bonds and producing a larger but not greater number of soluble fragments. In contrast, the protease from chloroquine-sensitive *P. berghei* might, like pronase, cleave most peptide bonds and thus produce large amounts of insoluble hemoglobin degradation products FP (Mahoney and Eaton, 1981a, 1981b).

In summary, the FP complex formation hypothesis states that the FP-chloroquine complex enhances its lytic activities against both erythrocytes and malarial parasites. FP as a receptor is responsible for the accumulation of chloroquine within the parasitized erythrocytes, and chloroquine resistance may be due to inaccessibility or absence of FP in the parasitized cells, resulting in impairment of the drug concentration mechanism within the cells and little or no formation of FP-chloroquine complex.

2. Lysosomotropic Hypothesis: The lysosomotropic nature of chloroquine, i.e., preference of the acid food vacuole of intraerythrocytic malaria parasites, inspired the hypothesis of the lysosomotropic mode of action of the antimalaria drug.

Lysosomotropic compounds are weak bases whose unprotonated form can translocate by free diffusion across biological membranes while their protonated forms cannot (Duve et al., 1974). The compound diffuses as a free base along its concentration gradient into the various compartments of the cell. The pH gradient between the extracellular medium and any particular cellular compartment is therefore the driving force for the accumulation of a lysosomotropic compound in this compartment (Yayon et al., 1984, 1985; Krogstad et al., 1985; Krogstad and Schlesinger, 1986). Concentration. of a lysosomotropic compound obviously will be higher in a compartment that is more acidic than the extracellular medium. Conversely, the drug will be less concentrated in those compartments in which the pH is higher than that of the medium (Ginsburg, 1990a). The pH of the parasite food vacuole is determined by the ATP-driven proton pump and proton leak. The faster the pumping rate and the slower the leak, the more acidic will be the food vacuole. The drug would act as a lysosomotropic agent and accumulate by virtue of its weak base properties into an acidic intracellular compartment of the parasite (Homewood et al., 1972a; Aikawa, 1972), where it inhibits the digestion of ingested host cell cytosol and, consequently, parasite growth.

An impairment in the activity of the vesicle proton pump could lead to resistance to chloroquine (Mahnalgi et al., 1989). The vacuolar pH of resistant strain is estimated to be proportionately higher than that of sensitive strains. Chloroquine accumulation in diverse parasite isolates dwindles with increased resistance to the drug (Vander Jagt et al., 1987).

It was demonstrated that, in a chloroquine-sensitive (N) strain of *P. berghei*, the chloroquine probes accumulated in the endocytic vacuoles where hemoglobin degradation is occurring. In a chloroquine-resistant (RC) strain of *P. berghei*, the probe was found scattered throughout the cytoplasm of the parasite (Moreau et al., 1986; Mahnalgi et al., 1989). Thus, resistance to chloroquine could be due to a mechanism by which the drug is accumulated in an intracellular compartment of the parasite where chloroquine does not act.

Warhurst (1986) proposed a membrane-permease hypothesis to explain the resistance to chloroquine. He suggested that chloroquine could be transported into the parasite cytoplasm an ATP-dependent pump, transferring protons from the by parasite cytoplasm into the erythrocyte cytoplasm (Warhurst and Thomas, 1978). Yayon and Ginsburg (1982) also reported that uninfected erythrocytes have symmetrical carrier systems for chloroquine. The lysosomes of the parasite which can be demonstrated to be acidic (internal pH approximately 5.0) will further concentrate the drug. In the absence of any change in proton distribution, changes in the quantity or quality or distribution of the postulated permease could lead to drug resistance: permease could be reduced in quantity or absent, the affinity of the permease for chloroquine could be reduced, or the permease might be present also in the lysosomal membrane (Warhurst, 1986).

Alkalinization of the food vacuole by chloroquine,

quinine and mefloquine was observed at a concentration which was 1-2 orders of magnitude larger than the IC50 of their antimalarial effect (Ginsburg et al., 1989). This means that vacuolar alkalinization is not the primary effect of antimalarial action of quinoline-containing drugs. Substitution of the chlorine atom of chloroquine by a proton produces a compound able to be targeted into the parasite food vacuole, which is less easily taken up by the parasite. The chlorine atom is significantly implicated in the uptake of chloroquine by the parasite which is independent of the weak base properties of the quinoline-containing drugs. The mechanism of uptake of chloroquine by the parasite is also apparently linked to structural characteristics of the quinoline ring (Veignie and Moreau, 1991). The hypothesis proposes that chloroquine may be concentrated into the lysosomes (i.e., the food vacuole) of the parasites by an ATP-dependent pump, where the drug acts by inhibition of digestion of ingested host elements and of growth of the parasites. Resistance to chloroquine could be due to an impairment in activity of the pump.

3. <u>P-glycoprotein Hypothesis</u>: This hypothesis is based on evidence linking chloroquine resistance of malaria parasites to the multi-drug resistance phenotype of mammalian tumour cells which is mediated by a protein molecule termed P-glycoprotein.

As is true for *P. berghei*, chloroquine-resistant *P. falciparum* parasites accumulate significantly less chloroquine than susceptible parasites. This was thought to

be the basis of their resistance (Macomber et al., 1966, Macomber and Sprinz, 1967; Fitch, 1970; Krogstad et al., 1985; Krogstad and Schlesinger, 1987). However, the initial rates of chloroquine uptake observed with chloroquinesensitive and chloroquine-resistant P. falciparum were indistinguishable (28.6 versus 29.1 fmol per 10⁶ parasitized cells per minute). Drug-resistant P. falciparum red parasites release chloroquine at a rate 40- to 50-fold greater than that of sensitive plasmodia (Krogstad et al., 1987). This efflux of chloroquine from the parasites explained the level of chloroquine resistance observed in P. falciparum and suggested that chloroquine resistance occurred by removal of the drug from its site of action. The rapid efflux phenotype has been observed in isolates of P. falciparum from Southeast Asia, South America and Africa (Krogstad et al., 1988).

Calcium antagonists, verapamil, vinblastine and daunomycin, were found to inhibit the efflux of chloroquine from the drug-resistant *P. falciparum* but not from the drugsensitive parasite (Martin et al., 1987; Krogstad et al., 1987). This suggests close similarities in the mechanism of resistance to chloroquine and the multidrug resistance (MDR) phenotype of mammalian tumor cells, (i.e., enhanced efflux of drug from resistant cells, less accumulation of drug in resistant cells and the reversal of drug resistance by calcium antagonists). The MDR phenotype of mammalian tumor cells is thought to be mediated by a membrane transport molecule called P-glycoprotein (Endicott and Ling, 1989). The P-glycoproteins of mammalian cells consist of homologous halves containing six hydrophobic transmembrane regions separated by two homologous hydrophilic regions, each of which contain an ATP binding site. Chloroquine efflux was inhibited reversibly by the removal of metabolizable substrate (glucose) and was also reduced by the ATPase inhibitor vanadate (Krogstad et al., 1992), implying that chloroquine efflux is an energy-requiring process. Presumably the hydrolysis of ATP provides the energy for transport or efflux across the membrane (Scheibel, 1992).

By a genetic cross between a chloroquine-resistant and a chloroquine-sensitive clone of P. falciparum, sixteen recombinant progeny were obtained that were derived from separate meiotic events. Eight of the 16 progeny exhibited the same absolute levels of resistance and drug efflux characteristics as the chloroquine-resistant parent while the other eight showed the same chloroquine response and slow efflux of the chloroquine-sensitive parent. No progeny exhibited a nonparental chloroquine phenotype; a simple genetic locus appeared to be responsible for the rapid chloroquine efflux mechanism (Wellems et al., 1990). Plasmodium falciparum contains at least two MDR-like genes (pfmdr 1 and pfmdr 2). One of these genes, pfmdr 1, was found to be expressed at a higher level and to be present in higher copy number in some drug-resistant P. falciparum, but not in any drug-sensitive isolates examined. Pfmrd 1 transcript levels were also increased in the resistant isolates. Pfmrd 1 was present on chromosome 5 which has extensive size

polymorphism particularly in chloroquine-resistant isolates. The full pfmrd 1 gene has been isolated and consists of a continuous open reading frame of 4257 nucleotides (Foote et al., 1989; Wilson et al., 1989). The predicted polypeptide encoded by this gene is 162 KDa, and it shows extensive homology with mammalian P-glycoproteins. A segment of pfmrd 2 gene also has been isolated from P. falciparum, and it consists of part of a putative ATP-binding domain. The polypeptide encoded by this segment is approximately 59% homologous to the same region of pfmrd 1 (Wilson et al., 1989). Foote et al. (1990) showed that the complete sequences of pfmrd 1 gene from chloroquine-sensitive isolates of P. falciparum are identical, and the pfmrd 1 sequence of the resistant isolates differed from that of the sensitive isolates at five key positions, resulting in amino acid substitutions. On the basis of these substitutions, they have correctly predicted the chloroquine-sensitive or chloroquineresistant status of a further 34 out of 36 isolates. It was concluded that a mutated pfmrd 1 gene is one of at least two mutated genes required for chloroquine resistance.

Based on the messenger (m)RNA expression of *pfmdr 1* and *pfmdr 2* genes in chloroquine-resistant and -sensitive isolates of *P. falciparum*, it was claimed that there is a positive correlation between levels of expression of both transcripts and drug resistance (Richardson et al., 1991). The *pfmdr 1* protein product (Pgh 1) is expressed throughout the erythrocytic asexual cycle. It is indeed present in the same stages where chloroquine acts. The subcellular

localization of the protein has been determined to be present on the membrane of the food vacuole in the trophozoite and on the membrane of the residual body, which is the remnant of the digestive vacuole of schizonts (Cowman et al., 1991). FAC8, a chloroquine-resistant line, has three copies of the pfmrd 1 on a 100 kb amplicon (Foote et al., 1989) and expresses approximately 3-fold more Pgh 1 than other P. falciparum isolates. Moreover, there is no increase in expression of Pgh 1 in most chloroquine-resistant isolates of P. falciparum that contain a single copy of the pfmrd 1 gene (Cowman et al., 1991). The target of chloroquine antimalarial activity is most likely within the food vacuoles of plasmodia. A molecule responsible for removal of chloroquine from its site of action may therefore be expected to be present on the membrane of the digestive vacuole. The time in the asexual erythrocytic cycle at which Pgh 1 is made and its subcellular localization would enable the pfmrd 1 gene to play a role in the chloroquine resistance phenotype. Chloroquine that concentrates in the digestive vacuole would be in the dicationic form, which is believed to be lipid insoluble. Consequently chloroquine is trapped and unable to move from this compartment. Pgh 1 in chloroquine-resistant parasites has amino acid changes at five key positions (i.e., Asn^{86} to Tyr^{86} , Tyr^{184} to Phe^{184} , $\operatorname{Ser}^{1034}$ to $\operatorname{Cys}^{1034}$, $\operatorname{Asn}^{1042}$ to Asp 1042 and Asp 1246 to Tyr 1246 ; Foote et al., 1990). These changes may allow the Pgh 1 to recognize chloroquine as a substrate for efflux and transport chloroquine out of the

vacuole into the cytoplasm of the parasite where the drug would either be monoprotonated or in an unchanged form. This would set up a concentration gradient that drives chloroquine out of both the parasite and the erythrocyte (Cowman, 1991).

Bray et al. (1992) found that chloroquine sensitivity was related to the energy-dependent rate of drug accumulation into chloroquine-resistant isolates of P. falciparum, but not to the rate of chloroquine efflux from these parasites. No linkage between rapid efflux of drug, phenotype of chloroquine resistance, multidrug resistance genes (pfmrd 1 and pfmrd 2) or amplification of these genes was found. Also, the genetic locus governing chloroquine efflux and resistance is independent of the known mdr-like genes (Wellems et al., 1990; Scheibel et al., 1989; Scheibel, 1992). Therefore it remains to be seen whether or not mdr genes or P-glycoproteins completely explain the resistance. In this regard, Wellems (1991) suggested that resistance in one clone of P. falciparum (Dd2) was determined not by mdr genes, but by a single gene, so far unidentified, located on chromosome 7. Martin (1993) proposed that chloroquine resistance can be expressed by malaria parasites in the absence of classical MDR characteristics (i.e., multiple copies of *pfmdr* and elevated levels of *pgh* 1) and chloroquine-resistant malaria parasites are not typical MDR cells.

In summary, the P-glycoprotein hypothesis assumes that chloroquine-resistant malaria parasites accumulate less of the drug by enhanced efflux from the parasites. Resistance is 24

thought to be mediated by P-glycoprotein as the MDR phenotype of mammalian tumor cells.

4. <u>DNA Intercalation Hypothesis</u>: The DNA intercalation hypothesis was popular from the 1950s to the early 1970s, and has recently been overlooked.

Parker and Irvin (1952) were the first to show that chloroquine forms a reversible complex with the DNA of beef spleen and a somewhat weaker complex with yeast RNA. The chloroquine is bound to DNA in two ways. The first is by the intercalation of the aromatic quinoline ring between adjacent base pairs of the DNA double helix, and the second by an interaction of the diaminopentane side chain of chloroquine with the phosphates of the complementary strands of DNA across the minor groove (Allison et al., 1965; O'Brien et al., 1966b). Indeed, the distance between the amine groups on this side chain corresponds to the distance between these phosphate groups. O'Brien and Hahn (1965) argued that these structural properties of chloroquine that enabled it to complex with DNA were identical to those that endowed it with its antimalaria action. The chloroquine congeners with shorter or longer distance between amino groups had less antimalarial effect. The 7-chloro ring substituent was necessary for interaction with 2-amino group of quanine, since the 7-deschloro analog of chloroquine bound to DNA more weakly than chloroquine itself (Stollar and Levine, 1963). It had only 7% of the antimalarial activity of chloroquine. Analogs with other halogen substituents at position 7, or with chlorine at positions 2, 3 or 6 (instead of position 7)

had decreased activity. Bulky substituents that might interfere with intercalation diminished antimalarial activity (Meshnick, 1990). Kwakye and Meshnick (1989) estimated that 0.03-1.0% of potential intercalation sites might be occupied when parasites are exposed to therapeutic concentrations of chloroquine through re-evaluation of the dissociation constants (KD).

In addition, chloroquine does not bind to all sequences of DNA with equal strength. The drug bound two to four times more avidly to poly(dG-dC) polynucleotides than to other sequences (Kwakye and Meshnick, 1990). This means that chloroquine might be toxic to the parasite by selectively accumulating in specific genes and inhibiting their expression at low concentrations. Chloroquine has been shown to inhibit RNA and DNA synthesis in *P. knowlesi* (Gutteridge et al., 1972) and to prevent the transition of DNA to the Z form *in vitro* at concentrations as low as 20 μ M (Kwakye and Meshnick, 1990).

Chloroquine, like other intercalators, may work by complexing with DNA, stabilizing the double helix against strand separation, inhibiting the DNA and RNA polymerase reactions, and restraining primarily DNA replication and secondarily RNA transcription from DNA (Hahn et al., 1966; O'Brien et al., 1966a, 1966b). Chloroquine may also affect dissimilation of ribosomal RNA, degradation of ribosomes, parasite membranes and orithine decarboxylase (Ciak and Hahn, 1966; Scheibel and Sherman, 1988).

Some questions about the DNA intercalation hypothesis

remain to be answered. How could chloroquine selectively bind to the parasite DNA as the drug appears to bind equally well to host DNA? How can it explain the stage specificity of chloroquine action? Why would effective antimalarial concentrations of chloroquine not affect other eukaryotes whose DNA is relatively richer in G and C (Ward, 1988; Ginsburg, 1990b)? Another questionable aspect of work on this hypothesis is that Hahn's studies were all done on a bacterium strain *Bacillus megaterium*, not on plasmodia.

This hypothesis proposes the following mode of action of chloroquine: chloroquine may intercalate into parasite's DNA, which results in stabilization of the double helix against strand separation, inhibition of the DNA and RNA polymerase activities, and prevention of DNA replication and RNA transcription from DNA. The basis of chloroquine resistance could be a reduced uptake or concentration of the drug in resistant parasites (Hahn et al., 1966). However, this hypothesis is no longer seriously considered in the search for an explanation of the chloroquine resistance of malaria parasites because there are no known differences between the DNAs of resistant and sensitive parasites (Krogstad et al., 1992), and the basis of chloroquine resistance is thought to be a reduced uptake or concentration of chloroquine in resistant parasites (Hahn et al., 1966).

5. <u>Calmodulin-Mediated Effector Proteins Hypothesis</u>: Works showing that calcium and calmodulin antagonists (e.g., verapamil, vinblastine and daunomycin) could reverse chloroquine resistance in *P. falciparum* fueled the calmodulin-mediated effector proteins hypothesis.

A calcium-modulated protein named calmodulin (CaM) (Cheung, 1971; Walsh and Hartshorne, 1983) has been found in all eukaryotic cells studied so far, but not in bacteria (Kippert, 1987). Nevertheless, calmodulin-like structures (Swan et al., 1987) and calmodulin-binding proteins (Iwasa et al., 1981) have been reported to be present in some prokaryotic cells. CaM is a multifunctional protein with a molecular mass of about 17 K Da containing 148 amino acids of which a high proportion are acidic residues. CaM proteins isolated from many sources have strikingly similar amino acid sequences. All mammalian CaMs are the same, although plant or invertebrate CaMs differ slightly in sequence (Watterson et al., 1980; Cheung 1980; Takagi et al., 1980). CaM contains four calcium (Ca^{2+}) -binding sites, each composed of twelve amino acids and flanked on each side by an 8-amino acid helix.

In the early 1980s there was a growing body of evidence that calcium (Ca²⁺) was extremely important to the malaria parasite. Using a Ca²⁺ chelating agent, ethyleneglycolbis (bamino-ethylether) N,N'-tetra-acetic acid (EGTA), Wasserman et al. (1982) showed Ca²⁺ to be indispensable for the normal growth of *P. falciparum* cultures, particularly during the maturation phase some 20-26 hours after erythrocyte invasion. Ca^{2+} is also essential in the process of reinvasion or final maturation of a full infective merozoite. Malaria parasites reportedly actively accumulate Ca^{2+} to levels 30 times those found in uninfected erythrocytes. This rate of influx is seven times that which occurs in normal red cells. Efflux of Ca^{2+} is also decreased in infected erythrocytes. The enhanced Ca^{2+} uptake is more pronounced with increasing levels of parasitemia and parasite maturity (Leida et al., 1981; Bookchin, 1981; Tanabe et al., 1982; Krungkrai and Yuthavong, 1983; Mikkelsen et al., 1984).

The ability of CaMs to bind calcium may be important in understanding drug resistance of malaria parasites. Following the stimulation of a cell, there is an increase in the intracellular free Ca^{2+} concentration due to movement of Ca^{2+}

through channels in the plasma membrane or due to its release from intracellular organelles. Each CaM molecule can bind as many as four Ca^{2+} . The binding of Ca^{2+} produces conformational changes to a more helical structure that exposes hydrophobic regions, which in turn can bind to and activate CaM-binding proteins or enzymes, resulting in a broad range of actions. On the other hand, if lipid-soluble CaM inhibitors are present, they may also bind to Ca^{2+} activated CaM, preventing the activation of target enzymes (Scheibel, 1992).

Quantitation of CaM in *P. falciparum* by Scheibel and coworkers (1987, 1989) supported these calcium requirements. Normal erythrocytes contained only 10 to 11 ng CaM per 10⁶ cells, whereas ring stage parasites contained about 18 ng CaM per 10^6 cells and schizonts up to twice the value found in rings. It appeared that CaM content increased with parasite maturity (Scheibel et al., 1987, 1989). In addition, using immuno-electron microscopy and autoradiography, Scheibel et al. (1989) identified the location of CaM within *P. falciparum*. In the mature malaria parasites, CaM was found scattered throughout the cytoplasm corresponding to the diffuse binding. In the merozoite, the CaM was concentrated at the apical end, underlying the plasma membrane. However, in both infected and uninfected erythrocytes, few CaM sites were demonstrated on the plasma membrane or in cytoplasm. This may in part explain the Ca²⁺ requirement observed by Wasserman et al. (1982) during merozoite invasion of erythrocytes in *P. falciparum* (Scheibel et al., 1987, 1989).

The *in vitro* growth of *P. falciparum* is inhibited to varying degrees by the Ca^{2+} antagonists. The growth of the human malaria parasite is also sensitive to CaM antagonists, in many instances to a greater degree than to the Ca^{2+} channel blockers. The degree of inhibition of the parasite is directly proportional to the known anti-CaM potency or degree of binding to CaM in mammalian tissues by these Ca^{2+} antagonists and CaM antagonists (Asano and Hidaka, 1984; Roufogalis, 1985). The rank order of activity of the clinically useful antimalarials within their class reflects their reported anti-CaM effect. This antagonism of biological response (parasitic growth) which parallels the anti-CaM 30

potency occurs at pharmacological doses and shows strong Ca²⁺ dependence of plasmodial growth. This points to parasitic CaM processes being related to growth inhibition by these agents (Johnson et al., 1980; Wasserman et al., 1982; Roufogalis, 1982, 1985; England, 1986).

Inhibition of the growth and development of Ρ. falciparum by a number of Ca²⁺ antagonists and CaM antagonists was described by several investigators (Tanabe et al., 1989; Geary et al., 1986; Panijpan and Kantakanit, 1983; Scheibel et al., 1987,1989; Kristiansen and Jepsen, 1985). The antimalarials, mepacrine, quinine and chloroquine, are also known to inhibit CaM (Asano and Hidaka, 1984; Roufogalis, 1982, 1985; Loffler et al., 1985; Nagai et al., 1987). Interaction of these drugs with CaM appears to involve two kinds of attachments. One is a hydrophobic interaction between a very large lipophilic (ring) portion of the drug and nonpolar regions of CaM. The other is electrostatic interaction between a positively charged amino group on the drug and a negatively charged acidic residue on CaM. This interaction with the Ca²⁺/CaM complex in turn inhibits the binding and subsequent activation by CaM of effector enzymes (Scheibel, 1992).

Drug resistance appears to be a result of drug binding to a receptor site that shares architectural features with CaM or CaM-mediated effector proteins. When the response (growth inhibition) to combinations of clinically effective antimalarials and CaM/Ca²⁺ inhibitors in the drug-resistant clone is compared to that in the drug-sensitive clone, a marked antagonism between these agents results in clones of resistant plasmodia which is not seen in sensitive clones. This antagonism suggests that the drugs are competing for the same receptor (Scheibel, 1992). That receptor appears to be expressed to a greater degree in the resistant clone and appears to be Ca^{2+}/CaM mediated.

It has been reported that in vitro synergy between the Ca²⁺ antagonist verapamil and the antimalarial anti-CaM drug chloroquine occurs in chloroquine-resistant but not chloroquine-sensitive clones of P. falciparum parasites (Martin et al., 1987). Similar results were observed in the P. chabaudi system in vivo, but verapamil increased the susceptibility to chloroquine of both susceptible and resistant strains (Tanabe et al., 1990), in contrast to what is seen in *P. falciparum*. There is evidence that the ability of CaM antagonists to reverse resistance is not necessarily related to Ca^{2+} channel blockade, but may instead be due to similar binding sites that exist on CaM as well as other pump proteins.

Warhurst and Thomas (1978) observed the distribution of CaM by following the binding of antimalarial mepacrine by fluorescence microscopy. The drug appeared to bind CaM in the living parasite. They also assumed that CaM may be a drug target in drug-resistant parasites (Warhurst, 1988). However, recently Robson et al. (1993) showed that CaM from *P*. *falciparum* has a high level of amino acid sequence identity (89%) with its mammalian counterpart. In addition, neither chloroquine nor quinine bind very well with CaM from *P. falciparum*. Robson and coworkers concluded that the difference between the malaria parasite and its host CaM are insufficient to merit this protein being chosen as a realistic target for antimalarial drugs. Other proteins more variant than CaM itself but mediated by Ca^{2+} or CaM may be likely candidates for drug action.

The hypothesis states that growth of malaria parasites depends strongly on Ca^{2+} and CaM, and that chloroquine resistance may be a result of the drug competing with Ca^{2+} for the same receptor. The receptor would be more strongly expressed and Ca^{2+}/CaM mediated in the resistant parasites. Reversion of chloroquine resistance with Ca^{2+} and CaMantagonists would be attributed to the fact that the parasites increase the ability to accumulate chloroquine by retarding efflux of preaccumulated drug.

6. <u>Parasite Detoxification Hypothesis</u>: This hypothesis maintains that many toxic xenobiotics and drugs are inactivated and eliminated from eukaryotic cells by cytochrome P450-dependent microsomal monooxygenases, and that chloroquine is metabolized by these enzymes in reactions that involved deethylation and hydroxylation (McChesney et al., 1967).

Agosin et al. (1976) first reported the presence of cytochrome P450 in parasites. They indicated that the epimastigote forms of *Trypanosoma cruzi* possess an active detoxifying system whose characteristics are those of a monooxygenase system linked to cytochrome P450 and whose activity is increased by phenobarbital. Pandey et al. (1986) also demonstrated the presence of cytochrome P450 in schizonts of *P. knowlesi*, a simian malaria parasite. Salganik et al. (1987) demonstrated increased activities of aryl hydrocarbon hydroxylase and aminopyrine N-demethylase in chloroquine-resistant *P. berghei* when compared with the enzymatic activities of chloroquine-sensitive parasites. The monooxygenase activity of the parasites was related to the degree of their drug-resistance. Subsequently, Ndifor et al. (1990) reported that chloroquine-resistant strains of *P. falciparum* and *P. berghei* had significantly higher activities of aminopyrine N-demethylase and ethoxycoumarin O-deethylase than those of the chloroquine-sensitive parasites.

Pretreatment of parasites with phenobarbital results in a significant increase in antipyrine N-demethylase and ethoxycoumarin O-deethylase activities in all the four strains of *P. falciparum* and *P. berghei* (Ndifor et al., 1990). Microsomal monooxygenase inhibitors, such as a copperlysine complex, metyrapone and a-naphthoflavone, considerably suppress the aryl hydrocarbon hydroxylase and ethoxycoumarin O-deethylase activities of *P. berghei* (Salganik et al., 1987; Ndifor et al., 1990). Use of these inhibitors could theoretically increase the sensitivity of malaria parasites to chloroquine and reverse the drug resistance of plasmodia. Rabinovich et al. (1987) showed that administration of a combination of chloroquine and the copper-lysine complex decreased the parasitemia levels of mice infected with a chloroquine-resistant strain of *P. berghei*; however, when given separately, chloroquine and the complex had no antimalaria effect. However, this hypothesis may offer a novel approach: the use of chloroquine synergists to overcome the drug resistance of malarial parasites.

hypothesis assumes that chloroquine-resistant The malaria parasites have enhanced cytochrome P450-dependent enzyme activities. Microsomal monooxygenase inhibitors could theoretically reverse the drug resistance of the parasites by increasing parent drug concentration. In addition, Howells and his colleagues found demonstrable succinic dehydrogenase activity in chloroquine-resistant P. berghei (RC strain) erythrocytic schizonts, but not in chloroquine-sensitive P. berghei (N strain). They conjectured that these parasites possess a functional citric acid cycle and that the presence of the respiratory pathway was in some manner associated with chloroquine resistance in the rodent malaria parasite (Howells, 1970; Howells and Peters, 1970; Howells and Homewood, 1971). But their further studies failed to confirm intraparasitic localization of succinic dehydrogenase in erythrocytic trophozoites of the parasites (Howells and Maxwell, 1973).

The mentioned hypotheses state that actions of chloroquine against malaria parasites may be implicated in formation of a toxic FP-chloroquine complex, inhibition of hemoglobin degradation in the food vacuole, and prevention of DNA repliction and RNA transcription by DNA-intercalation. Thus the mechanism of resistance to chloroquine may be attributed to reduced ability to accumulate chloroquine in resistant parasites by decreased drug uptake or concentration rate, enhanced drug efflux from the parasites, and enhanced cytochrome P450-dependent drug- metabolizing enzyme activity of the parasites. However, these hypotheses are still controversial, and the mode of action of chloroquine and the mechanism of drug resistance are not completely clear. But few studies have been conducted to investigate the role of the host's detoxification mechanisms, namely, the hepatic microsomal mixed function oxidases, in antimalarial drug resistance.

C. Mixed Function Oxidase and Parasitic Infections: The hepatic microsomal mixed function oxidase system (MFOS) plays important role in an biotransformation of various xenobiotics. such as antimalarial drugs. Parasitic infections, including malaria, can modulate the activity of the MFOS system. Review of properties of the MFOS system and its role in drug metabolism, the effect of malaria and other parasitic infections on the activity of the MFOS of their hosts, and the role of the MFOS system on insecticide resistance in insects is helpful to understanding the mode of action of chloroquine and the mechanism of resistance to the antimalaria drug in malaria parasites.

1. <u>Properties of Cytochrome P450 and Its Role on Drug</u> <u>Metabolism</u>: Most drugs are metabolized in two phases. In phase I, one or more polar groups are introduced into the parent compound through oxidation, reduction or hydrolysis.

In phase II, the metabolites are conjugated with an endogenous substrate to yield products which are hydrophilic and readily eliminated in urine or bile. Most hydrophobic compounds are metabolized through the MFOS. The system is located in the membranes of the endoplasmic reticula. It is comprised of cytochrome P450 and NADPH-cytochrome P450 reductase. Cytochrome P450 is a hemeprotein with heme as the prosthetic group linked to an apoprotein. Several P450s have been separated, purified and characterized from mammalian tissues, including human livers. Many are substrate-specific and regulated by xenobiotics. Cytochrome P450, so named because in its reduced form it binds to carbon monoxide and then absorbs light with an absorbance maximum at about 450 nanometers, is the terminal oxidase of the MFOS. It accepts electrons from NADPH and binds oxygen molecules. Subsequently one oxygen atom oxidizes the drug and the other is reduced to form water. Cytochrome P450 reductase is a flavoprotein which transfers electrons from NADPH to cytochrome P450. Maintenance of the structural integrity of endoplasmic reticulum, location of the MFOS activities, and access of lipid soluble xenobiotics to the membrane-bound P450 require phospholipid (Kappas and Alvares, 1975; Tekwani et al., 1988).

Cytochrome P450 is present in mammals, bacteria, plants, fungi, arthropods, fish and parasites. In mammals its activities can be detected in the gastrointestinal tract, lungs, skin and kidneys, but it occurs predominantly in the liver. More than 200 drugs, insecticides, chemical carcinogens and other environmentally derived chemicals are known to induce cytochrome P450-dependent enzymes in experimental animals, and many foreign chemicals have been shown to induce these enzymes in humans. Cytochrome P450s also metabolize endogenous substrates, such as steroid hormones and fatty acids.

The MFOS is highly inducible. Its activity can be greatly increased by exposure to a wide variety of environmental agents and drugs which act as substrates for the system. In the elaboration of the reaction mechanism of the monooxygenation with xenobiotics, use of enzyme inducers and inhibitors also has proven to be of considerable value (Netter, 1987). Inducers of hepatic monooxygenases have been categorized into two main groups depending upon the mechanism of enzyme induction (Conney, 1967). One group, of which phenobarbital (PB) and the insecticide DDT are prototypes, enhances the metabolism of a large variety of substrates. PB is a potent inducer of ethylmorphine and benzphetamine N-demethylases. In rats, this group of enzyme inducers has been shown to markedly increase liver cytochrome P450 content and associated enzyme activities. A second major group of enzyme inducers includes polycyclic aromatic hydrocarbons, such as benzo(a)pyrene and 3-methylcholanthrene (3-MC). This group enhances the metabolism of only a few xenobiotics, e.g. aryl hydrocarbons, and induces the synthesis of cytochrome (Alvares, 1982). The mechanism of induction of P448 drug-metabolizing enzymes by xenobiotics involves stimulation of mRNA synthesis followed by de novo synthesis of new enzyme

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protein. Induction by PB also appears to produce stabilization of the enzyme protein and decreases the rate of degradation of mRNA and of microsomal protein, including NADPH-cytochrome P450 reductase (Ress, 1979).

Some chemicals or metabolic products of parasites, such as malaria pigment, may act as inhibitors and thus be responsible for the decrease of cytochrome P450 and associated enzyme activities (Alvares et al., 1984, Saxena et al., 1987). By far the most common way of inhibiting the reaction of a particular substrate is the competitive inhibition by alternative substrates. The inhibiting chemicals bind to the same site and are metabolized, or they may be activated by cytochrome P450 and then covalently bind to the heme moiety to cause an irreversible destruction of the hemeprotein and alteration of the enzyme (Netter, 1987). These inhibitors are known as "suicide inhibitors".

Cytochrome P450 exists in multiple forms, often exhibiting specific but overlapping substrate specificities. The differences in substrate specificity, positional specificity and stereo-specificity of the various forms of cytochrome P450 play an important role in regulating the balance between activation and inactivation of a given chemical (Lu and West, 1980). The metabolism of some test i.e., benzphetamine, substrates, benzo(a)pyrene, ethylmorphine, aminopyrine and aniline, are routinely used to assess hepatic drug-metabolizing activities in vitro. Activity of these enzymes gives an index of cytochrome P450-dependent N-dealkylation and hydroxylation reactions

which are involved in chloroquine metabolism.

2.Effect of Malaria Infection on Hepatic Drug-Metabolizing Enzymes in Their Hosts: Malaria infection is known to cause host hepatomegaly, to alter hepatic ultrastructure, and to disturb the normal physiological functions of the liver (Fletcher and Gilles, 1988; Boonpucknavig and Boonpucknavig, 1988). In this regard, electron microscopic studies revealed that malaria infections appear to cause a change in smooth-surfaced endoplasmic reticulum (SER), such that it appears very sparse and vesicular (Rosen et al., 1967). It is the SER that is involved in the metabolism of many foreign compounds (Fouts, 1961; Remmer and Merker, 1963; Fouts et al., 1966),

In addition to alterations in physiological and biochemical parameters in the liver, malaria infection greatly affects the activities of hepatic drug-metabolizing enzymes of the hosts, including phase I biotransformation processes and phase II conjugation reactions. Previous investigations have revealed that malaria infections suppress cytochrome P450 content and associated enzyme activities in experimental animal models. These results are summarized in Table 2. *Plasmodium berghei* and *P. yoelii* significantly lowered cytochrome P450 content and activities of ethylmorphine N-demethylase, aminopyrine N-demethylase, aniline hydroxylase and benzo(a)pyrene hydroxylase. (McCarthy et al., 1970; Sharma et al., 1978; Alvares et al., 1984; Singh et al., 1985; Saxena et al., 1987; Srivastava et al., 1991). The cytochrome P450 content and the associated enzyme <u>Table.2</u> Alteration in the host's hepatic microsomal MFOS during malaria infections.^a

Spcies	Experim.	Cyt.P450	b P450	EMD	APD	AH	BPH	Reference
	hosts		reduc	e.				
	(strain)							
P.yoelii	Mice	11.11	61.32		24.75	51.15	6.90	Saxena et al,
	(Swiss)							1987
P.berghei	Mice	70.36		68.72			62.78	Alvares et
	(NIH/NMRI)							al, 1984
P.berghei	Mice	65.19		55.84			78.41	
	(A/J)							
P.berghei	Mice	9.91			20.78	48.63	11.11	Srivastava
	(Swiss)							et al, 1991
P.berghei	Rat	61.40		27.91		62.50		McCarthy et
								al, 1970
P.berghei	Mice				55.03	30.41		Sharma et al,
	(Swiss)							1978
P.berghei	Mice				39.23	23.96		Singh et al,
	(Swiss)							1985

^aValues are for the infected host as a percentage of the control values.

^bCyt.P450=Cytochrome P450; P450 reduc.=Cytochrome P450 reductase; EMD=Ethylmorphine N-demethylase; APD=Aminopyrine N-demethylase; AH=Aniline hydroxlase; BPH=Benzo(a)pyrene hydroxylase. activities declined along with a concomitant rise in parasitemia (McCarthy et al., 1970; Saxena et al., 1987; Srivastava et al., 1991). In addition, Emudianughe et al. (1985) also showed a considerable decrease in the ability of livers from animals infected with *P. berghei* to convert benzoic acid to hippuric acid, a reaction which involves the phase II drug-metabolizing enzymes. These reductions paralleled increases in parasitemia. Singer (1963) argued that malaria infections reduced the coenzyme A (CoA) content of liver, thus reducing the amount of CoA available for activation of benzoic acid to benzoyl CoA. Benzoic acid binds with CoA prior to conjugation with glycine to form hippuric acid.

Although malaria infection reduced the activity of the microsomal enzymes, it did not prevent the hepatic cells from responding to induction by PB. Parasitized animals treated with barbiturate showed a marked increase in the metabolism of ethylmorphine and aniline (3.2- and 5.8-fold respectively) when compared to infected animals not receiving PB (McCarthy et al., 1970). When the infected mice were cleared of parasitemia by treatment with chloroquine, the altered levels of cytochrome P450 and related enzyme activities returned to almost normal (Saxena et al., 1987; Srivastava et al., 1991). It is clear that malaria infection decreases the hepatic microsomal drug metabolising enzyme activities of mice, and that such derangements appear to be reversible.

Alvares et al. (1984) suggested that the mechanism which may be responsible for the impairment of liver monooxygenases

during malaria infection is either the release of pharmacologically active substances or the production of the malaria pigment by the malaria parasites. The depression of cytochrome P450-dependent enzymatic activities by such pharmacologically active substances may be due to a perturbation of one or more components of the immune system. Insect venom, as well as Mycobacterium butyricum, markedly depressed cytochrome P450-dependent enzymatic activities and enhanced the activity of hepatic microsomal heme oxygenase, an enzyme which catalyzes the breakdown of heme, the prosthetic group of cytochrome P450, to biliverdin (Eiseman et al., 1982). This decreased hepatic monooxygenase activity may be a common property of all immunomodulators (Barnes et al., 1979). The parasites release heme or degradative products of hemoglobin containing heme. These are then transported into the liver cells and cause marked decreases liver cytochrome P450 content due to heme-induced in induction of microsomal heme oxygenase, which converts heme to biliverdin and subsequently to bilirubin (Muller-Eberhard et al., 1983).

3. Effect of Parasites other than Malaria Parasites on <u>Hepatic Drug-Metabolizing Enzymes in Their Hosts</u>: Other parasites demonstrate similarities to malaria parasites with regard to biosystematic status, host environments, and biological and biochemical characteristics. Therefore, it is of interest to examine the host effects of these other parasites on hepatic microsomal MFOS.

Many parasites exert a profound effect on the host MFOS

by altering the microsomal P450-dependent drug-metabolizing enzymes. Ten species or subspecies of various parasites impair cytochrome P450 and associated enzyme activities in the host liver. These parasites, listed in Table 3, belong to four classes: Nematoda, Trematoda, Mastigota and Rhizopoda. Unlike malaria parasites, some of these (e.g., *S. mansoni, F. hepatica, L. donovani* and *En. histolytica*), directly reside or deposit their eggs in the host's liver. The liver is the main site of the parasitic infection, and a severe and generalized impairment of hepatic drug-metabolizing ability occurs (Cha and Edwards, 1976; Facino et al., 1984; Coombs et al., 1990; Singh et al., 1989; Kumar et al., 1983).

In addition, these parasites cause structural damage. The intrahepatic migration of F. hepatica induces direct liver cell damage, blood stasis, portal eosinophilic infiltration, tortuous migration tunnels (Galtier et al., 1983), and ultrastructural alterations of endoplasmic reticulum (Facino et al., 1981). Toxic excretions of the flukes may lead to localized necrosis and ischaemic areas in which activities of drug-metabolizing enzymes associated with membranes of the endoplasmic reticulum have been shown to be affected (Galtier et al., 1983; Facino et al., 1981, 1984). Residence of L. donovani in hepatic Kupffer cells and granulomatous reaction within liver resulting from infection of S. mansoni may stimulate hypertrophy and hyperplasia of the Kupffer cells. The activated Kupffer cells release inflammatory mediators or cytolytic substances, which in turn cause impairment of MFOS activities in adjacent hepatocytes

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<u>Table.3</u> Alteration in the host's hepatic microsomal MFOS during parasitic infections.^a

Species	Experim.	Cyt.b	P450	APD	BPD	AH	BPH	References
	host	P450	reduc.					
Ancylostoma	Golden	35.60	100	37.38		112	32.38	Tekwani et
ceylanicum	hamster							al., 1990
Nippostrogylu	s Rat	48.51	86.51	34.84		51.52		Takwani et
brasiliensis	(Duckery)							al., 1987
Brugia	Rat	55.36	44.14	46.15		34.51		Srivastava
malayi								et al., 1986
Dipetalonema	Mastomys	42.94	106	58.46		57.32		Srivastava
viteae matalensis							et al., 1985	
Schistosoma	Mice			96.70		81.38		El-Bassiouni
mansoni	(Swiss)							et al., 1984
S. mansoni	Mice	41.80	38.30	27.10		23.50	21.00	Cha & Edward,
	(Swiss)							1976
Fasciola	Cattle	53.68	61.13	51.83		55.19		Facino et
hepatica								al., 1984
F. hepatica	Rat	60.34	95.29	13.64	46.67	59.04		Galtier et
	(Sprague-I	Dowey)						al.,1985,1986
Leishmania	Golden		95.73	83.94		61.57	69.74	Singh et
donovani	hamster							al., 1989
L. donovani	Mice	61.80	70.00	44.66	41.15	45.90		Coombs et
	(BALB/c)							al., 1990
Trypanosoma	Rat		110	59.43		46.50	83.33	Emerole et
brucei	(Wistar)							al., 1983
T. brucei	Mice	69.92				60.77	63.77	Shertzer et
gambiense	(Swiss)							al., 1981
T. brucei	Field	39.53	118	46.26	46.46	54.28	38.96	Shertzer et
gambiense	vole							al., 1982
Entamoeba	Golden	23.31		10.29		42.32		Kumar et
histolytica	hamster							al., 1983

^aValue are for the infected host as a percentage of the control values.

^bCyt.P450=Cytochrome P450; P450 reduc.=Cytochrome P450 reductase; APD=Aminopyrine N-demethylase; BPD=Benzophetamine N-demethylase; AH=Aniline hydroxylase; BPH=Benzo(a)pyrene hydroxylase. (Singh et al., 1989; Kyegombe et al., 1986).

Cha et al. (1980a, 1980b) studied athymic nude mice infected with pairs of male and female S. mansoni, in which the eosinophil population surrounding hepatic egg granuloma was dramatically reduced, and thymus-intact mice infected with either female or male S. mansoni, in which an accumulation of schistosome pigment without egg deposition occurred in the mouse livers. They found no depression of hepatic drug metabolizing enzymatic activities in the athymic nude mice, nor in either the female or male schistosome infections. They concluded that the depression of hepatic drug metabolizing function which occurred in the thymusintact mice infected with pairs of female and male S. mansoni (Cha and Edwards, 1976) was a response to the egg-induced inflammation rather than to direct effects of schistosome eggs or to deposition of schistosome pigment. They suggested that a likely candidate for a role in the depression of hepatic drug-metabolizing enzyme activities is the eosinophil, with its high content of peroxidase and variety of hydrolytic enzymes.

Parasites such as A. ceylanicum, N. brasiliensis, B. malayi, D. viteae, T. brucei and T. brucei gambiense do not live in and do not directly injure liver tissues. More specific and selective effects are encountered, and cytochrome P450 is also adversely affected in these parasitic infections. But other associated microsomal components, e.g., NADPH-cytochrome P450 reductase, are not significantly altered. This is in contrast to the parasitic infections such as hepatic amoebiasis, acute schistosomiasis, fascioliasis and visceral leishmaniasis (Tekwani et al., 1988; Cha and Edwards, 1976; Facino et al., 1984; Coombs et al., 1990). Hosts showed marked liver pathology accompanied by a reduction of both cytochrome P450 and cytochrome P450 reductase.

In filarial worm infections (B. malayi and D. vetene), the suppression of drug-metabolizing enzymes was most dramatic during the patent phase of the infection when the microfilariae are released into the circulation. The microsomal MFOS activities returned to normal when the release of microfilaria subsided (Srivastava et al., 1985; Srivastava et al., 1986). These studies indicate that filarial infections may release some toxic and/or pharmacologically active substances into the circulation. Intestinal infection by hookworm (A. ceylanicum) may also increase formation and release of lipid peroxides into the circulation. All of these may be involved in the impairment of MFOS activities. The impairment of cytochrome P450-dependent monooxygenase activities during hookworm infection appears to be specific, affecting mainly the catalytic site of cytochrome P450. The binding capacity of the iron atom of the heme is not altered. Pharmacological/ immunological mediators from parasites or host-parasitic interactions may be involved in selective denaturation and may exert their effect directly on cytochrome P450, or through alterations in the phospholipid environment of the MFOS (Ghezzo et al., 1986, Peterson and Renton, 1986).

In spite of the impairment of the MFO system during parasitic infections, the system can still be induced by certain chemical inducers (Galtier et al., 1987). Responsiveness of cytochrome P450 and associated enzyme activities to PB and 3-MC treatment was neither lost nor delayed during schistosomiasis, trypanosomiasis and hookworm infections (Table 4). Treatment of the host with 3-MC produced a marked increase in benzo(a)pyrene hydroxylase activity in livers of infected animals. In contrast, treatment with PB preferentially induced the activities of aminopyrine N-demethylase in infected animals. As expected, cytochrome P450 contents in livers of infected animals can be induced by both of PB and 3-MC. A complete restoration of hepatic drug metabolizing capacity followed a cure of schistosome infection with schistosomicidal drugs (Cha and Bueding, 1978; El-Bassiouni et al., 1984). Apparently the loss in cytochrome P450 and associated enzyme activities due to parasitic infection may be attributed to an accelerated degradation of cytochrome P450, rather than decreased synthesis of this hemeprotein (Facino and Carini, 1982). In other words, the synthesis of the components was preserved during this infection. Neither PB treatment nor 3-MC treatment induced the MFOS in rats infected with Fasciola hepatica (Table 4). This may be due to direct residence of the parasites in their host's liver and severe injury of the liver cells due to intrahepatic migration of the parasites (Facino et al., 1981).

Impairment of the MFOS during parasitic infections may
Species	Experim.P	arameter	PE	3 Treatm	nent	3-MC I	reatment	t
	host		Contr	. Infec	. Treat. ^a	Contr.	Infec.	Treat.
Ancylostoma	d _{Golden}	Cyt.P450 ¹	0 1.4	0.5	<u>2.1</u> ^c	1.2	0.4	1.1
ceylanicum	hamster	APD	1.5	0.6	2.5	1.6	0.5	0.2
		BPH	0.3	0.1	0.2	0.3	0.1	0.9
Schistosoma	^e Mice	Cyt.P450	67.6	35.0	166.0	63.0	37.2	64.7
mansoni	(Swiss)	APD	55.2	15.7	125.7	61.3	12.8	16.4
		BPH	1382	506	1310	1368	489	4523
Fasciola ^f	Rat	Cyt.P450	0.6	0.4	0.8	0.6	0.4	0.7
hepatica	(Sprague	APD	0.4	0.1	0.3	0.4	0.1	0.1
	Dawley)	BPH	1.5	0.7	2.4	1.5	0.7	0.5
Trypanosoma ^g	Rat	APD	198.2	117.8	324.5	198.2	125.0	202.0
b. brucei	(Wistar)	BPH	1.8	1.5	2.5	1.8	1.4	12.9

<u>Table.4</u>. Effect of inducers on P450-dependent drug metabolizing enzymes in parasitic infections

^aContr.=Control without infection and treatment group; Infec.=Infection without treatment group; Treat.=Treatment with infection group.

^bCyt.P450=Cytochrome P450; APD=Aminopyrine N-demethylase; BPH=Benzo(a)pyrene hydroxylase.

^CUnderlined data in treatment groups markedly increased comparing with that in infection groups and higher than that in control groups.

d_{Data} taken from Tekwani et al., 1990.

^eData taken from Cha, 1978.

^fData taken from Galtier et al., 1985.

gData taken from Emerole et al., 1983.

limit the ability of the host to metabolize drugs and xenobiotics, leading to their accumulation and adverse reactions in the host. In addition, impairment of the MFOS by parasitic infections may also affect the metabolism of endogenous substances. This derangement in MFOS activities may have severe pharmacological, physiological, and toxicological manifestations in the infected host.

D. Role of the mixed function oxidase system in insecticide resistance in insects: The MFOS is known to occur in over twenty species of insects, principally in the Diptera and Lepidoptera. In insects, an increasing number of biochemical functions have been ascribed to this enzyme system including the metabolism of a wide variety of endogenous substrates (i.e., insect hormones) and the many xenobiotics (i.e., oxidation of insecticides). Cytochrome P450 was first demonstrated in insects by Ray (1965, 1967). As in other animal groups, insect monooxygenases were found in many tissues and organs, and the highest concentrations were generally found in the midgut, fat body and malpighian tubules. At the sub-cellular level, insect monooxygenases were found in both the endoplasmic reticulum (Cassidy et al., 1969; Gilbert and Wilkinson, 1974) and mitochondria (Weirich et al., 1985). Like the mammalian system, insect microsomal monooxygenase systems consist of two components, the hemeprotein cytochrome P450 and the flavoprotein NADPH-cytochrome P450 reductase. The former exists as multiple isozymes displaying overlapping substrate specificities. Insect monooxygenases catalyze a wide variety

of biochemical oxidations resulting in detoxification or, occasionally, activation (Hodgson, 1985). These reactions have been classified as desulphuration and ester cleavage, epoxidation, aliphatic hydroxylation, aromatic ring hydroxylation, N-, O- and S-dealkylation, sulphoxidation, dehydrogenation and dioxole ring cleavage (Kulkarni and Hodgson, 1980).

Insect cytochrome P450 can be inducible by many types of endogenous and exogenous compounds. A number of similarities and differences exist between insects and mammals in their responses to traditional mammalian inducers of cytochrome P450. In both mammals and insects, PB and the cyclodiene insecticides are good inducers of cytochrome P450 (Yu and Terriere, 1972, 1973, 1977; Moldenke and Terriere, 1981). Hormonal induction of cytochrome P450 has been demonstrated in insects. In the housefly, cytochrome P450 and heptachlor epoxidase were induced by juvenile hormones and ecdysterone (Yu and Terriere, 1971). A genetic variation between the strains of Drosophila melanogaster investigated was observed. After PB treatment, the cytochrome P450 content and the various enzyme activities varied from non-responsiveness to a 4- to 5-fold increase in D. melanogaster (Hallstrom et al., 1984). PB caused a slight increase in the amount of cytochrome P450 and a marked increase in the epoxidase activities in the insecticide-susceptible strain of the housefly (Musca domestica), while the opposite occurred in the insecticide-resistant strain of housefly (Agosin 1982).

In the last several decades, many species of insects

have acquired resistance to insecticides. This resistance is inherited and has proven to be the biggest single barrier to successful chemical control of insects. Many behavioral, physiological and biochemical mechanisms have been implicated in this insecticide resistance (Hodgson, 1983, 1985; Brattsten et al., 1986). In many instances, the resistance to insecticides is due at least in part to increased levels of the MFOS. Changes in oxidative metabolism appear to be one of the major biochemical mechanisms in the resistance to carbamate insecticides, and are also involved in detoxication of organophosphates, DDT and pyrethroids.

The phenomenon that high levels of MFOS activities for a given insecticide are often paralleled by high LD_{50} values for the same insecticide has been mostly studied in houseflies. Cytochrome P450 content, the activity of Baygon (or Propoxur, a carbamate insecticide) hydroxylation and the LD₅₀ value for Baygon in insecticide-resistant strains of the housefly are 2-, 4- and 28.5-fold greater than those in insecticide-susceptible strains of the housefly, respectively (Agosin, 1982). Low levels of microsomal monooxygenase activity are usually associated with susceptibility to insecticides, whereas high monooxygenase activity correlated with varying degrees of insecticide resistance (Table 5). Higher P450 levels as well as differences in P450 catalytic and spectral characteristics have also been observed in insecticide-resistant strains of Μ. domestica. Such "qualitative" changes may result either from the increased expression of specific P450 genes or from the selection of

Strain	Su	bstrate Mon	ooxygenase	Activity	Cyt.P450	LD50
		(pm	ol/fly	(nmol/nmol	(nmol/fly)	(µg/fly)
			/min)	Cyt.P450)		
acbco	(S)C	Aldrin	18.70	0.43	0.04	<10 ^b
Fc	(R) ^d		183.30	1.83	0.10	>1000 ^b
Rutgers	(R)		573.80	4.94	0.12	>1000 ^b
NAIDM	(S)	Baygon	31.25	0.64	0.05	0.35
Rutgers	(R)	(Propoxur)	124.58	1.07	0.12	>10
Fc	(R)		81.60	0.82	0.10	2.70
CSMA	(S)	Diazinon	3.80	0.10	0.04	0.05
Rutgers	(R)		22.02	0.19	0.12	7.10
CSMA	(S)	EPN	4.79	0.12	0.04	2.49
Rutgers	(R)		8.35	0.07	0.12	21.79

<u>Tabel.5</u> Cytochrome P450 content, monooxygenase activity and resistance to insecticides in *Musca domestica* a

a. Data taken from Agosin, 1985.

b. LD₅₀ for Baygon (Propoxur).

c. S indicates insecticides-sensitive strain of Musca domestica.

d. R indicates insecticides-resistant strain of Musca domestica.

flies carrying a mutant P450 gene that would confer a selective advantage during insecticide exposure (Feyereisen et al., 1989). Eldefrawi et al. (1960) observed that a carbaryl-sesamex combination could reverse insecticide resistance, because sesamex, as an insecticide synergist, inhibited monooxygenase activity both *in vivo* (Sun and Johnson, 1960) and *in vitro* (Hodgson and Casida, 1960, 1961). Two protein bands, P450-A and P450-B, account for most of the cytochrome P450 in *D. melanogaster*. P450-A is ubiquitous among strains tested, whereas P450-B is unique to certain strains. A positive correlation was seen between resistance to phenylurea, P450-B expression, and dimethylnitrosamine demethylase activity. This indicated that P450-B expression was related to insecticide resistance (Waters and Nix, 1988).

SPECIFIC AIMS

Rodents and their malaria parasites have been widely employed as useful animal models in studies involving chemotherapy and drug resistance. In the present study, a *Plasmodium berghei*-mouse system was used as a model for the study of the interaction between malaria parasites and mammalian hosts. Studies to date on *P. berghei* have shown that there is an inverse relationship between resistance to chloroquine and virulence of infection. Experimental manipulation of *P. berghei* to select for chloroquine resistance is also associated with loss of virulence (Peters, 1964, 1968a; Jacobs, 1965; Thompson et al., 1965).

Preliminary studies from our laboratory showed that there was a very different growth pattern of parasites in mice infected with P. berghei RC strain (chloroquineresistant) and of Ρ. berghei N strain (chloroquinesensitive). The increase in the number of RC parasites in the infected mice proceeded more slowly than in the N strain. It was therefore of interest to determine the growth rate of both strains in infected mice using different inocula, and to determine the optimal inoculum doses of the parasites that could result in a predetermined level of parasitemia within similar time intervals. RC and N strains of malaria parasites break down hemoglobin into globin fragments and malarial pigment, hemozoin, which is taken up by the liver and spleen. It was also of interest to determine if these strains have differential effects on the hemoprotein, cytochrome P450, and associated drug-metabolizing enzymes in the livers of their

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hosts. These enzyme activities were determined using mice infected with both active and inactivated parasites. Inactivation was accomplished through irradiation with cobalt-60. Appropriate cobalt-60 doses needed to be determined, since there were no data available regarding differences in radiation doses required to kill the erythrocytic stages of chloroquine-resistant or chloroquinesensitive strains of *P. berghei*.

On the basis of these general aims, the following specific studies were to be carried out.

I. <u>Comparison of growth curves and virulence between</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei</u>

A. A comparison was made of growth curves in mice infected with chloroquine-sensitive or chloroquine-resistant *P. berghei* using different inoculum doses.

B. A comparison was made of virulence of the two strains based on infection rates and mortality of infected mice, and prepatent and patent periods of infection.

C. We determined inoculum doses of both strains of *P*. *berghei* needed to achieve a certain level of parasitemia in the infected mice.

II. <u>Comparison of the radiation sensitivity of the</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei</u>

A. An appropriate radiation dose that kills the erythrocytic stages of both strains of *P. berghei* was determined.

B. Survival of the two strains of P. berghei using

different radiation doses was compared.

III. <u>Determination of the effect of infection with irradiated</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei on cytochrome P450 and associated enzyme activities</u>

We determined differences, if any, in cytochrome P450 and associated enzyme activities between livers of mice injected with killed (by irradiation) chloroquine-sensitive or chloroquine-resistant strains of *P. berghei*, with irradiated non-parasitized erythrocytes or with phosphate buffer solution (PBS) controls. The comparison of hepatic cytochrome P450 and associated enzyme activities of mice injected with killed chloroquine-sensitive or chloroquineresistant strains of *P. berghei*, or irradiated nonparasitized erythrocytes was useful in determining if parasitic and erythrocytic protein cause a nonspecific alteration in liver mixed function oxidases.

IV. Effect of infection with chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* on cytochrome P450 and associated enzyme activities

We examined possible differences in liver cytochrome P450 and associated enzyme activities among mice infected with chloroquine-sensitive or chloroquine-resistant strains, and uninfected mice.

V. <u>Observation of alteration in hepatic cytochrome P450</u> <u>isozymes from mice infected with chloroquine-sensitive and</u> <u>chloroquine-resistant strains of P. berghei</u>

The cytochrome P450 system consists of several P450 isozymes. The effect of infection of mice with the chloroquine-resistant and chloroquine-sensitive strains on

the composition of cytochrome P450 isozymes was studied using SDS-PAGE gel electrophoresis.

VI. <u>Examination of gross changes in liver and spleen weights</u> of mice infected with the chloroquine-sensitive and <u>chloroquine-resistant strains of *P. berghei*</u>

Malaria parasites catabolize hemoglobin in red blood cells. The malaria pigment so formed and/or its breakdown products are taken up by the liver and spleen of their hosts. Since differences exist in the catabolism of hemoglobin by the various strains of *P. berghei*, these may affect the gross weight of these organs. It therefore was of interest to determine the gross weights of these organs in mice infected with either strain of the parasite. These were correlated with parasites which did produce pigment (N or drug-sensitive strain) and those that did not produce pigment (RC or drugresistant strain).

MATERIALS AND METHODS

A. <u>Animals and Parasites</u>: Female ICR mice, weighing 25 to 30 grams, were obtained from the Department of Animal Resources, Division of Veterinary Medicine, Walter Reed Army Institute of Research. They were provided with Rodent Laboratory Chow 5001 and an automatic water system. The mice were maintained at 21-23 °C with 43% relative humidity and a 12-hour light schedule (6:00 a.m. to 6:00 p.m.).

Plasmodium berghei N strain (chloroquine-sensitive) and P. berghei RC strain (chloroquine-resistant) parasites were obtained from Dr. David Walliker, Division of Biological Sciences, University of Edinburgh, UK. The P. berghei RC strain, derived from P. berghei N strain, was produced by treating infected mice with increasing doses of chloroquine parenterally and selecting for surviving parasites (Peters, 1965b). The ED_{50} of chloroquine (the dose which limits parasitemia to 50% of the level achieved in the chloroquine untreated group by a specified day) in each strain was determined with a four day suppressive test (Peters, 1965b).

Thirty mice were divided into six groups of five. Each mouse was infected with 10^7 red blood cells parasitized with *P. berghei* RC strain or *P. berghei* N strain diluted with PBS in a volume of 0.1 ml. The parasitized cells were administered intraperitoneally on day zero. Chloroquine was dissolved in a solution of one part 70% ethyl alcohol and three parts sterile PBS (pH 7.5). One of five chloroquine dosages was administered intraperitoneally to the infected

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mice once daily from day 0 to day 3. Untreated, infected controls were injected intraperitoneally with the solution used to dissolve the chloroquine. On day 4, blood from the tail vein was examined microscopically for parasitemia in Giemsa-stained thin blood smears. Elimination rates of parasitemia in chloroquine-treated groups were adjusted based on the parasitemia of the untreated control group. The ED50 was calculated using the probit method (Delaunois, 1973).

Infection of Mice: Mice were intraperitoneally в. injected with 0.25 ml of blood parasitized with P. berghei RC strain or P. berghei N strain which had been stored in -80°C freezer. To maintain its resistance against chloroquine, the donor mice infected with P. berghei RC strain received chloroquine at a dosage of 60 mg/kg daily by intraperitoneal injection. From day 2 post-injection, the parasitemia of the infected donor mice was monitored daily by microscopic examination of Giemsa-stained thin blood smears. When the parasitemia reached 10 to 20%, the blood of the donor mouse was drawn by cardiac puncture. Red blood cell densities were counted using a hemacytometer, and the density of parasitized erythrocytes was calculated. To prepare desired inoculum doses, the parasitized erythrocytes were diluted with sterile PBS (pH 7.5), and administered to mice by intraperitoneal injection in a total volume of 0.1 ml.

C. <u>Irradiation of Parasites and Erythrocytes</u>: Blood of infected mice with the chloroquine-sensitive or the chloroquine-resistant strain of *P. berghei* was drawn by cardiac puncture when the parasitemia reached 10 to 20%. Nonparasitized red blood cells were taken from uninfected mice of the same batch. One ml. of parasitized blood from the sensitive or the resistant strain and nonparasitized blood were put into separate 25 cm² polystyrene tissue culture flasks. The flasks containing the parasitized or nonparasitized blood were exposed to a cobalt-60 source at the required radiation doses using a Cobalt-60 Irradiator Model 220 (Atomic Energy of Canada LTD.) and housed in the Division of Instrumentation, Walter Reed Army Institute of Research. Radiation doses were calculated with the equation: $A_t = A_o e - 0.693 t/t_{1/2}$. In the equation, $A_t = activity$ of radiation at time t, A_o=starting activity of radiation, t=time and $t_{1/2}$ =half time.

D. Treatment of Animals:

1. Comparison of Growth Curve and Virulence between Chloroquine-sensitive and Chloroquine-resistant Strains of *P*. berghei: Eighty mice were divided into sixteen groups of five. Eight groups were infected with *P*. berghei RC strain and eight with *P*. berghei N strain, by intraperitoneal injection. Inocula of the RC strain and the N strain were 10^{0} , 10^{1} , 10^{2} , 10^{3} , 10^{4} , 10^{5} , 10^{6} and 10^{7} parasitized erythrocytes/mouse diluted in a volume of 0.1 ml with PBS. From the first day post-inoculation, blood from the tail vein was microscopically examined daily for parasitemia in Giemsastained thin blood smears, until the death of infected mice or the disappearance of the parasites from the blood smears. The parasitemia was expressed as a percentage of parasitized erythrocytes based on counting 200 red blood cells. If no malaria parasite was found in 100 microscopic fields (x1,000), parasitemia was considered to be negative. The experiment was repeated three times.

2. Comparison of Sensitivity to Irradiation between <u>Chloroquine-sensitive</u> and Chloroquine-resistant strains of *P*. berghei: Sixty-four mice were divided into eight groups of eight. Three groups were injected with irradiated P. berghei RC strain, another three groups with irradiated P. berghei N strain, and the remaining two groups were control mice infected with non-irradiated P. berghei RC strain or P. berghei N strain. Red blood cells parasitized with P. berghei RC strain or P. berghei N strain had been irradiated with gamma radiation provided by cobalt-60; radiation doses were 10, 15 and 20 kilorads. Inocula of both the irradiated and the non-irradiated P. berghei RC strain or N strain consisted of 10⁶ parasitized erythrocytes for each mouse diluted with PBS in a volume of 0.1 ml. From the first day postinoculation, blood from the tail vein of each mouse was examined microscopically for parasitemia. The parasitemia from mice injected with the irradiated or the non-irradiated P. berghei RC strain was determined every six days and that from mice injected with the irradiated or the non-irradiated P. berghei N strain every two days, since there were differences in growth rate and patent period between both strains of the parasites.

3. Effect of Infections with Irradiated Chloroquinesensitive and Chloroquine-resistant Strains of *P. berghei* on Cytochrome P450 and Associated Enzyme Activities: Sixty mice were separated into five groups of twelve. Red blood cells parasitized with *P. berghei* RC strain or *P. berghei* N strain,

irradiated with a cobalt-60 source at 20 kilorads to kill the parasites, were injected into treated mice using an inoculum of 10⁶ RC strain parasitized red blood cells per mouse, or 10^2 N strain parasitized red blood cells per mouse. These inoculation doses were chosen to match the inoculation doses used in experiments measuring cytochrome P450 and enzyme activity. Another two groups of mice were inoculated with normal red blood cells irradiated with a cobalt-60 source at 20 kilorads, using an inoculum of 10^6 or 10^2 red blood cells per mouse. Mice injected with PBS (pH 7.5) were used as controls in this set of experiments. Ten or thirteen days after injection, the mice were weighed and killed, and their livers and spleens were excised and weighed. Serum levels of glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) were determined from blood samples, and hepatic cytochrome P450 content and P450-dependent enzyme activities were determined from liver samples that had been frozen at -20°C for seven days or less.

4. Effects of Chloroquine-sensitive and Chloroquineresistant Strains of *P. berghei* Infections on Cytochrome P450 and Associated Enzyme Activities: Thirty-six mice were divided into three groups of twelve. One group was infected with *P. berghei* RC strain, one with *P. berghei* N strain, and the third group of uninfected mice were used as controls. The inoculum of the *P. berghei* RC strain was 10⁶ parasitized red blood cells per mouse diluted in a volume of 0.1 ml PBS, and that of the *P. berghei* N strain 10² parasitized red blood cells per mouse diluted in a volume of 0.1 ml PBS. The day of inoculation was considered as day zero. The parasitemia of the infected mice was monitored by microscopic examination of Giemsa-stained thin blood smears obtained from tail veins every other day beginning on day 2 post-inoculation. After day 8 post-inoculation, blood smears were examined for parasitemia every day. The mice were killed when their parasitemia reached 20-30%, usually between days 9 and 13 post-inoculation. Livers and spleens were weighed, and the blood used for assays of SGOT and SGPT level, and livers for assays of cytochrome P450 content and P450-dependent enzyme activities. All livers were frozen at -20°C and analyzed within a week after being removed.

E. Preparation of Hepatic Microsomes: Livers from two mice were pooled, weighed and homogenized in 3 volumes of 1.15% KCl solution (w/v) in a glass tube with a teflon homogenizer. The homogenate was centrifuged in a Sorvall RC5- B centrifuge at 9,000 x g for 20 minutes. The 9,000x g supernatant was collected and centrifuged in a Beckman L5-50 centrifuge at 105,000x g for one hour to sediment the microsomes. The 105,000x g supernatant was discarded and the pellets were washed by resuspending them in 1.15% KCl solution and centrifuging at 105,000x g for one hour again. The microsomal pellets obtained were suspended in 0.1 M potassium phosphate buffer (pH 7.4) to get a 25% microsomal suspension (liver equivalent to 250 mg, wet weight/ml of 0.1 M phosphate buffer) and assayed for cytochrome P450 content, benzphetamine N-demethylase and benzo(a)pyrene hydroxylase activities, and protein content (Alvares et al., 1984). All

enzyme assays were performed at 0-4°C.

F. Enzyme Assays:

1. <u>Cytochrome P450</u>: Cytochrome P450 was measured using the carbon monoxide (CO) difference spectrum of reduced microsomes, and cytochrome P450 content was assayed using microsomes equivalent to 100 mg of liver, wet weight. The hemeprotein content was determined by the method of Omura and Sato (1964) using an extinction coefficient of 91 mM^{-1} cm⁻¹ between 450 and 500 nm.

2. Benzphetamine N-demethylase: Benzphetamine is Ndemethylated by liver microsomes with the methyl group $(-CH_3)$ converted to HCHO, which is measured colorimetrically. N-demethylase activity was determined using hepatic microsomes equivalent to 250 mg liver, wet weight. The incubation mixture contained 10 μ mol MgCl₂, 37.5 μ mol semicarbazide HCl to trap HCHO, 200 μ mol of Na₂HPO₄-KH₂PO₄ buffer (pH 7.4) and an NADPH regenerating system, 2 units of glucose-6-phosphate dehydrogenase, 2 μmol of NADP and 20 μmol of glucose-6-phosphate. The samples were incubated for 15 minutes at 37°C. HCHO in the presence of the Nash's reagent forms a yellow chromogen which has an absorbance at 412 nm. N-demethylase activity was measured as described by Alvares and Mannering (1970), using the modified Nash's reagent described by Anders and Mannering (1966).

3. <u>Benzo(a)pyrene hydroxylase</u>: The reaction mixture contained 3 μ mol MgCl₂, 1 μ mol NADPH, 100 μ mol Na₂HPO₄-KH₂PO₄ buffer (pH 7.4), liver microsomes equivalent to 4 mg liver, wet weight, and 100 μ mol of benzo(a)pyrene in a total volume

of 1.0 ml. The samples were incubated in the dark for 5 minutes at 37°C. The reaction was stopped by the addition of 1.0 ml cold acetone. The amount of phenolic metabolites formed was measured as described by Nebert and Gelboin (1968). The fluorescence of the products formed was determined using a Perkin Elmer MPF-44A fluorescence spectrophotometer, slit width 6 nm, excitation wavelength of 396 nm and emission wavelength of 522 nm. The fluorescence intensity of the phenolic products formed was determined using 3-hydroxybenzo(a)pyrene as a standard.

4. Serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT): In each treatment, sera pooled from two mice were assayed. Activities of SGOT and SGPT were assayed using a KODAK EKTACHEM 500 Analyzer (Eastman Kodak Company, Rochester, NY) located in the Clinical Pathology Laboratory, Division of Pathology, Walter Reed Army Institute of Research. To assay SGPT, alanine and a-ketoglutaric acid were added to serum samples to form pyruvic acid. To assay SGOT, aspartic acid and aketoglutaric acid were added to serum samples to form oxaloacetic acid. The oxaloacetic or pyruvic acid formed in reactions the above was reacted with 2, 4 dinitrophenylhydrazine to form phenylhydroazones. The color intensity of the reaction products was read at 340 nm wavelength (Frankel, 1970).

G. <u>Protein content of liver microsomes</u>: Protein determinations were performed by Lowry's method (1951), using the Folin phenol reagent. Liver microsomes equivalent to 1 mg liver, wet weight, were reacted with the Folin phenol reagent following alkaline copper treatment. The color obtained was measured at 750 nm using a Gilford 250 UV/VIS spectrophotometer. Bovine serum albumin (BSA, 0.4 mg/ml) was used as a standard. Standard curves were generated using 0.1 to 0.4 mg BSA/ml.

SDS-PAGE Electrophoresis: Sodium dodecyl sulfateн. polyacrylamide gel (SDS-PAGE) electrophoresis was carried out as described by Laemmli (1970). The stacking gel contained 6% and the separating gel contained 9% acrylamide. Three liver microsomal samples from each group of mice (2 infected and 1 uninfected) were selected for running the gel electrophoresis. Each sample was diluted to 60 micrograms of microsomal protein in 30 microliters of the sample buffer to give a final concentration of 0.0625 M Tris HCl (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue as the dye. The diluted samples were boiled for 5 minutes in a water bath to denature the proteins and applied to the bottom of the wells of the gel plate. Dalton Mark VII-L (SIGMA Chemical Company, St. Louis, MO) was used as a molecular weight mark. Electrophoresis was carried out at 60 V during stacking and at 120 V during separation until the tracking dye reached the bottom of the gel. Protein bands were stained with 0.25% Coomassie Blue and placed on a slowly rocking platform at room temperature for two hours. The stained gel plate was destained in a methanol/acetic acid solution on a slowly rocking platform at room temperature overnight. After destaining, the gel was dried using a Gel Dryer at 80°C for 120 minutes.

I. Statistics: Differences between experimental groups

were calculated using analysis of variance (ANOVA) or the Student's t test, with a p value of 0.05 or less as the level of significance.

RESULTS

A. <u>Comparison of growth rate and virulence between</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei</u>: At all inoculum doses studied, the growth of the P. <u>berghei</u> chloroquine-sensitive strain was much faster than that of the P. berghei chloroquine-resistant strain. In the former, peak parasitemia (over 60%) occurred 7 to 13 days post-infection (Fig. 1 and 2). But the parasitemia of the P. berghei chloroquine-resistant strain peaked at a maximum of 20% after 18 days post-infection (Fig. 3 and 4).

Mean patent period of infection was defined as the interval from appearance of malaria parasites in the blood stream of the mice to death of the mice or to disappearance of the parasites from the blood stream of the mice. This in mice infected with the chloroguine-sensitive period strain was significantly shorter than that in mice infected with the chloroquine-resistant strain (t=12.26, DF=14, P<0.01). They were 7.28 and 18.67 days, respectively (Table 6). Even though only one parasite was inoculated, all the mice infected with the chloroquine-sensitive strain were dead following peak parasitemia; mortality was 100%. In contrast, most of mice infected with the chloroquine-resistant strain were free of blood parasites in about 30 days, and their mortality was only 2.50% (Table 6). Various inoculum doses were used, but the size of inoculum did not have a significant effect on the patent period of infection (Fig.5) nor on the mortality of infected mice (Fig. 6) in either

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Fig.2 Growth curves of chloroquine-sensitive strain of *P. berghei* at inoculum doses of 10^{4} - 10^{7} parasites. Each value represents the mean for 15 animals.



Fig.3 Growth curves of chloroquine-resistant strain of *P.berghei* at inoculum doses of $10^{0}-10^{3}$ parasites. Each value represents the mean for 15 animals.



Fig.4 Growth curves of chloroquine-resistant strain of *P. berghei* at inoculum doses of 10^4-10^7 parasites. Each value represents the mean for 15 animals.

<u>Table.6</u> Prepatent period, patent period of infection, infection rate, and mortality of mice infected with either the chloroquine-resistant or the chloroquine-sensitive strain of *P. berghei.*^a

Parameters	Sensitive strain	Resistant strain	
Prepatent period ^b	3.60	4.52	
Days	<u>+</u> 0.98	<u>+</u> 1.16	
Patent period ^C	7.28	18.67	
Days	<u>+</u> 0.27 ^d	<u>+</u> 0.89	
Infection rate	82.50	78.33	
%	<u>+</u> 10.15	<u>+</u> 11.32	
Mortality %	100.00 ^d <u>+</u> 1.75	2.50	

a. Each value represents mean \pm S.E. of 8 means.

b. Prepatent period indicates interval between inoculation of mice and appearance of malaria parasites in blood stream of the mice.

- c. Patent period indicates interval from appearance of malaria parasites in blood stream of mice to death of the mice or to disappearance of the parasites from the blood stream of the mice.
- d. Value was significantly different from that obtained from mice infected with resistant strain (P<0.01).



Fig.5 Patent period of infection with the *P. berghei* chloroquine-sensitive and the chloroquine-resistant strains

Each value represents the mean \pm S.E. for

15 animals. N strain indicates the chloroquine-sensitive strain. RC strain indicates the chloroquine-resistant strain. Patent period indicates interval from appearance of malaria parasites in blood stream of mice to death of mice or disappearance of parasites from the blood stream.



Fig.6 Mortality of mice infected with the *P. berghei* chloroquine-sensitive or the chloroquine-resistant strain.

Each value was calculated with 15 animals. N strain indicates chloroquine-sensitive strain. RC strain indicates chloroquineresistant strain. strain of *P. berghei*. The virulence of *P. berghei* chloroquine-sensitive strain was higher than that of *P. berghei* chloroquine-resistant strain in mice.

There was no significant difference in mean prepatent period of infection, i.e., interval between inoculation of mice with the malaria parasites and appearance of the parasite in the blood stream of the mice, between mice infected with the chloroquine-sensitive and chloroquineresistant strains. Their prepatent periods were 3.60 days and 4.52 days, respectively (Table 6). In both strains of *P. berghei*, there was a significant negative correlation between the prepatent period of infection and the logarithm of the number of parasitized red blood cells inoculated with a correlation coefficient (r) of -0.99 for the sensitive strain and -0.97 for the resistant strain (P<0.01). When the inoculum size was increased by a factor of 10, the prepatent period of infection decreased by approximately one day in the chloroquine-sensitive strain (Table 7 and Fig. 7).

Mean infection rate of mice with the chloroquinesensitive strain was not significantly different from that of mice infected with the chloroquine-resistant strain. The former was 82.50% and the latter 78.33% (Table 6). There also was a significant positive correlation between the infection rate of mice and the logarithm of the number of parasitized red blood cells injected into the mice in both of the strains of *P. berghei* (Table 7, 8 and Fig. 8). Their correlation coefficients (r) were 0.82 in mice infected with the sensitive strain and 0.86 in mice infected with the resistant strain (P<0.01). <u>Table.7</u> Prepatent period, patent period of infection, infection rate, and mortality of mice infected with the chloroquine-sensitive strain of *P. berghei* at various dosages.

Dosage	Prepatent	Patent	Infection	Mortality
Parasites	period ^a	period ^b	rate	of infected mice
/mouse	Days ^C	Days ^C	ક્ષ	8
100	7.33 <u>+</u> 0.33	6.00 <u>+</u> 0.58	20	100
101	6.22 <u>+</u> 0.15	7.56 <u>+</u> 0.90	60	100
10 ²	5.85 <u>+</u> 0.19	6.77 <u>+</u> 0.38	87	100
10 ³	4.36 <u>+</u> 0.32	7.07 <u>+</u> 0.29	93	100
104	2.87 <u>+</u> 0.19	7.73 <u>+</u> 0.38	100	100
10 ⁵	1.73 <u>+</u> 0.23	8.00 <u>+</u> 0.40	100	100
106	0.40 <u>+</u> 0.16	8.27 <u>+</u> 0.40	100	100
107	0	6.80 <u>+</u> 0.31	100	100

- a. Prepatent period indicates interval between inoculation of mice and appearance of malaria parasites in blood stream of the mice.
- b. Patent period indicates interval from appearance of malaria parasites in blood stream to death of mice.
- c. Each value represents mean \pm S.E. of 15 determinations.



Fig.7 Prepatent period of infection with the *P. berghei* chloroquine-sensitive or the chloroquine-resistant strain.

Each value represents the mean \pm S.E. for

15 animals. N strain indicates chloroquinesensitive srain. RC strain indicates chloroquine-resistant strain. Prepatent period indicates interval between inoculation of mice with malaria parasites and appearance of the parasites in blood stream. <u>Table.8</u> Prepatent period, patent period of infection, infection rate, and mortality of mice infected with the chloroquine-resistant strain of *P. berghei* at various dosages.

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Dosage Parasites	Prepatent period ^a	Patent period ^b	Infection rate	Mortality of infected mice	
/mouse	Days ^C	Days ^C	8	8	_
100	8.00	17.75 <u>+</u> 3.77	26.67	0	
101	7.60 <u>+</u> 0.40	15.20 <u>+</u> 1.46	33.33	0	
10 ²	7.10 <u>+</u> 0.50	18.90 <u>+</u> 1.63	66.67	0	
10 ³	6.27 <u>+</u> 0.44	15.93 <u>+</u> 0.61	100	0	
104	4.80 <u>+</u> 0.47	17.53 <u>+</u> 1.20	100	0	
10 ⁵	1.93 <u>+</u> 0.30	21.93 <u>+</u> 2.70	100	0	
106	0.47 <u>+</u> 0.22	20.53 <u>+</u> 1.67	100	13.33	
107	0	21.60 <u>+</u> 1.76	100	6.67	

a. Prepatent period indicates interval between inoculation of mice and appearance of malaria parasites in blood stream.

- b. Patent period indicates interval from appearance of malaria parasites in blood stream to death of mice or to disappearance of parasites from blood stream.
- c. Each value represents mean \pm S.E. of 15 determinations.



Fig.8 Infection rate of mice infected with the *P. berghei* chloroquine-sensitive or the chloroquine resistant strain.

Each value was calculated with 15 animals.

N strain indicates chloroquine-sensitive strain. RC strain indicates chloroquineresistant strain. In comparing different inoculum doses, mice infected with 10^6 parasites of the *P. berghei* chloroquine-resistant strain achieved the highest peak of 20% parasitemia at 18 days post-infection (Fig. 3). However, with inoculum doses of the chloroquine-sensitive *P. berghei*, parasitemia of mice reached 20% in 10 days post-infection or longer (Fig. 2). Dosage of 10^2 parasitized red blood cells had the highest infection rate of the mice, 87% (Table 7).

B. <u>Comparison of sensitivity to irradiation of the</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei</u>:

Fifteen kilorads of radiation from a cobalt-60 source killed all 10^6 parasites of chloroquine-sensitive *P. berghei*, determined by an infection rate of zero when mice were injected with the irradiated parasites. In contrast, the infection rate of mice injected with chloroquine-resistant *P. berghei* irradiated at 15 kilorads was 12.50%, indicating that some of the parasites survived. It required 20 kilorads of radiation from cobalt-60 source to kill all 10^6 parasites of chloroquine-resistant *P. berghei* (Table 9 and Fig. 9).

For the chloroquine-resistant strain, the relation between infection rates of mice and radiation doses received by the parasites was linear, with an equation of Y=111.07-5.42X (R=0.91). In both the chloroquine-resistant and the chloroquine-sensitive strains, the patent period of infection was decreased with an increased radiation dose, but the prepatent period of infection was increased (Table 9). 82

<u>Table.9</u> Vitality of irradiated chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* at various radiation doses.^a

Strain	Radiation doses Kilorad	Infection rate %	Mortality %	Prepatent period Days	Patent period Days
Resistant	20	0	0	0	0
strain	15	12.50	0	12	12
	10	87.50	0	9 <u>+</u> 0.65	14.14 <u>+</u> 1.08
	0 b	100	12.50	0	26.63 <u>+</u> 2.69
Sensitive	20	0	0	0	0
strain	15	0	0	0	0
	10	100	100	6.50 <u>+</u> 0.19	7.63 <u>+</u> 1.39
	0 b	100	100	0	9.25 <u>+</u> 0.82

a. Each value represents mean \pm S.E. of 8 determinations.

b. A radiation dose of zero means non-irradiated parasites were used as control.



Fig.9 Infection rate of irradiated chloroquineresistant and chloroquine-sensitive *P. berghei* at various radiation doses. Each bar was calculated with 8 animals.
C. Effect of infection with irradiated chloroquinesensitive and chloroquine-resistant strains of *P. berghei* on cytochrome P450 and associated enzyme activities: In all of the mice injected with the chloroquine-sensitive or chloroquine-resistant strain of *P. berghei* irradiated at 20 kilorads with the cobalt-60, parasitemia was checked at the time of sacrifice to confirm that no malaria parasites were found in their blood smears.

There were no significant differences in the size and color of the livers among the mice injected with irradiated Ρ. parasites of either strain, irradiated berghei erythrocytes, or PBS. Mean liver weights ranged from 45.85 to 48.61 mg/g body weight (Table 10 and Fig. 10). As shown in Table 10 and Fig. 11, mean spleen weights from mice infected with irradiated chloroquine-sensitive or chloroquineresistant strains of *P. berghei* or 10⁶ irradiated red blood cells were increased by 24-37% compared to mice injected with 10² irradiated erythrocytes or PBS (P<0.05). No significant differences in mean spleen weights were observed between the mice injected with the irradiated chloroquine-sensitive strain or the irradiated chloroquine- resistant strain of P. berghei or 10⁶ irradiated red blood cells, or between mice injected with 10² irradiated red blood cells and PBS. There were also no significant differences in mean microsomal protein concentrations of livers among the variously treated mice when compared to mice injected with PBS (Fig. 12). Their mean hepatic microsomal protein concentrations ranged from 27.11 to 31.61 mg/g liver weight (Table 10).

<u>Table.10</u> Hepatic microsomal protein concentration, average liver weight and average spleen weight of mice injected with irradiated *P. berghei* (chloroquine-sensitive or chloroquineresistant strain), irradiated erythrocytes, or PBS.^a

Inocula	Dosage	Protein	Liver weight	Spleen weight	
	Parasites	concentration	mg/g body wt.	mg/g body wt.	
	/mouse	mg/g liver wt			
b			40 61 1 20	4 70.0 20 g	
IR ~	100	27.87 <u>+</u> 0.81	48.61 <u>+</u> 1.32	$4.70 \pm 0.39^{\circ}$	
IS ^c	10 ²	28.65 <u>+</u> 2.15	48.21 <u>+</u> 0.84	5.20 <u>+</u> 0.33 ^g	
IE.6 ^d		27.11 <u>+</u> 2.06	46.25 <u>+</u> 1.80	4.72 <u>+</u> 0.30 g	
IE.2 ^e		31.61 <u>+</u> 2.20	45.85 <u>+</u> 1.19	3.85 <u>+</u> 0.17	
PBS f		29.89 <u>+</u> 0.60	47.05 <u>+</u> 1.04	3.81 <u>+</u> 0.19	

a. Livers from 2 mice were pooled for each determination. Each value represents mean <u>+</u> S.E. of at least 6 determinations.

b. IR indicates irradiated P. berghei chloroquine-resistant strain.

c. IS indicates irradiated P. berghei chloroquine-sensitive strain.

d. IE.6 indicates 10⁶ irradiated erythrocytes.

e. IE.2 indicates 10^2 irradiated erythrocytes.

f. PBS indicates phosphate buffer solution.

g. Value was significantly different from that obtained from mice injected with 10^2 irradiated erythrocytes or PBS (P<0.05).



Fig. 10 Mean liver weights of mice injected with irradiated rodent malaria parasites, irradiated rodent red blood cells, or PBS.

Each bar represents the mean \pm S.E. for 12 animals. IR indicates mice injected with irradiated chloroquine-resistant strain. IS indicates mice injected with irradiated chloroquine-sensitive strain.IE.6 indicates mice injected with 10⁶ of irradiated mouse red blood cells.IE.2 indicates mice injected with 10² of irradiated mouse red blood cells. PBS indicates mice injected with phosphate buffer solution.





Each bar represents the mean \pm S.E. for 12 animals. See legend of Fig.10 for abbreviations used in this figure. Asterisk represents a value significantly different from value obtained from mice injected with 10^2 of irradiated red blood cells of mice or PBS control (P<0.05).



Fig.12 Microsomal protein concentrations of livers from mice injected with irradiated rodent malaria parasites, irradiated rodent erythrocytes, or PBS

Each bar represents the mean \pm S.E. for 6 determinations. See legend of Fig.10 for abbreviations used in this figure.

The hepatic cytochrome P450 content of mice injected with irradiated chloroquine-sensitive or chloroquineresistant strains of *P. berghei*, as well as irradiated red blood cells, was decreased by about 15% compared to mice injected with PBS (P<0.05). However, there was no significant difference in hepatic cytochrome P450 content between mice injected with irradiated strains of *P. berghei* (chloroquineresistant and -sensitive) and mice injected with irradiated red blood cells. Their cytochrome P450 contents were 0.64-0.66 nmol/mg protein (Table 11 and Fig. 13).

Hepatic benzo(a)pyrene hydroxylase activity of mice injected with the irradiated chloroquine-sensitive or chloroquine-resistant strain of *P. berghei* or 10^2 irradiated red blood cells was decreased by 23-30% compared to mice injected with PBS (P<0.05). However, there was no significant difference in hepatic benzo(a)pyrene hydroxylase activity between groups of mice injected with irradiated *P. berghei* parasites or irradiated red blood cells. The benzo(a)pyrene hydroxylase activity in these groups of mice ranged from 0.18 to 0.23 pmol OHBP formed/mg protein/minute (Table 11 and Fig. 14)

There was no significant difference in hepatic benzphetamine N-demethylase activity among mice injected with irradiated *P. berghei* parasites, irradiated red blood cells or PBS, except for mice injected with 10² irradiated red blood cells. Their benzphetamine N-demethylase activities ranged from 0.22 to 0.26 µmol HCHO formed/mg protein/hour. The hepatic benzphetamine N-demethylase activity of mice 90

<u>Table.11</u> Cytochrome P450 contents and monooxygenase activities in livers of mice injected with irradiated *P*. *berghei* (chloroquine-sensitive or chloroquine-resistant strain), irradiated erythrocytes, or PBS.^a

Inocula	Dosage	Cytochrome P450	Benzo(a)pyrene	Benzphetamine
	Parasites	nmol/mg protein	hydroxylase	N-demethylase
	/mouse		pmol OHBP/mg	µmol HCHO/mg
			protein/min.	protein/hr.
IR b	106	0.64 <u>+</u> 0.02 g	0.18 <u>+</u> 0.01 g	0.24 <u>+</u> 0.01
IS ^c	10 ²	0.64 <u>+</u> 0.02 g	0.20 <u>+</u> 0.03 g	0.23 <u>+</u> 0.02
IE.6 ^d		0.66 <u>+</u> 0.04 ^g	0.23 <u>+</u> 0.02	0.22 <u>+</u> 0.02
IE.2 ^e	1	0.66 <u>+</u> 0.01 g	0.18 <u>+</u> 0.02 g	0.19 <u>+</u> 0.02 g
PBS f		0.77 <u>+</u> 0.02	0.26 <u>+</u> 0.02	0.26 <u>+</u> 0.01

- a. Livers from 2 mice were pooled for each determination. Each value represents mean \pm S.E. of 6 determinations.
- b. IR indicates irradiated P. berghei chloroquine-resistant strain.
- c. IS indicates irradiated P. berghei chloroquine-sensitive strain.
- d. IE.6 indicates 10⁶ of irradiated erythrocytes.
- e. IE.2 indicates 10^2 of irradiated erythrocytes.
- f. PBS indicates phosphate buffer solution.
- g. Value was significantly different from that obtained from mice injected with PBS (P<0.05).



Fig.13 Hepatic microsomal cytochrome P450 content of mice injected with irradiated *P. berghei* (chloroquine-resistant or chloroquine-sensitive strain), irradiated erythrocytes, or PBS. Each bar represents the mean <u>+</u> S.E. for 6 determinations. See legend of Fig.10 for abbreviation used in this figure. Asterisk represents a value significantly different from value obtained with PBS control (P<0.05).





Each bar represents the mean \pm S.E. for 6 determinations. See legend of Fig.10 for abbreviation used in this figure. Asterisk represents a value significantly different from value obtained with PBS control (P<0.05). injected with 10^2 irradiated red blood cells was decreased by 27% compared to mice injected with PBS (Table 11 and Fig. 15).

Serum glutamic-pyruvic transaminase (SGPT) levels of mice injected with the irradiated resistant strain of *P. berghei* were about twice those of the other groups of mice (P<0.05; Table 12 and Fig.16). Serum glutamic-oxalacetic transaminase (SGOT) levels of mice injected with the irradiated resistant strain were 30% greater than that of mice injected with 10^6 irradiated mouse erythrocytes (P<0.05). (Table 12; Fig. 17). There was no significant difference in SGPT and SGOT levels between mice injected with the irradiated sensitive *P. berghei* and mice injected with irradiated mouse erythrocytes or PBS (Table 12; Fig. 16 and 17). However, the SGOT levels of mice injected with 10^2 irradiated mouse erythrocytes were significantly greater than those of mice injected with 10^6 irradiated mouse erythrocytes (P<0.05) (Table 12; Fig. 17).

D. Effect of infection with chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* on cytochrome <u>P450 and associated enzyme activities</u>: The ED_{50} of *P. berghei* RC strain and *P. berghei* N strain were 102.93 mg/kg (Y=7.66-1.32X, R²=0.56) and 0.95 mg/kg (Y=4.95-1.80X, R²=0.74), respectively. The index of chloroquine resistance for the *P. berghei* RC strain was 108.35. In all of the experiments, the average parasitemia of variously infected mouse groups ranged from 20.55% to 23.83%. There were no significant differences



Fig.15 Hepatic benzphetamine N-demethylase activity of mice injected with irradiated *P. berghei* (chloroquine-resistant or chloroquine-sensitive strain), irradiated erythrocytes, or PBS

Each bar represents the mean \pm S.E. for 6 determinations. See legend of Fig.10 for abbreviation used in this figure. Asterisk represents a value significantly different from value obtained with PBS control (P<0.05).

Table.12 SGOT and SGPT levels of mice injected with the irradiated P. berghei (chloroquine-sensitive or chloroquineresistant strain), irradiated erythrocytes, or PBS.^a

Groups	Dosage parasites/mouse	Parasitemia %	SGPT Units/liter	SGOT Units/liter	
Irradiated resistant strain	10 ⁶	0	78.67 ^b <u>+</u> 12.70	210.17 ^C <u>+</u> 19.80	
Irradiated Sensitive strain	10 ²	0	36.33 <u>+</u> 7.30	192.17 <u>+</u> 17.41	
10 ⁶ irradiat erythrocyte	ed es		37.20 <u>+</u> 0.97	160.40 <u>+</u> 10.68	
10 ² irradiat erythrocyte	ed es		38.83 <u>+</u> 6.95	216.00 ^d <u>+</u> 25.96	
PBS			45.25 <u>+</u> 1.78	171.63 <u>+</u> 6.85	

- a. Each value represents mean \pm S.E. of at least 5 determinations.
- b. Value was significantly greater than that from all other groups of mice studied (P<0.05).
- c. Value was significantly greater than that from mice injected with 10^6 irradiated erythrocytes (P<0.05).
- d. Value was significantly greater than that from mice injected with 10^6 irradiated erythrocytes or PBS (P<0.05).





Each bar represents the mean \pm S.E. of at least 5 determinations. See legend of Fig.10 for abbreviations used in this figure. Asterisk represents value significantly different from values of IS, IE.6, IE.2 and PBS (P<0.05).





Each bar represents the mean \pm S.E. of at least 5 determinations. See legend of Fig.10 for abbreviations used in this figure. Asterisk represents value significantly different from value of IE.6 (P<0.05). Cross represents value significantly different from value of IE.6 and PBS (P<0.05). in the average parasitemia among the infected mouse groups (P>0.05) (Table 13 and 14).

Upon microscopic examination, differences in morphologic features of the erythrocytic stages were found between the chloroquine-sensitive and chloroquine-resistant strains. In the chloroquine-sensitive strain, dark-brown malarial pigment was found scattered within the blue cytoplasm of immature schizonts and late trophozoites (Fig. 18A), and a mass of golden brown pigment was located in the center of a mature schizont, which was surrounded by merozoites (Fig. 18B). In contrast, no pigment was found within the cytoplasm of immature schizonts and late trophozoites in the chloroquineresistant strain. Unstained vacuoles were observed in the blue cytoplasm (Fig. 19A). In mature schizonts, merozoites were found to surround a large unstained vacuole, but no pigment was found in the cytoplasm (Fig. 19B).

Differences in the size and color of livers and spleens were observed among mice infected with chloroquine-resistant or chloroquine-sensitive *P. berghei* and uninfected mice (Fig. 20 and Table 13). Livers and spleens from mice infected with the resistant strain were the largest; those from mice infected with the sensitive strain were intermediate and those from the uninfected control were the smallest. The color of livers and spleens of mice infected with the resistant strain was reddish brown; those from the sensitive strain were chocolate brown and those of uninfected mice brownish (Fig. 20). When compared to the uninfected controls, the average liver weight from mice infected with the 99

<u>Table.13</u> Hepatic microsomal protein concentration, average liver weight and average spleen weight of mice infected with the chloroquine-sensitive, the chloroquine-resistant strain of *P. berghei*, and uninfected mice.

Strain	Parasitemia	Protein concentration	Liver weight	Spleen weight
	୫	mg/g liver	mg/g body wt.	mg/g body wt.
Experiment 1 ^a	ı			
Resistant strain	22.33 <u>+</u> 0.85	23.80 <u>+</u> 1.08 ^C	79.15 <u>+</u> 2.06 ^d	42.34 <u>+</u> 1.97 ^d
Sensitive strain	23.83 <u>+</u> 0.91	25.38 <u>+</u> 0.98 ^C	60.30 <u>+</u> 1.60 ^C	10.14 <u>+</u> 0.35 ^C
Uninfected mice		36.44 <u>+</u> 1.78	47.00 <u>+</u> 1.20	3.30 <u>+</u> 0.12
Experiment 2 ^k)			
Resistant strain	21.79 <u>+</u> 0.96	27.41 <u>+</u> 1.55 ^C	65.16 <u>+</u> 1.76 ^d	
Sensitive strain	21.17 <u>+</u> 1.15	28.34 <u>+</u> 0.99 ^C	55.69 <u>+</u> 1.05 ^c	N. D. ^e
Uninfected mice		38.38 <u>+</u> 1.40	42.28 <u>+</u> 0.69	
a. Livers f	rom 2 mice wer	e pooled for ea	ch determinatio	n. Each value
represen	nts mean <u>+</u> S.E.	of 6 determina	tions.	
b. One liver was assayed for each determination. Each value				
represents mean <u>+</u> S.E. of at least 12 determinations				

- c. Value was significantly different from value obtained from uninfected mice (P<0.01).
- d. Value was significantly different from value obtained from mice infected with the chloroquine-sensitive strain and uninfected mice (P<0.01).</p>
- e. N. D. indicates not determined.

<u>Table.14</u> Cytochrome P450 contents and monooxygenase activities in livers of mice infected with the chloroquine-sensitive, the chloroquine-resistant strain of *P. berghei*, and uninfected mice.

Strain	Parasitemia	Cytochrome P450	Benzo(a)pyrene hydroxylase	Benzphetamine N-demethylase
	*	nmol/mg protein	pmol OHBP/mg	µmol HCHO/mg
			protein/min.	protein/hr.
Experiment 1	 la			
Resistant strain	22.33 <u>+</u> 0.85	0.57 <u>+</u> 0.02	0.21 <u>+</u> 0.01	0.25 <u>+</u> 0.01 ^d
Sensitive strain	23.83 <u>+</u> 0.91	0.48 <u>+</u> 0.02 ^C	0.19 <u>+</u> 0.01 ^C	0.18 <u>+</u> 0.01
Uninfected mice		0.61 <u>+</u> 0.03	0.25 <u>+</u> 0.02	0.15 <u>+</u> 0.01
Experiment 2	3p			
Resistant strain	21.79 <u>+</u> 0.96	0.34 <u>+</u> 0.06(9)		0.26 <u>+</u> 0.04(6) ^d
Sensitive strain	21.17 <u>+</u> 1.15	0.39 <u>+</u> 0.15(4)	N. D. ^e	0.14 <u>+</u> 0.01(6)
Uninfected mice		0.54 <u>+</u> 0.08(8)		0.14 <u>+</u> 0.01(6)

- a. Livers from 2 mice were pooled for each determination. Each value represents mean <u>+</u> S.E. of 6 determinations.
- b. One liver was assayed for each determination. Each value represents mean <u>+</u> S.E. of the number of determinations indicated in brackets.
- c. Value was significantly different from value obtained from uninfected mice (P<0.05).
- d. Value was significantly different from value obtained from mice infected with chloroquine-sensitive strain and uninfected mice (P<0.01).</p>
- e. N. D. indicates not determined.

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Fig.18 Microscopic photographs of chloroquinesensitive *Plasmodium berghei* (N strain) (X 1,000). The thin blood smear was stained with Giemsa. In picture A, an immature schizont and late trophozoites contain dark brown malaria pigment. In picture B, a mature schizont (arrow) contains a mass of golden brown malaria pigment.



Fig.19 Microscopic photographs of chloroquineresistant *Plasmodium berghei* (RC strain) (X 1,000). The thin blood smear was stained with Giemsa. In picture A, an immature schizont and late trophozoites contain clear vacuoles but not malaria pigment. In picture B, a mature schizont without malaria pigment (arrow) is observed.



Fig. 20 Photograph of liver (bottom) and spleen (top) of a mouse infected with chloroquineresistant P. berghei (A), chloroquine-sensitive P. berghei (B) and uninfected mouse (C).

resistant strain was increased by 54-68% (P<0.01) and that from mice infected with the sensitive strain was increased by 28-31% (P<0.01). In comparing average liver weights between mice infected with either strain, the liver weights were 17-31% larger in the mice with the resistant strain (P<0.01). The spleen weights of mice infected with the resistant strain were increased about 13-fold and those from mice infected with the sensitive strain were increased about 3-fold compared to the uninfected controls (P<0.01) Moreover, in comparing average spleen weights between mice infected with either strains, the mean spleen weight was 4.2-fold larger in mice with the resistant strain. (P<0.01) (Table 13 and Fig. 21).

Protein concentration of liver microsomes from mice infected with the resistant strain or the sensitive strain was significantly less than from the uninfected mice (P<0.01). The protein concentration from mice infected with the resistant strain was only 65-71% of that from the uninfected controls, and not significantly different from that of mice infected with the sensitive strain, which was 70-74% of that from the uninfected mice (Table 13 and Fig. 22).

The hepatic cytochrome P450 content, expressed as nmol P450/mg protein, of mice infected with the sensitive strain was significantly decreased (P<0.01) compared to that of the uninfected controls. While the cytochrome P450 content of mice infected with the resistant strain was also decreased when compared to the controls, the difference was not statistically significant (Table 14 and Fig. 23).



Fig.21 Liver and spleen weights of mice infected with chloroquine-resistant or chloroquinesensitive *P. berghei* and uninfected mice. Each bar represents the mean \pm S.E. for 12 animals. Asterisk represents value significantly different from the organ weights of uninfected mice (P<0.01).





Each bar represents the mean \pm S.E. for 6 determinations. Asterisk represents value significantly different from value of uninfected mice (P<0.01).



Fig.23 Hepatic cytochrome P450 content of mice infected with the chloroquine-resistant, the chloroquine-sensitive *P. berghei*, and uninfected mice.

Each bar reparesents the mean \pm S.E. of 6 determinations. Livers from 2 mice were pooled for each determination. Asterisk represents value significantly different from value of uninfected mice (P<0.01). Hepatic benzo(a)pyrene hydroxylase activity of mice infected with the sensitive strain was decreased by 25% compared to that of the uninfected controls (P<0.05). Although the hydroxylase activity of mice infected with the resistant strain was also decreased, the difference was not statistically significant when compared to the uninfected controls (Table 14 and Fig. 24).

The hepatic benzphetamine N-demethylase activity of mice infected with the resistant strain was about 76% greater than that of the uninfected mice and about 59% greater than that of the mice infected with the sensitive strain (P<0.01). The hepatic N-dealkylase activity of mice infected with the sensitive strain was not significantly different from that of the uninfected controls (Table 14 and Fig. 25).

Data expressed as weight of whole liver are summarized in Table 15. The liver weight of mice infected with the resistant strain was 38% greater than that of mice infected with the sensitive strain and 56% greater than that of the uninfected controls (P<0.01). Although the livers of mice infected with the sensitive strain appeared to be larger than that of the uninfected mice, the differences were not statistically significant. Total amounts of microsomal protein, cytochrome P450 content and benzo(a)pyrene hydroxylase activity of mice infected with the sensitive strain were significantly lower than those obtained from livers of uninfected mice and mice infected with the resistant strain (P<0.01, Table 15). There were no significant differences for these parameters between mice





Each bar represents the mean \pm S.E. of 6

determinations. Livers from 2 mice were pooled for each determination. Asterisk represents value significantly different from value of uninfected mice (P<0.01).



Fig.25 Hepatic benzphetamine N-demethylase activity of mice infected with the chloroquine-resistant, the chloroquine-sensitive P. berghei, and uninfected mice.

Each bar represents the mean \pm S.E. of 6

determinations. Livers from 2 mice were pooled for each determination. Asterisk represents value significantly different from value of mice infected with the sensitive strain and uninfected mice (P<0.01). <u>Table.15</u> Total amount of cytochrome P450 content and monooxygenase activities in livers of mice infected with the chloroquine-sensitive, the chloroquine-resistant strain of *P*. *berghei*, and uninfected mice.^a

Strain Pa	rasitemia %	Liver weight g	Microsomal protein mg/liver	P450 ^b nmol /liver	BPHase pmol OHBP/ liver/min.	BPDase µmol HCHO/ liver/hr.
Resistant	22.33	1.74 d	41.29 ^c	23.55	c 8.77	c 10.29 d
strain	<u>+</u> 0.85	<u>+</u> 0.06	<u>+</u> 1.73	<u>+</u> 1.73	<u>+</u> 0.66	<u>+</u> 0.49
Sensitive	23.83	1.26	31.89	15.32	6.14	5.61
strain	<u>+</u> 0.91	<u>+</u> 0.04	<u>+</u> 1.25	<u>+</u> 0.67	<u>+</u> 0.47	<u>+</u> 0.34
Uninfected		1.11	40.21 c	24.44	c 10.22	c 6.13
mice		<u>+</u> 0.05	<u>+</u> 0.94	<u>+</u> 1.03	<u>+</u> 0.70	<u>+</u> 0.40

- a. Livers from 2 mice were pooled for each determination. Each value represents mean<u>+</u>S.E. of 6 determinations.
- b. P450 indicates cytochrome P450 content; BPHase indicates benzo(a)pyrene hydroxylase activity and BPDase indicates benzphetamine N-demethylase activity.
- c. Value was significantly greater than value obtained from mice infected with the sensitive strain of *P. berghei* (P<0.01).
- d. Value was significantly greater than value obtained from mice infected with the sensitive strain of *P. berghei* and uninfected mice (P<0.01).</p>

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infected with the resistant strain and the uninfected controls. Total amount of benzphetamine demethylase activity of mice infected with the resistant strain was 83% greater than that of mice infected with the sensitive strain and 68% greater than that obtained using the uninfected controls (P<0.01). There was no significant difference in total amount of benzphetamine demethylase activity between mice infected with the sensitive strain and the uninfected controls.

Serum glutamic-pyruvic transaminase (SGPT) enzyme levels of mice infected with the resistant strain were about twice the SGPT levels of the control mice (P<0.05), whereas, the SGPT levels of mice infected with the sensitive strain was 3fold greater than the serum levels of uninfected mice (P<0.05). Serum glutamic-oxalacetic transaminase (SGOT) levels of mice infected with the sensitive strain or the resistant strain were increased by 76% and 61% respectively compared to those of the uninfected mice (P<0.05; Table 16 and Fig. 26).

The SDS-PAGE electrophoresis gel of hepatic microsomes from the infected and uninfected mice is shown in Fig. 27. The electrophoretic pattern indicated that the band in the 45 kDa molecular weight region was darker in mice infected with the resistant strain than those from mice infected with the sensitive strain or uninfected mice.

E. <u>Hepatic cytochrome P450 content and monooxygenase</u> activities of mice infected with the chloroquine-sensitive or <u>chloroquine-resistant strain of P. berghei compared to that</u> <u>obtained from mice injected with irradiated parasites or</u> 113

<u>irradiated red blood cells</u>: In order to exclude nonspecific <u>Table.16</u> SGOT and SGPT levels of mice infected with the chloroquine-sensitive, the chloroquine-resistant strain of

Strain	Parasitemia	SGPT	SGOT
	%	Units/liter	Units/liter
Resistant strain	21.73	83.24	291.00
	<u>+</u> 0.95	<u>+</u> 3.80 [°]	<u>+</u> 16.05 ^C
Sensitive strain	22.25	142.32	317.00
	<u>+</u> 1.13	<u>+</u> 12.06 ^b	<u>+</u> 27.34 ^C
Uninfected mice		44.00 <u>+</u> 1.24	180.53 <u>+</u> 13.38

P. berghei, and uninfected mice^a

a. Each value represents mean <u>+</u> S.E. of at least 19 determinations.

- b. Value was significantly greater than that from mice infected with the resistant strain and uninfected mice (P<0.05).
- c. Value was significantly greater than that from uninfected mice.





Each bar represents the mean \pm S.E. for at least 19 animals. Asterisk represents value significantly different from value obtained with uninfected mice (P<0.05).



Fig.27 SDS-PAGE of liver microsomes from mice infected with chloroquine-resistant or chloroquine-sensitive *P. berghei* and uninfected mice. Well R contained microsomes from mice infected with the resistant strain, wells N from mice infected with the sensitive strain and wells C from the uninfected mice respectively. Well M contained molecular weight standards: bovine albumin 66K; egg albumin 45K; glyceraldehyde-3-p-dehydrogenase 36K; bovine carbonic anhydrase 29K and bovine pancrease trypsinogen 24K.

effects of injected rodent malaria parasites' protein and the rodent erythrocytes' protein on cytochrome P450 and associated enzyme activities in the infected mice, comparisons were made. Data obtained from mice infected with chloroquine-sensitive or chloroquine-resistant strains of *P. berghei* were expressed as total amount per liver and compared with those from mice injected with irradiated rodent malaria parasites or irradiated rodent red blood cells. Results are summarized in Tables 17 and 18.

In mice infected with the chloroquine-resistant strain, all determined parameters except cytochrome P450, i.e., total liver weight (Fig. 28), total spleen weight (Fig. 29), microsomal protein (Fig. 30), benzo(a)pyrene hydroxylase activity (Fig. 32) and benzphetamine N-demethylase activity (Fig. 33), were significantly increased by 40-70%, 8-10 fold, 25-27%, 35-50% and 30-65%, respectively, when compared to those in mice injected the irradiated parasites or the irradiated erythrocytes (P<0.05). Total hepatic cytochrome P450 content of mice infected with the resistant strain was not significantly different from those of mice injected with the irradiated parasites or the irradiated erythrocytes (Fig. 31). Compared to mice injected with PBS, total liver weight, total spleen weight, microsomal protein and benzphetamine N-demethylase activity of mice infected with the resistant strain were significantly enhanced by 50%, 10 fold, 20% and 15%, respectively (P<0.05)(Fig. 28-30 and 33), but their cytochrome P450 content (Fig. 31) and benzo(a)pyrene hydroxylase activity (Fig. 32) were not significantly changed.

In mice infected with the chloroquine-sensitive strain,

<u>Table.17</u> Comparison of total hepatic microsomal protein, liver weights and spleen weights of mice infected with the chloroquine-sensitive or the chloroquine-resistant strain of *P. berghei* to that obtained from mice injected with irradiated *P. berghei* (chloroquine-sensitive or chloroquineresistant strain), irradiated erythrocytes, or PBS.^a

Parasitemia	Microsomal	Liver weight	Spleen weight
۶	protein mg/liver	grams	grams
22.33 <u>+</u> 0.85	41.29 <u>+</u> 1.73	1.74 <u>+</u> 0.06 ^d	0.93 <u>+</u> 0.04 ^d
23.83 <u>+</u> 0.91	31.89 <u>+</u> 1.25	1.26 <u>+</u> 0.04	0.21 <u>+</u> 0.01 ^f
	40.21 <u>+</u> 0.94 ^C	1.11 <u>+</u> 0.05	0.08 <u>+</u> 0.002
0	33.03 <u>+</u> 1.95	1.19 <u>+</u> 0.06	0.12 <u>+</u> 0.01
0	32.42 <u>+</u> 1.74	1.14 <u>+</u> 0.03	0.12 <u>+</u> 0.01
	32.77 <u>+</u> 2.60	1.24 <u>+</u> 1.11	0.12 <u>+</u> 0.01
	32.49 <u>+</u> 3.24	1.02 <u>+</u> 0.05 ^e	0.09 <u>+</u> 0.01
	34.18 <u>+</u> 1.19	1.17 <u>+</u> 0.05	0.09 <u>+</u> 0.002
	Parasitemia % 22.33±0.85 23.83±0.91 0 0 0 	Parasitemia Microsomal protein mg/liver % mg/liver 22.33±0.85 41.29±1.73 23.83±0.91 31.89±1.25 40.21±0.94 ^C 0 33.03±1.95 0 32.42±1.74 32.77±2.60 34.18±1.19	Parasitemia Microsomal protein mg/liver Liver weight grams 22.33±0.85 41.29±1.73 1.74±0.06 ^d 23.83±0.91 31.89±1.25 1.26±0.04 40.21±0.94 ^c 1.11±0.05 0 33.03±1.95 1.19±0.06 0 32.42±1.74 1.14±0.03 32.77±2.60 1.24±1.11 32.49±3.24 1.02±0.05 ^e 34.18±1.19 1.17±0.05

a. Each value represents mean <u>+</u> S.E. of 6 determinations.

b. RS=chloroquine-resistant P. berghei; SS=chloroquine-sensitive
 P. berghei; UC=uninfected mice (control); IR=irradiated chloroquine-resistant strain; IS=irradiated chloroquine-sensitive strain;
 IE.6=10⁶ irradiated erythrocytes; IE.2=10² irradiated erythrocytes and PBS=phosphate buffer solution.

- c. Value was significantly different from those of SS, IR, IS, IE.6, IE.2 and PBS (P<0.05).
- d. Value was significantly different from those of SS, UC, IR, IS, IE.6, IE.2 and PBS (P<0.05).</p>
- e. Value was significantly different from those of SS and IE.6 (P<0.05).</p>
- f. Value was significantly different from those of UC, IR, IS, IE.6, IE.2 and PBS (P<0.05).</p>

<u>Table.18</u> Comparison of total amount of cytochrome P450 and monooxygenase activities in livers of mice infected with the chloroquine-sensitive or the chloroquine-resistant strain of *P. berghei* to that obtained from livers of mice injected with irradiated *P. berghei* (chloroquine-sensitive or chloroquineresistant strain), irradiated erythrocytes, or PBS.^a

Inocula ^b	Parasitemia	Cytochrome P450	Benzo(a)pyrene	Benzphetamine
			hydroxylase	N-demethylase
	ક	nmol/liver	pmol OHBP/liver	μ mol/HCHO/liver
			/min.	/hr.
RS	22.33 <u>+</u> 0.85	23.55 <u>+</u> 1.73	8.76 <u>+</u> 0.66 ^e	10.28 <u>+</u> 0.499
SS	23.83 <u>+</u> 0.91	15.32 <u>+</u> 0.67 ^C	6.13 <u>+</u> 0.47	5.61 <u>+</u> 0.34 ^h
UC		24.43 <u>+</u> 1.03	10.22 <u>+</u> 0.70 ^f	6.13 <u>+</u> 0.40
IR	0	21.27 <u>+</u> 1.60	6.03 <u>+</u> 0.38	7.75 <u>+</u> 0.41 ⁱ
IS	0	20.88 <u>+</u> 1.89	6.54 <u>+</u> 1.22	7.13 <u>+</u> 0.44
IE.6		21.84 <u>+</u> 2.59	7.41 <u>+</u> 0.55	6.99 <u>+</u> 0.62
IE.2		21.35 <u>+</u> 2.35	5.82 <u>+</u> 0.99	6.24 <u>+</u> 0.20
PBS		26.76 <u>+</u> 1.04 ^d	10.70 <u>+</u> 0.67 ^f	8.80 <u>+</u> 0.46j
a. Each va	alue represent	ts mean <u>+</u> S.E. o	of 6 determinatio	ns.
b. RS=chlo	proquine-resis	stant P.berghei;	SS=chloroquine-	sensitive
P. bergi	hei. UC=uninfo	ected mice (cont	rol); IR=irradia	ted chloroquine-
resistar	nt strain; IS:	=irradiated chic	proquine-sensitiv	e strain;
IE.6=10	0° irradiated	erythrocytes; 1	E.2=10 ² irradiate	ed erythrocytes
	vas significa	aller solution. atly different f	from those of RS.	UC. TR. TS.
IE.6.	IE.2 and PBS	(P<0.05).		00, 11, 10,
d. Value v	was significan	ntly different f	from those of IR,	IS and IE.2
(P<0.05	b). Wag significat	atly different f	from those of SS	TR TC and
IE.2 (1	P<0.05).	, arrecent i		IN, 15, and
f. Value v	was significan	ntly different f	from those of SS,	IR, IS, IE.2

- and IE.6 (P<0.05). g. Value was significantly different from those of SS, UC, IR, IS,
- IE.6, IE.2 and PBS (P<0.05).
 h. Value was significantly different from those of IR, IS ans IE.6
 (P<0.05).</pre>
- i. Value was significantly different from those of UC and IE.2 (P<0.05).
- j. Value was significantly different from those of SS, UC, IS, IE.6 and IE.2. (P<0.05).



Fig.28 Comparison of total liver weights of mice infected with the *P. berghei* resistant strain or sensitive strain to that of mice injected with irradiated rodent malaria parasites, irradiated erythrocytes, or PBS.

Each bar represents the mean \pm S.E. of 6 determinations. RS indicates mice infected with chloroquine-resistant strain. SS indicates mice infected with chloroquine-sensitive strain. UC indicates control uninfected mice. IR indicates mice injected with irradiated chloroquine-resistant strain. IS indicates mice injected with irradiated chloroquine-sensitive strain. IE.6 indicates mice injected with 106 mouse irradiated erythrocytes. IE.2 indicates mice injected with 102 mouse irradiated erythrocytes. PBS indicates mice injected with phosphate buffer solution for control. Asterisk represents value significantly different from value of SS, IR, IS, IE.6, IE.2, UC and PBS (P<0.05). Cross represents value significantly different from value of SS and IE.6 (P<0.05).


Fig.29 Comparison of total spleen weights of mice infected with the *P. berghei* resistant strain or sensitive strain to that of mice injected with irradiated rodent malaria parasites, irradiated erythrocytes, or PBS.

Each bar represents the mean <u>+</u> S.E. of 6 determinations. See legend of Fig.28 for abbreviations used in this figure. Asterisk represents value significantly different from value of SS, IR, IS, IE.6, IE.2, UC and PBS (P<0.05). Cross represents value significantly different from value of UC, IR, IS, IE.6, IE.2 and PBS (P<0.05).





Each bar represents the mean \pm S.E. of 6 determinations. See legend of Fig.28 for abbreviations used in this figure. Asterisk represents value significantly different from value of SS, IR, IS, IE.6 IE.2 and PBS (P<0.05).



Fig.31 Comparison of total hepatic cytochrome P450 content of mice infected with the *P. berghei* resistant strain or sensitive strain to that of mice injected with irradiated rodent malaria parasites, irradiated erythrocytes, or PBS.

Each bar represents the mean \pm S.E. of 6 determinations. See legend of Fig.28 for abbreviations used in this figure. Asterisk represents value significantly different from value of RS, UC, IR, IS, IE.6, IE.2 and PBS (P<0.05). Cross represents value significantly different from value of IR, IS and IE.2 (P<0.05).



Fig.32 Comparison of total hepatic benzo(a)pyrene hydroxylase activities of mice infected with the *P. berghei* resistant strain or sensitive strain to that of mice injected with irradiated rodent malaria parasites, irradiated erythrocytes, or PBS. Each bar represents the mean \pm S.E. of 6 determinations. See legend of Fig.28 for abbreviations used in this figure. Asterisk represents value significantly different from value of SS, IR, IS and IE.2 (P<0.05). Cross represents value significantly different from value of SS, IR, IS, IE.6 and IE.2 (P<0.05).



Fig.33 Comparison of total hepatic benzphetamine N-demethylase activities of mice infected with the *P. berghei* resistant strain or sensitive strain to that of mice injected with irradiated rodent malaria parasites, irradiated erythrocytes, or PBS.

Each bar represents the mean <u>+</u> S.E. of 6 determinations. See legend of Fig.28 for abbreviations used in this figure. Asterisk represents value significantly different from value of SS, IR, IS, IE.6, IE.2, UC and PBS (P<0.05). Cross represents value significantly different from value of RS, IR, IS, IE.6 and PBS (P<0.05). Triangle represents value significantly different from value of RS, SS, UC, IS, IE.6 and IE.2 (P<0.05). Spot represents value significantly different from value of RS, SS, UC, IE.2 and PBS (P<0.05).

total spleen weight was 1-2 fold greater than that of mice injected with the irradiated parasites or the irradiated erythrocytes (P<0.05) (Fig. 29). In contrast, the total cytochrome P450 content (Fig. 31) and benzphetamine Ndemethylase activity (Fig. 33) were significantly decreased by 25-30% and 20-30%, respectively, compared to mice injected with irradiated rodent malaria parasites or irradiated erythrocytes (P<0.05). However there were no significant differences in total liver weight (Fig. 28), microsomal protein (Fig. 30), or benzo(a)pyrene hydroxylase activity (Fig. 32) between mice infected with the chloroquinesensitive strain and mice injected with the irradiated parasites or the irradiated erythrocytes. Compared to mice injected with PBS, total spleen weight of mice infected with the chloroquine-sensitive strain was increased by 10 fold (Fig. 29), and cytochrome P450 content (Fig. 31), benzo(a)pyrene hydroxylase activity (Fig. 32) and benzphetamine N-demethylase activity (Fig. 33) were all increased by about 40% (P<0.05). Total liver weight (Fig. 28) and microsomal protein (Fig. 30) were not significantly changed.

There were no significant differences in total liver weight (Fig. 28), total spleen weight (Fig. 29), cytochrome P450 content (Fig. 31) and benzo(a)pyrene hydroxylase activity (Fig. 33) between uninfected controls and mice injected with PBS. However, a comparison of livers from uninfected controls showed that the total microsomal protein (Fig. 30) was 20% greater and the benzphetamine N-demethylase activity (Fig. 33) was 30% less than that of mice injected with PBS (P<0.05). When compared to mice injected with the irradiated malaria parasites or the irradiated erythrocytes, total liver weight, total spleen weight and cytochrome P450 content of uninfected control were not significantly changed (Fig. 28, 29 and 31), and their microsomal protein (Fig. 30) and benzo(a)pyrene hydroxylase activity (Fig. 32) were increased by 20-25% and 40-75%, respectively (P<0.05). There was no significant difference in benzphetamine N-demethylase activity between uninfected controls and mice injected with the irradiated chloroquine-sensitive strain or irradiated erythrocytes, although the enzyme activity of uninfected with irradiated chloroquine-resistant strain (P<0.05) (Fig. 33).

DISCUSSION

A. <u>Differences in growth rate and virulence between</u> <u>chloroquine-sensitive and chloroquine-resistant strains of P.</u> <u>berghei and determination of suitable inocula</u>:

Previous studies revealed that acquisition of resistance chloroquine was usually associated with a to lowered virulence of the parasite in mice (Peters, 1964, 1968a; Jacobs, 1965; Thompson et al., 1965 and, Rabinovich and Tikhomirova, 1973.). Peters (1965a) observed that infection with chloroquine-sensitive P. berghei (N strain) followed an acute course with death of all the infected mice by the 9th day post-infection. The course of infection with chloroquineresistant P. berghei (RC strain) was much slower. Some mice infected with the RC strain died by the end of the third week of infection, but most mice recovered spontaneously by about the 30th day. In the present studies, the growth of chloroquine-resistant P. berghei (RC strain) was also much slower than that of chloroquine-sensitive P. berghei (N strain). Infection with the N strain reached parasitemia of over 60% in 7 to 13 days post-infection. In contrast, infection with the RC strain reached a parasitemia of only 20% in 18 days post-infection. Mortality of mice infected with the N strain was 100% with survival of only 10.9 days (S.E.=0.85 days), even when only one parasite was inoculated per mouse. Survival was much greater for mice infected with the RC strain, with only 2.5% mortality and most mice recovering (i.e., complete absence of parasitemia) without medication within 30 days post-infection.

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As in many infectious diseases, severity of the outcome of malaria infection is influenced by the number of parasites in the original inoculum, virulence of the particular strain of parasite, and the immunological ability of the host to defend itself against the parasites. Virulence of the parasites depends on their rate of replication or growth and on their inherent pathogenicity. Host defenses depend on genetic factors, immunological factors, nutritional status and, for humans, socio-environmental factors (Peters, 1968a; Greenwood et al., 1991; Lines and Armstrong, 1992). Differences in mortality between mice infected with the N strain and the RC strain may be attributed to the rapid growth of the N strain. The N strain killed the hosts before they had time to produce an effective immunological response (Peters, 1968a).

Taylor et al. (1951) found that the prepatent period of infections with *P. gallinaceum* in chickens was in inverse relation to the logarithm of the number of parasitized erythrocytes inoculated. Warhurst and Folwell (1968) also reported that the relation between prepatent period and the logarithm of the number of parasitized cells injected was found to be linear in chloroquine-sensitive and chloroquineresistant strains of *P. berghei*. In the present studies, the mortality of infected mice and the patent period of infection were not affected by the size of the inoculum. In contrast, the inoculum size of the parasites had a significant effect on infection rate and prepatent period of the infection. There was a significant positive correlation between the infection rate and the inoculum size and a significant negative correlation between the prepatent period and the inoculum size. There were no significant differences in either mean infection rate or mean prepatent period of the infection between both strains. These results imply that infection rate and prepatent period might depend more on the quantity of parasites inoculated than on the virulence of the parasites. On the other hand, the dissimilarity of the two strains' effects on mortality and patent period are probably inherent in the virulence of the strains.

Large inoculum size might be important in two ways. Large groups of the parasites inoculated by several mosquitoes would tend to contain a wider range of genetic variants, and would thus be more likely to include a variant the host has not yet encountered. Alternatively, when a new variant is encountered, the likelihood that parasitemia will reach detectable densities before checked by immune responses may depend on how many of the new variants are present in the inoculum (McGregor, 1965; Marsh, 1992; Lines and Armstrong, 1992). In addition, the size of inoculum would have some influence on the time that elapses before a level of parasitemia is detectable (Greenwood et al., 1991). The larger the size of an inoculum, the higher the infection rate of mice and the shorter the prepatent period of infection. Once the mouse is infected, surviving parasites would tend to express their inherited properties of virulence as a roughly constant mortality and patent or survival period for a particular strain. The mortality rate and patent (or survival) periods of infection could be better measurements of parasite's virulence. We observed that the mortality of mice infected with the RC strain was significantly lower than that of the N strain, and that the patent period of infection with the RC strain was significantly longer than that of the N strain. Rabinovich and Tikhomirova (1973) explained that reduction of virulence in the chloroquine-resistant strain of *P. berghei* could be the result of the metabolic cost of resistance as well as the parasite's increased preference for reticulocytes, which constitute a less favorable environment for development.

The size of the inoculum of chloroquine-resistant parasites affected the peak parasitemia reached in the mice. Unexpectedly, those which received 10^7 parasites reached a lower parasitemia than those which received 10^6 or 10^5 parasites. Mortality of mice inoculated with 107 parasites was also lower than that of mice inoculated with 10^6 parasites. One possible explanation for these phenomena is that a protective factor had been produced in the donor hosts and then transfered with their body fluids, since mice inoculated with more parasites received more total volume of body fluids from donor mice. We did not detect any antibody against the RC strain of P. berghei using an indirect fluorescent antibody assay in mice recovered from infection with RC strain, even though these mice were protected from subsequent challenge with the RC strain. Further study of potential protective factors could be a valuable topic for chloroquine resistance research.

Based on analysis of the growth curves following different inocula of each strain of *P. berghei*, we determined

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the most appropriate experimental inoculation rates as 10^6 parasites per mouse for the RC strain and 10^2 parasites per mouse for the N strain. Mice receiving 10^6 parasites of the RC strain reached their highest mean peak parasitemia (20%) at 18 days post-infection. Mice receiving 10^2 parasites of the N strain inoculum achieved 20% mean parasitemia by the 10th day post-infection. In the present studies, mice infected with 10^6 parasites of the RC strain achieved a mean of 22% parasitemia 12 days post-infection and mice infected with 10^2 parasites of the N strain achieved a mean of 24% parasitemia at 10 days post-infection.

B. <u>Sensitivity of both strains of P. berghei to</u> radiation and the effect of irradiated parasites on cytochrome P450 and associated enzyme activities in the <u>murine host</u>:

In early investigations on immunity against malaria parasites, various stages of inactivated parasites were inoculated into animals with the intention of inducing immunity. Among the methods of inactivation were ultra-violet light, formalin fixation, drug-treatment, and X and gamma radiations (Hughes and Dixon, 1980). In most of the investigations, animals were immunized with radiationattenuated sporozoites of *P. berghei*, *P. yoelii* or *P. cynomolgi*. In a few instances, erythrocytic forms of *P. berghei* were irradiated and an attenuated variant obtained. Waki et al. (1982) reported that a dose of 20 Krads from Xray source abolished infectivity of erythrocytic stages of *P*.

berghei NK65 strain. Parasitemia increased more slowly in mice inoculated with P. yoelii nigeriensis erythrocytic stages irradiated at 10 Krads compared to mice infected with unirradiated parasites. The peak parasitemia in mice inoculated with the irradiated materials was less than 0.5% (Waki et al. 1986). Few studies on radio-sensitivity of drugresistant strains of malarial parasites have been published. In our studies, differences in radiation sensitivities of chloroquine-sensitive and chloroquine-resistant strains of P. berghei were determined. A dose of 15 Krads from a cobalt-60 source (gamma radiation) killed all chloroguine-sensitive P. berghei as judged by an infection rate of zero. However, 15 Krads of radiation only partially eradicated the chloroquineresistant P. berghei, documented by a residual infection rate of 12.5%. We found that the chloroquine-resistant strain of Ρ. is less sensitive to radiation than berghei the chloroquine-sensitive strain. Allison and Eugui (1982) suggested that parasites in young erythrocytes are less susceptible to irradiation than those in mature erythrocytes because the young erythrocytes have an adequate level of enzymes that remove free radicals. X- and gamma radiation primarily produce damage by creation of reactive, charged molecules (free radicals). Since free radicals contain unpaired electrons, they are very reactive and can oxidize or reduce the biological molecules within the cell (Prasad, 1984). The tendency of chloroquine-resistant P. berghei to preferentially reside in immature erythrocytes (Peters, 1968b; Fitch, 1977) might be one reason for its diminished radio-sensitivity.

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On the other hand, slower growth of the chloroquineresistant *P. berghei* could also contribute its decreased radio-sensitivity. According to the Law of Bergonie and Tribondeau, X-rays are more damaging to cells which have greater reproductive activity. Using cell death as the criterion, rapidly dividing cell systems are more radiosensitive than non-dividing systems (Prasad, 1984). The observed required radiation dosage in Waki et al.'s work (20 Krads) may be higher than the value we observed (15 Krads) because they used a larger inoculation (10^7 irradiated parasites)than we did (10^6 irradiated parasites), and because they did not test 15 Krads. However, we found that 20 krads would kill both chloroquine-sensitive and chloroquineresistant *P. berghei* at an inoculum of 10^6 parasites.

In our studies, liver weight, spleen weight, hepatic microsomal protein concentration, hepatic cytochrome P450 content, benzo(a)pyrene hydroxylase activity and benzphetamine N-demethylase activity were determined. These did not differ significantly between mice injected with irradiated parasites of the chloroquine-sensitive and chloroquine-resistant strains, even though the inoculum dose of the RC strain was four orders of magnitude (10⁴) greater than that of the N strain. These results might indicate that quantitative differences in parasitic antigens used in the present studies do not have a direct effect on differences in host response induced by viable organisms. Although there were significant differences in mean spleen weight, hepatic cytochrome P450 content, and benzo(a)pyrene hydroxylase activity between mice injected with irradiated doses of either strain of the parasites and mice injected with PBS, the observation that irradiated erythrocytes induced significant differences in these parameters from PBS-injected controls suggests that the effect may be a general one dependent on total protein inoculated. The reason that benzphetamine N-demethylase activity of mice injected with 10^2 irradiated erythrocytes was significantly less than that of control mice injected with PBS is not clear. It should be noted that benzo(a)pyrene and benzphetamine are metabolized by different cytochrome P450 isozymes (Hodgson and Levi, 1987).

Serum glutamic-pyruvic transminase (SGPT) and serum glutamic-oxalacetic transminase (SGOT) levels of all treatment groups of mice were normal (i.e., SGPT: 28-132 units/liter serum; SGOT: 59-247 units/liter serum; Bogin, 1992) although SGPT and SGOT levels of mice injected with irradiated RC strain parasites tended to be significantly greater than those of the other treatment groups.

C. Effect of infection with chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* on cytochrome <u>P450 and associated enzyme activities</u>, and a possible <u>mechanism for chloroquine resistance</u>:

1. <u>Gross changes in liver and spleen weights of mice</u> <u>infected with the chloroquine-sensitive and the chloroquine-</u> <u>resistant strains of P. berghei</u>:

Hepatomegaly and splenomegaly are commonly found in malaria infection. Saxena et al. (1987) reported that the

average weight of livers from mice infected with P. yoelii nigeriensis was 18% greater than those of normal mice. Singh et al. (1985) also determined that the mean liver weight of mice infected with P. berghei was increased by 79% and mean spleen weight was increased 10-fold. In our experiments, the mean liver weight of mice infected with the chloroquinesensitive strain of P. berghei was 28-31% greater and mean spleen weight was about 3-fold greater than that of uninfected mice. In the experiments of Singh et al. (1985), parasitemia in infected groups reached levels of 9-13%, which are lower than ours (21-24%), but they found greater liver and spleen weights. Perhaps this is related to differences in the strains of P. berghei and strains of mice used in the two studies. We also observed the effect of infection with chloroquine-resistant P. berghei on liver and spleen weights of infected mice to be very high; liver weights increased by 17-31% and spleen weights increased 4-fold compared to mice infected with the chloroquine-sensitive N strain. An increase in size and weight of livers and spleens from mice infected with the N strain appears due to the malaria pigments of the parasites which might stimulate activation and proliferation of phagocytic cells in the livers and spleens.

In acute malaria infections, the color of enlarged livers and spleens may range from dark red to brown depending on the amount of malaria pigment deposited. The microscopic picture of the enlarged liver shows distinct hypertrophy and hyperplasia of Kupffer cells with active phagocytosis. Kupffer cells or circulating macrophages in the sinusoids are seen phagocytosing malaria pigment, parasitized and non-

parasitized erythrocytes, and cell debris. Microscopically, numerous infected red cells, showing all stages of asexual parasite development, are seen in the cords and sinuses of the enlarged spleen. Also, there is an abundance of active phagocytic cells, of which the major elements are macrophages, in the splenic cords and sinuses. Malaria pigment and infected and non-infected erythrocytes, as well as nuclear debris, were contained in these cells. During the acute stages of disease, spleen enlargement is usually moderate, but in chronic malaria the spleen may increase to more than 20 times its normal size (Boonpucknavig and Boonpucknavig, 1988; Srichaikul, 1959; Schnitzer et al., 1972; Aikawa et al., 1980). In our studies, the color of enlarged livers and spleens of mice infected with the N strain, containing abundant malaria pigments, were chocolate brown. Although no malaria pigments were visible in the erythrocytic stages of the RC strain, size and weight of livers and spleens of the infected mice were increased and their colors were reddish brown. The elements, other than the malaria pigments in erythrocytic stages of the RC strain, which could cause enlargement of liver and spleen of their hosts, remain to be determined in further studies.

2. Effect of infection with chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* on cytochrome P450 and associated enzyme activities:

As in many parasitic infections, malaria infection may decrease microsomal protein concentration, cytochrome P450 content, and associated enzyme activities in livers of their hosts. Previous investigations have shown that infection with *P. berghei* decreased microsomal protein concentrations in livers by 14-20% (Sharma et al., 1979; Singh et al., 1985), cytochrome P450 contents by 30-90%, and benzo(a)pyrene hydroxylase activities by 20-90% (Alvares et al., 1984; Srivastava et al., 1991). In our studies, hepatic microsomal protein concentrations of mice infected with chloroquinesensitive *P. berghei* were decreased 25-30%, cytochrome P450 content by 20-30% and benzo(a)pyrene hydroxylase activities by 25% when compared to these parameters in the uninfected mice.

There were interesting contrasts in the concentrations of liver microsomal protein and enzymes when expressed as amounts in the whole liver. When expressed per gram liver, microsomal protein of mice infected with the RC strain was significantly less than that of the uninfected controls. There were no significant differences between mice infected with the RC and N strains in the cytochrome P450 content and benzo(a)pyrene hydroxylase activity when these parameters were calculated on the basis of per milligram of microsomal protein. When calculated on the basis of whole liver, however, mice responded to the RC strain infection with more cytochrome P450 content and benzo(a)pyrene hydroxylase activity than mice infected with the N strain. The results expressed per gram of liver would have underestimated the effect because liver weights of mice infected with the RC strain were much greater than those of uninfected and N strain infected mice. We believe that the results expressed as total amount of whole liver are more suitable for the present studies. By this reckoning, hepatic cytochrome P450

and associated enzyme activities were inhibited by infection with the chloroquine-sensitive *P. berghei* but not by infection with the chloroquine-resistant *P. berghei*. Expressed as either activity per mg microsomal protein or total activity per liver, benzphetamine N-demethylase activity of mice infected with the RC strain was significantly greater than for uninfected or N strain infected mice.

Hepatic cytochrome P450 consists of several isozymes which exhibit specific but overlapping substrate specificities (Lu and West, 1980). Our results showed that amounts of cytochrome P450 content and total the benzo(a)pyrene hydroxylase activity of mice infected with the strain were unchanged but their benzphetamine N-RC demethylase activity was markedly enhanced compared to uninfected mice. This strongly suggests that cytochromes P450 2B1 and 2B2 isoenzymes are increased, whereas cytochrome P450 1A1 is not. Cytochromes P450 2B1 and 2B2 are involved in Ndealkylation reactions (Hodgson and Levi, 1987; Huang et al., 1976) whereas cytochrome P450 1A1 is involved in the metabolism of polycyclic aromatic hydrocarbons such as benzo(a)pyrene (Guengerich and Shimada, 1993). This is further suggested by the increase in the 45 kDa band in the SDS-PAGE gel electrophoresis of hepatic microsomes from mice infected with the RC strain in our studies. Cytochromes P450 2B have lower molecular weights than cytochrome P450 1A1 (Ryan and Levin, 1990).

Alvares et al. (1984) reported that there was no significant decrease in cytochrome P450-dependent enzymatic

activities during the exoerythrocytic (liver) stage of the malaria infection. The malaria parasites were no longer in liver cells at the time the cytochrome P450 enzymatic activities were depressed (during erythrocytic stage of malaria infection). These investigators suggested that malaria parasites release pharmacologically active substances or malaria pigment which might in turn induce heme oxygenase. This enzyme breaks down heme into biliverdin and bilirubin. The induction of heme oxygenase could result in a decrease in cytochrome P450 and cytochrome P450-dependent enzyme activities. The mechanism for such suppression might also involve one or more components of the immune system, since immunomodulators, e.g., endotoxin, mitogens, and venom, depress hepatic monooxygenases by enhancing heme oxygenase activity in the liver.

Malaria infection also could result in a loss of the structural and functional integrity of the smooth endoplasmic reticulum involved in the metabolism of many foreign compounds. This could occur by direct effect of malaria parasites or by secondary effects of the infection, e.g., hypoxia and hemolysis (McCarthy et al., 1970). However, Peterson et al. (1986) believed it more likely that the depression of cytochrome P450 in the parenchymal cells results indirectly from the phagocytic action occurring in the Kupffer cells. Phagocytozing Kupffer cells release a factor which is transferred to the hepatic parenchymal cells and lowers cytochrome P450 and P450-dependent enzymatic activities.

We found that cytochrome P450 and associated enzymatic

activities were decreased in livers of mice infected with the N strain but not in mice infected with the RC strain. This may be related to production of malaria pigment in the N strain and absence of the pigment in the RC strain. In addition, injection of irradiated RC strain parasites caused total hepatic benzo(a)pyrene hydroxylase activity of the injected mice to be significantly less than that of mice infected with the virulent RC strain and the uninfected controls. It also caused their total benzphetamine Ndemethylase activity to be significantly less than that of mice infected with the RC strain. These findings imply that metabolic products of the RC strain might protect cytochrome P450-dependent enzymes from impairment, and improve the enzymatic activities. Total benzo(a)pyrene hydroxylase activity of mice injected with irradiated N strain or infected with the N strain was signicantly decreased compared to the uninfected controls. But there was no significant difference in the enzymatic activity between mice injected with irradiated N strain and mice infected with the N strain. Whether impairment of the enzymatic activity can be attributed to metabolic products of the N strain or to the parasite's protein needs to be resolved in further studies. The following questions remain: (1) Why does infection with chloroquine-resistant P. berghei enhance benzphetamine Ndemethylase activity in the murine hosts? (2)What differences are there in malaria pigment and other metabolic products between the two strains of P. berghei? (3) What induces enhanced benzphetamine N-demethylase activity in mice infected with the RC strain?

glutamic-pyruvic transaminase (SGPT) is Serum predominantly found in liver, but serum glutamic-oxalacetic transaminase (SGOT) is found in several organs and tissues including the liver and red blood cells. In general, SGPT and SGOT elevations are associated with acute liver cell injury. SGPT is generally accepted as being more specific for liver lesions than SGOT. During the course of malaria infection, disturbances of liver function have been observed. The degree of liver damage is dependent to some extent on the species of malaria parasite involved and the severity of malaria infection (Fletcher and Gilles, 1988). Sadun et al. (1966) reported a significant increase in SGPT but not SGOT levels in human patients with P. falciparum infection. Significant increases in both SGPT and SGOT were seen in Aotus monkeys infected with a Ugandan and a Vietnamese strain of P. falciparum (Schnell et al., 1969). In our experiments, SGPT and SGOT levels in mice infected with the N strain were 3fold and 2-fold higher, respectively, than those in the uninfected controls (P<0.01). In mice infected with the RC strain, only the SGOT level was significantly greater than in the uninfected control, but the SGPT level was in the normal range. These results revealed that chloroquine-sensitive P. berghei caused more severe liver injury than did chloroquineresistant P. berghei.

3. <u>Alteration in isozymes of hepatic cytochrome P450</u> <u>from infected mice</u>:

The electrophoretic pattern on SDS gels of the proteinstaining bands from microsomal preparations has been used to determine qualitative changes in the composition of cytochrome P450 isozymes. The minimum molecular weight of mouse liver microsomal cytochrome P450 isozymes in SDS gels was estimated to be 48-55 kDa (Ryan and Levin, 1990). We found in the 45 kDa molecular weight region of the SDS gels, a band from mice infected with the RC strain was stronger in protein staining intensity than the corresponding band from either mice infected with the N strain or uninfected mice. This phenomenon appears to be consistent with differences in benzphetamine N-demethylase activity among mice infected with the RC strain versus the N strain and uninfected mice. Whether the protein at the location of the band is the isozyme responsible for benzphetamine N-demethylation remains to be determined.

4. <u>A possible mechanism of chloroquine resistance in</u> <u>malarial parasites</u>:

Although several hypotheses about chloroquine resistance have been presented in the literature, the mechanisms responsible for the resistance in malaria parasites have not been completely elucidated. Some researchers observed the phenomenon of differences in chloroquine concentration between parasitized and unparasitized erythrocytes, and between erythrocytes parasitized with chloroquine-sensitive and chloroquine-resistant strains. Erythrocytes parasitized by chloroquine-sensitive *P. berghei* contain 25 times more chloroquine than do normal erythrocytes (Hahn et al., 1966). Also, the concentration of chloroquine is 2-3 times higher in erythrocytes containing chloroquine-sensitive plasmodia than in those containing chloroquine-resistant *P. berghei* (Hahn et al., 1966; Macomber et al., 1966; Macomber and Sprinz, 1967).

Some researchers presented hypotheses to explain this phenomenon. The FP hypothesis identifies FP as the receptor on the parasites for the accumulation of chloroquine. Inaccessibility or absence of FP in resistant strains of plasmodia may be attributed to low concentration of chloroquine in the parasites (Chou et al., 1980; Fitch, 1989). The lysosomotropic hypothesis states that chloroquine could be transported into the parasite cytoplasm and further concentrated into the lysosome of the parasite by an ATPdependent proton pump. An impairment in the activity of the proton pump could lead to differences in the effective drug concentrations (Warhurst, 1986; Mahnalgi et al., 1989). Krogstad et al. (1987) determined that drug-resistant P. falciparum released chloroquine at a rate 40-50 times greater sensitive parasites. The P-glycoprotein than that of hypothesis assumes that the efflux of chloroquine may be mediated by a membrane transport molecule called Pglycoprotein. The enhanced efflux of the drug from resistant parasites and the lowered accumulation of drug in resistant parastites could be implicated in chloroquine resistance in the malaria parasites (Endicott and Ling, 1989; Martin et al., 1987; Krogstad et al., 1987).

Elimination of chloroquine in laboratory animals has been shown to be inhibited by administration of SKF-525A, a cytochrome P450 inhibitor. This strongly indicates the possible involvement of cytochrome P450-dependent enzymes in the metabolism of chloroquine (Gaudette and Coatney, 1961). The metabolite N-desethyl-chloroquine is the second most important excretory product following the administration of chloroquine (McChesney and Fitch, 1984). Desethyl-chloroquine is more polar and has less antimalarial activity than chloroquine (Kuroda, 1962). Chloroquine also undergoes a hydroxylation reaction most probably catalyzed by a different form of cytochrome P450. In urine samples, unchanged chloroquine levels were 59% of the parent drug and desethylchloroquine levels were 38% of the drug (McChesney et al., 1966). In man and other mammals, N-dealkylation is the primary pathway for chloroquine metabolism (Fig. 34).

Our studies indicated that infection of chloroquineresistant *P. berghei* infection may enhance cytochrome P450dependent dealkylation of chloroquine, and speed the metabolism of chloroquine and the excretion of the antimalaria drug. In so doing, the drug pressure faced by the malaria parasites becomes weaker. Whether the enhancement of benzphetamine N-demethylase activity by infections with chloroquine-resistant *P. berghei* increases the metabolism of chloroquine and whether this postulated mechanism of chloroquine resistance in rodent malaria may be related to infections with human malaria parasites(*P. falciparum*) remains to be determined in further studies.

In summary, we determined that, unlike chloroquinesensitive *P.berghei* infection, chloroquine-resistant *P. berghei* infection dose not impair cytochrome P450 content and associated enzyme activities, but enhances benzphetamine Ndealkylase activity in the rodent host. These findings imply speeding of chloroquine elimination from the rodent host by N-dealkylation and decreasing of drug pressure on the parasites. This may be a mechanism for chloroquine resistance



Fig.34 Pathway involved in the biotransformation of chloroquine in mammals (taken from McChesney and Fitch, 1984).

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in malaria parasites. However, whether these findings can be extended to chloroquine-resistant human malaria parasites needs to be determined in further studies, because there are differences in virulence and production of malaria pigment between chloroquine-resistant *P. berghei* and chloroquineresistant *P. falciparum*.

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