GENETICS OF DRUG RESISTANCE IN RODENT MALARIA

ROSE ANN PADUA, B.Sc.

Submitted to the University of Edinburgh

as a Thesis in Fulfilment of the Requirement

for a degree of

Doctor of Philosophy

Institute of Animal Genetics, University of Edinburgh

February, 1980.

CONTENTS

Page

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Geoffrey Beale and Dr David Walliker for their encouragement, useful advice and critical appraisal of the manuscript. In addition, I would like to thank my colleagues, Dr Virgilio Rosario and Dr Andrew Tait, for many useful suggestions, and Robin Gray for his help in the production of this thesis.

I thank Richard Fawcett for his technical assistance in carrying out some of the electrophoresis gel runs reported in this thesis and Edie Joyce for the maintenance of the animal house.

I certify that, apart from the assistance mentioned above, all of the experiments presented in this thesis were planned and carried out by myself.

This work has been supported by the Medical Research Council.

February, 1980.

ABSTRACT

Chloroquine-resistance was studied in the rodent malaria parasite, Plasmodium chabaudi. Parasite lines exhibiting different degrees of resistance were obtained by submitting a line already resistant to a low level of the drug to a gradual increase of drug pressure. The chloroquine-resistant lines were found to be stable after mosquito transmission and after multiple blood passage in the absence of the drug. The inheritance of the high level of chloroquine-resistance (resistant to 30 mg kg^{-1} chloroquine for 6 days) was examined by crossing this resistant line with a drug sensitive (sensitive to 3 mg kg^{-1} chloroquine for 6 days) line. The progeny of the cross showed intermediate levels of chloroquine-resistance suggesting that the high level of resistance was due to more than one mutation. Competition studies between different lines of P. chabaudi revealed that the highly resistant line was at a selective advantage over the sensitive forms and that cross products were favoured over parental resistant lines.

A mefloquine-resistant line was established by a gradual increase in drug pressure. This resistance was variable in its stability after mosquito transmission and after blood passage without drug pressure. A clone was obtained which was stable on blood passage in the absence of the drug (to 15 mg kg^{-1} Mefloquine for 4 days).

SUMMARY

(1) Chloroquine-resistance has been studied in the rodent malaria parasite Plasmodium chabaudi. Parasite lines exhibiting different degrees of resistance were obtained by submitting a line already resistant to a low level of the drug to a gradual increase of drug pressure.

(2) Lines showing different levels of chloroquine-resistance were produced; low (3CQ) intermediate (15CQ) and high (30CQ) These levels could be distinguished by drug tests using varying doses of chloroquine and by using other drugs to which chloroquine appeared to be cross-resistant; these included mefloquine, mepacrine and quinine.

(3) Tests using chloroquine administered for four and six days were compared. These experiments indicated that the duration of drug treatment as well as the dose administered was important for antimalarial activity. Chloroquine administered at a high dose (30 mg Kg^{-1}) for four days did not eliminate chloroquine-sensitive parasites whereas lower doses administered for six days did eradicate chloroquine-sensitive parasites.

(4) The chloroquine-resistant lines selected were found to be stable after mosquito transmission and after multiple blood passage in the absence of the drug.

(5) The inheritance of the high level of chloroquine resistance (30CQ) was examined by crossing this resistant line with a drug sensitive line. These two lines were polymorphic for two enzyme markers

and differed in their response to pyrimethamine. The progeny of the cross were cloned and each clone was examined for drug response and enzyme types. Clones which were classified as sensitive (to 3 mg Kg^{-1} for 6 days), intermediate (3CQ) or 15 CQ) or highly resistant (30CQ) were detected. The presence of clones showing intermediate levels of chloroquine resistance suggested that the high level (30CQ) was due to more than one mutation. Blood mixtures of the highly resistant (30CQ) and the drug sensitive line did not generate parasites with intermediate levels of chloroquine-resistance.

(6) In vitro chloroquine drug tests were conducted using a chloroquine-sensitive and the highly resistant (30CQ) lines. In the presence of chloroquine, the resistant parasites formed vacuoles and their development appeared to be arrested at a specific stage whereas sensitive parasites continued to develop in the presence of the drug forming parasites which appeared abnormal. Inoculations from cultures into uninfected mice revealed that the resistant parasites remained viable at all but the highest of the doses of chloroquine used whereas the sensitive parasites showed a reduced viability after exposure to the drug.

(7) Competition studies were carried out in which known proportions of two lines differing in specific characters were injected into mice. After varying periods of time the infections were examined to determine whether the proportions of each line had changed. The highly resistant (30CQ) line appeared to possess a selective advantage in competition with the chloroquine-sensitive line from which it was derived. However, in

competition with the chloroquine-sensitive line used in the cross the 30CQ line appeared to grow as well as the sensitive line; neither line was at a selective advantage. A highly resistant clone derived from the progeny of the cross was at a selective advantage over the parental (30CQ) highly resistant line obtained by selection.

(8) A mefloquine-resistant line was established by a gradual increase in drug pressure. This line was resistant to a high level of the drug (30MF).

(9) This mefloquine-resistant line appeared to lose its resistance after multiple blood passage in the absence of the drug; although the parasites survived the treatment, they emerged progressively later following drug treatment. After mosquito transmission, a lower level of mefloquine-resistance (15MF) was obtained.

(10) The resistance of the lower level of resistance (15MF) was variable in its stability after mosquito transmission. In one case the parasites seemed unstable, after a separate mosquito transmission the line was stable in its resistance; this resistance was stable after blood passage in the absence of the drug.

1. INTRODUCTION

1.1 The Malaria Problem

The worldwide malaria morbidity is at least one hundred million cases per year; the mortality, although considerably lower is still about one million; most of those affected are the young. At present 21 per cent of the world's 2,048 million people who live in malarious areas are in countries from which malaria has been eradicated. Some 62 per cent are living where antimalarial measures are being applied. Nevertheless, there are still about 352 million people living in places where no antimalarial control activities are being undertaken (WHO, 1976; Wernsdorfer, 1979).

In several Asian countries malaria has come back to territories from where it was once eradicated and there is little indication that this trend will not continue. This may be partly due to problems of migration (Bruce-Chwatt, 1970, 1973) as well as increasing problems with chemotherapy.

In the absence of a vaccine, chemotherapy has been the only practical measure we have had against malaria, especially against the most important human species, Plasmodium falciparum. Reviews by the World Health Organization (WHO) Scientific Groups (1967, 1973) emphasise the role drugs play in the control and eradication of malaria in endemic areas. The development of drug resistance to chemotherapy has obviously been of great concern. The eradication programmes using insecticides against the vector and antimalarial drugs against the parasite have been retarded by the diminishing effects of these chemicals due to the increasing development of drug resistance.

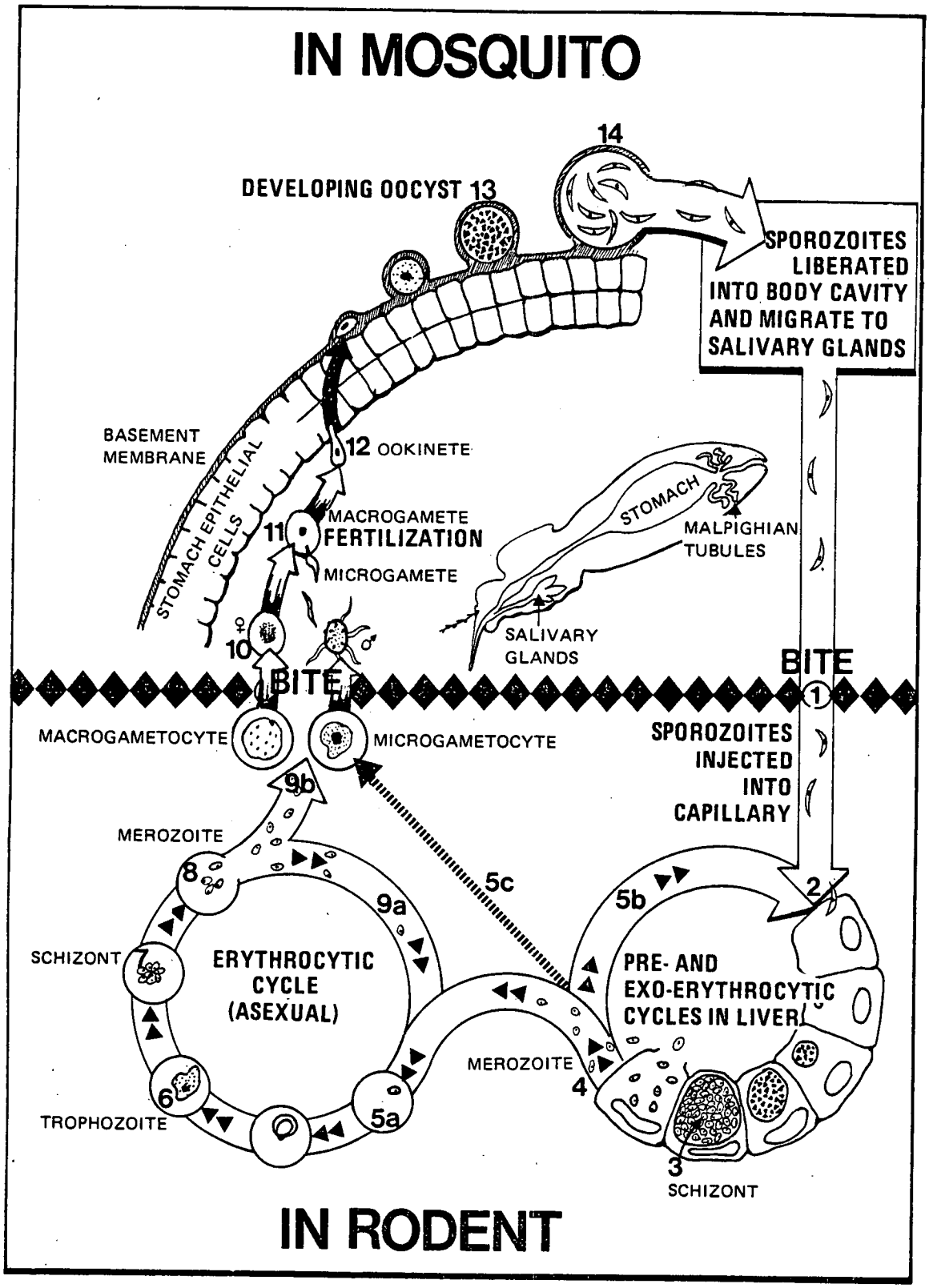
Drug resistance has been in existence for some time. It was

predicted that at least in the case of trypanosomes, resistance to any type of drug active against them could arise (Ehrlich, 1910). This belief is certainly true of antimalarial drugs where resistance to all existing antimalarial drugs has been developed either in nature or in laboratories (see reviews by Peters, 1970, 1974).

There are several definitions of drug resistance. In order that comparative tests can be undertaken throughout the world, the WHO (1965, 1973) defined resistance as a "drug-parasite interaction in which there is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of the drug given in doses equal or higher than those usually recommended, but within the limits of tolerance of the subject". In other words the parasite loses its sensitivity to the drug by a change in the parasite rather than the host. Any alteration in the host's mechanisms are presumed to be parasite mediated.

The increased incidence of drug resistant malaria in the Far East prompted massive drug screening programmes by the United States of America in the 1960s in view of their military involvement in the area. Malaria to American soldiers in Vietnam was as devastating as wounds from action (Tigert, 1966). These drug screening programmes have resulted in new compounds which are currently undergoing trials (Schmidt, 1978). In addition to drug screening there has been increasing interest in the study of the underlying mechanism of drug resistance which has stimulated research on the biology of the parasite at various levels, using a variety of techniques.

Fig.1 Lifecycle of Rodent Malaria Parasites



1.2 Life cycle

The life cycle of rodent malaria is summarised in Figure 1. The parasite undergoes a cyclical development which involves an asexual phase in a mammal and a sexual phase in the insect vector. In the mammalian host two cycles of division occur, tissue schizogony (exoerythrocytic, which takes place in the liver) and blood schizogony (erythrocytic). An infection is initiated by the inoculation of sporozoites into the blood by infected female mosquitoes (1). Liver parenchyma cells are invaded by sporozoites(2) which then develop into schizonts by nuclear division(3).The schizonts mature (42 - 72 hours after mosquito bite) releasing between 2,000 - 20,000 merozoites into the blood stream (4). The time of exoerythrocytic schizogony varies from one species to another (Garnham, 1966). Merozoites can either invade erythrocytes (5a) or undergo secondary exoerythrocytic cycles of development in the liver (5b, 2-4). Merozoites which invade erythrocytes form trophozoites (6) which mature, undergoing several nuclear divisions to form schizonts (7). The schizonts contain several merozoites which vary in number from species to species (4-18 in murine plasmodia); these merozoites are released following the breakdown of the host cells (8). Some merozoites reinvade other red blood cells, thus continuing the asexual cycle in the blood (5a). Alternatively, some merozoites develop sexual forms called macro-and microgametocytes (9b). It has been shown that merozoites from hepatic schizonts can develop directly into gametocytes (5c) (Killick-Kendrick & Warren, 1968).

In the midgut of the insect host the gametocytes give rise to the female (macro-) gamete while the male (micro-) gametocyte undergoes exflagellation liberating the male (micro-) gamete which fertilizes

the female gamete (10 & 11). Exflagellation is a process whereby the motile microgametes are formed; the microgametes lash about continuously and one by one break away from the parent body. This takes between 10 - 30 minutes according to species and environmental temperature. The zygote formed following fertilization is called an ookinete (12), which penetrates the midgut wall of the mosquito to form an oocyst. On the outside of the midgut the ookinete develops into an oocyst (13) which matures over a period of 8 - 15 days depending on environmental temperatures, forming sporozoites. The number of sporozoites varies according to species from more than 1,000-10,000 (Garnham, 1966). When mature, the sporozoites are liberated into the body cavity (14) from which they migrate to the salivary glands where they accumulate before being injected into another mammalian host when the female mosquito again feeds (1).

1.3 Antimalarial Drugs

There are numerous reviews on drug resistance in Protozoa, e.g. Bishop, 1958; Schnitzer, 1963, 1966. Drug resistance in Plasmodia has been reviewed by many authors including Schmidt, 1969; Peters, 1970, 1974; Warhurst, 1973; Carter & Diggs, 1977.

There are five main groups of antimalarial drugs. These are:

1. Quinine, mepacrine and 4-Aminoquinolines, e.g. chloroquine, 4-Quinoline methanols, e.g. mefloquine.
2. 8-Aminoquinolines, e.g. primaquine.
3. Sulphonamides, e.g. sulphadiazine.
4. Antifolates, e.g. pyrimethamine, proguanil.

These drugs share general structural similarities and mechanisms of antimalarial action. They also overlap in the area of attack in the parasite life cycle. The first class which includes quinine and chloroquine are almost without effect against sporogonic and pre-erythrocytic stages. However, they act against the asexual blood parasites and are hence referred to as blood schizontocides, inhibiting blood schizogony. The 8-aminoquinolines are effective against all stages of the parasite including the liver forms. The sulphonamides and antifolates are effective against actively dividing parasites. These drugs are therefore active against all stages of the parasite life cycle except gametocytes. 4-Quinolinemethanols are active against asexual blood forms; there is no evidence for activity against pre-erythrocytic stages of the parasite.

Reports of drug resistance refer to resistance of blood forms of the parasite. Drug resistance to the first four classes are prevalent (see reviews above). This investigation is mainly concerned with drug resistance involving the 4-aminoquinoline, chloroquine and the 4-quinolinemethanol, mefloquine. Pyrimethamine-resistance is used as a marker for genetic analyses.

1.4 Drug Resistance in Human Malaria

There are four main species of malaria parasites; these are: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. These four human species of malaria parasites show variation in their response to drugs (Peters, 1970). Chloroquine-resistance at present only concerns *P. falciparum*. As chloroquine-resistance has only recently emerged in Africa, these strains may differ from their Far Eastern or South American counterparts where chloroquine-resistant strains are widespread.

In contrast, resistance to other drugs such as proguanil and pyrimethamine has arisen rapidly and is widespread. Four years after its introduction proguanil-resistance emerged (Field & Edeson, 1949) and only two years after its use resistance was widespread. The first report of pyrimethamine-resistance was in 1952 (McGregor & Smith, 1952), the same year as its first use in the field. Many reports of pyrimethamine-resistance followed (summarised by Peters, 1970).

Chloroquine-resistance was first suspected in 1960 in Venezuela (Maberti, 1960). The first confirmed case of chloroquine-resistance in P. falciparum was from Colombia in 1961 (Moore & Lanier, 1961; Young & Moore, 1961). This was followed by other reports from the Far East (Young et al., 1963; Montgomery & Eyles, 1963; Eyles et al., 1963; Powel et al., 1964; Harinasuta, 1965; Sandosham et al., 1966; Ebisawa & Fukuyama, 1975; Hall et al., 1975a). It has spread as far as Sabah (Clyde et al., 1973b), the Philippines (Clyde et al., 1971; Ramos et al., 1971; Shute et al., 1972; Valera & Shute, 1976), Indonesia (Clyde et al., 1976a), Australasia (Simpson & Williams, 1978) and as far West as Rangoon in Burma (Clyde et al., 1973a). Reports of chloroquine-resistant P. falciparum from South America also followed (da Silva et al., 1961; Rodrigues, 1961; Box et al., 1963; Godoy et al., 1975). Several claims of chloroquine-resistance in Africa have been made (Schwendler, 1965; Lasch & N'Guyen, 1965; Jeffrey & Gibson, 1966; Stevenson, 1966; Hipoo & McCallum, 1967; Beausoleil, 1968; Wolfe & Huddleston, 1969; Ricosse et al., 1969; Bruce-Chwatt, 1974). However, the tests used did not meet the WHO criteria for drug resistance and were therefore unconfirmed. Studies by Dennis et al. (1974) on an Ethiopian strain, Ansdell et al. (1974) on a Nigerian strain, a case report by Pillay & Bhoola (1975) in

South East Africa, a review by Olatunde (1977) and studies by Kahn & Maguire (1978), although unconfirmed indicate that chloroquine-resistant P. falciparum may exist in Africa. Recent confirmed case reports of chloroquine-resistant P. falciparum from Kenya supports this view (Fogh, 1979; Stille, 1979).

Although chloroquine-resistance did not appear for more than a decade after its use for therapy in 1945, once developed it has spread rapidly throughout parts of the world and its progress is anxiously being followed into Africa which, until recently,had been free of chloroquine-resistance. Warnings of the possibility of the development of chloroquine-resistance came as early as 1946 (Most et al., 1946) and 1948 (Earle & Berliner, 1948). The latter authors had forseen the possibility of developing chloroquine-resistant P. falciparum by using low doses of the drug which were active against another human malaria species P. vivax but were less effective against P. falciparum. As shown by work with rodents (Rosario, 1976a,b) this would be analogous to selection for resistance using a low pressure method. Unwittingly ideal conditions for the selection of chloroquine-resistant P. falciparum had been present.

1.5 Chloroquine-resistance in the Laboratory

In the primary screening of drugs in vivo, animals provide us with a useful tool. Rodent malaria is a good model for the demonstration of blood schizontocidal action and avian systems are useful in the study of causal prophylactic activity (see review by Peters, 1970, 1974). The adaptation of the human malaria P. falciparum to the owl monkey has resulted in a valuable model for the evaluation of more promising

compounds (see reviews by Schmidt, 1969, 1973, 1978). Schmidt (1978) showed that this host offers a possible model for human malaria. Chloroquine-resistant strains of P. falciparum were classified and fourteen potential antimalarial drugs were tested. This work on P. falciparum and P. vivax infections in the owl monkey (Schmidt, 1978) will probably never be repeated due to the large numbers of animals used.

The first report of chloroquine-resistance in a malaria parasite was in 1956 in the avian species P. gallinaceum (Ray & Sharma, 1956) and in the rodent species P. berghei (Ramakrishnan et al., 1957). Both sets of authors used a low pressure method to induce resistance; this involved selection using a low drug dose and prolonged passaging under drug pressure. There have also been numerous attempts to select for chloroquine-resistance in various malaria species which have been unsuccessful (Thompson et al., 1948 with P. lophurae; Seaton, 1951; Bishop & McConnachie, 1952 with the avian P. gallinaceum; Kollert, 1963 with P. relictum; Schmidt et al., 1949 with P. cynomolgi).

A great number of studies have been undertaken using rodent malaria species. Unlike simian models, rodents are readily available at low costs and large numbers which render them the most suitable laboratory model. The four species of rodent malaria have innate differences in their response to chloroquine as follows:

(1) P. yoelii - This species is naturally resistant to high doses of chloroquine (Warhurst & Killick-Kendrick, 1967; Peters, 1968c; Carter, 1972).

(2) P. berghei - This species is usually sensitive to chloroquine. Resistance to chloroquine has been obtained by a variety of means. A

low pressure method was used by Ramakrishnan et al. (1957), Kollert (1963), Peters (1965) and Jacobs (1965). High doses were used by Sautet et al. (1959) and Benazet (1965). Hawking and Gammage (1962) selected for resistance by blocking the immune system of the host and by adding drug in the diet.

Another method attempted by Hawking (1966) involved the administration of a single high dose to mice with high parasitaemias; shortly after drug treatment (1 and 5 hours) the animals were exsanguinated and the blood inoculated into clean mice. However, this procedure was unsuccessful in producing resistance.

It must be stressed that the chloroquine-resistant lines obtained have mainly been unstable in the absence of the drug (Ramakrishnan et al., 1957; Kollert, 1963; Jacobs, 1965; Thompson et al., 1965; Peters, 1965; Hawking, 1966). Peters (1968a,b,c) has carried out studies of the loss of chloroquine-resistance during blood and mosquito passage. The occurrence of a stable resistant line in P. berghei developed by Peters et al. (1969) was later found to be a subspecies of P. yoelii (Peters et al., 1978) which is naturally resistant to the drug.

(3) P. vinckei - This species is naturally sensitive to chloroquine. Only one successful report of the development of stable chloroquine-resistance in this species has been reported by Powers et al. (1969). Using a low pressure method a line resistant to chloroquine was established from a strain which was already resistant to pyrimethamine.

(4) P. chabaudi - Peters (1970) has shown that this species has a similar sensitivity to chloroquine as P. berghei. The only previous

report of stable chloroquine-resistance before the work presented here is that of Rosario (1976a,b).

The development of *in vitro* culture techniques for drug tests is likely to provide a quick and easy assay in field work. Recent developments include a microtechnique (Rieckmann *et al.*, 1978) which is a refinement of earlier attempts (Rieckmann *et al.*, 1968). Earlier short term culture techniques enabled various workers to study new drugs (Trager, 1971; Gutteridge *et al.*, 1972; Siddiqui *et al.*, 1972).

One of the most significant developments is that of the continuous culture *in vitro* of human malaria parasites (Trager & Jensen, 1976; Jensen & Trager, 1978). Drug tests using parasites from continuous cultures have already started (Richards & Maples, 1979), which indicate that chloroquine-resistant parasites can be distinguished from sensitive forms by these methods. A study by Nguyen-Dinh and Trager (1978) suggests that an African strain of *P. falciparum* has the genetic capability to develop chloroquine-resistance. They used a continuous cultivation method to select for a level of chloroquine-resistance in *P. falciparum* comparable to the naturally occurring resistant strains in South East Asia.

1.6 Chloroquine-Mode of Action and Mechanism of Resistance

Extensive studies have been done to investigate the mechanism of action of chloroquine and it has been shown to inhibit a very wide range of functions including nucleic acid and protein synthesis as well as respiration (Schellenberg & Coatney, 1961; Van Dyke *et al.*, 1969; Gutteridge *et al.*, 1972; Theakston *et al.*, 1972). Three main sites of action have been proposed:-

(1) DNA and RNA directly or on synthesis.

(2) Inhibition of proteolytic enzymes/stimulation of autophagic vacuole formation.

(3) Inhibition of oxidative metabolism.

There is evidence that chloroquine binds or interacts with DNA (Allison et al., 1965; Cohen & Yielding, 1965; Hahn et al., 1966; Gutteridge et al., 1972). Chloroquine also binds to polynucleotides (Blodgett & Yielding, 1968; Morris et al., 1970). It has been shown to degrade large species of rRNA of P. knowlesi (Warhurst & Williamson, 1968). It has been suggested that RNA is rendered more sensitive to hydrolysis by ribonucleases when bound to chloroquine (Wichard & Holbrook, 1970). In addition, the drug inhibits nucleic acid and protein synthesis (Warhurst, 1969; Gutteridge & Trigg, 1972; Warhurst et al., 1972). The ease with which chloroquine interacts with nucleic acids has led a number of workers to conclude that this is the basis of their antimalarial action. However, chloroquine has a similar affinity for the DNA of P. knowlesi and its host (Gutteridge et al., 1972). Furthermore, the host and parasite nuclear chromatin structure and composition of rodent malaria parasites have been found to be similar (Bahr & Mikel, 1972). Therefore the primary action of chloroquine is unlikely to be at this level, although it may be the site of lethal action.

Chloroquine induces pigment clumping and autophagic vacuole formation (Macomber & Sprinz, 1967; Warhurst & Hockley, 1967); during vacuolation autolysis occurs inside the cytosome that is formed on exposure to chloroquine which leads to the cytoplasmic destruction of parasites as a result of leakage of enzymes (Warhurst & Williamson, 1970). Protein synthesis, as well as RNA synthesis, is essential for the stimulation of clumping and vacuolation (Homewood et al., 1971; Warhurst & Robinson, 1971; Warhurst et al.,

1971; Warhurst & Baggaley, 1972; Warhurst *et al.*, 1972,1974). This process is therefore, inhibited by high concentrations of chloroquine.

Two explanations have been suggested to account for the way chloroquine acts to bring about sequestration within vacuoles and cytoplasmic destruction. Homewood *et al.* (1972a,b) suggest that the drug may act by inhibiting haemoglobin digestion in plasmodia. The theory given is that the pH of the lysosome, which is usually maintained at an acidic level is raised by the consumption of hydrogen ions during protonation of chloroquine. In *P. berghei* pigment clumping can be induced by raising the pH without chloroquine (Homewood *et al.*, 1972c). An alternative explanation is based on the observation that chloroquine is found to be concentrated in lysosomes of mammalian cells (Allison & Young, 1964; Fedorko *et al.*, 1968) which can be considered to be analogous to the digestive vacuole of the parasites. This concentration could be a function of lysosomal activity due to the direct inhibition of proteolytic enzymes (De Duve *et al.*, 1974) and is energy dependent (Polet, 1970).

In examining chloroquine-induced pigment clumping and vacuolation, Warhurst (1973) postulated clumping sites, where at low concentrations of chloroquine, the drug is associated. It was suggested that the clumping receptors were not lost from chloroquine-resistant parasites but were present in a modified form which was still accessible to suitable drugs; these sites may be localized in the digestive vacuole membrane (Warhurst & Thomas, 1975a). Using electron microscopy ^{3}H-chloroquine was found to be localized in pigment containing digestive vacuoles of *P. berghei* (Aikawa, 1972). Another antimalarial drug, mepacrine, was also found to be localized at the host-parasite interface and on the membrane of digestive vacuoles (Warhurst & Thomas, 1975b).

Chloroquine is concentrated in parasitized cells to a greater extent than in uninfected red blood cells (Macomber et al., 1966; Polet & Barr, 1969; Fitch, 1969, 1970). It has been suggested that the parasites require energy for accumulation of the drug (Fitch et al., 1974b,c,1975a); Plasmodia infected cells use higher quantities of glucose than uninfected cells (Momen, 1979). Fitch attributed such accumulation to the existence of binding sites in the parasites. These 'binding sites' should not be confused with 'clumping sites' which are thought to be separate sites as the affinities of amino-alcohol antimalarials for the two sites differ (Warhurst & Thomas, 1975a). Three classes of binding sites were proposed based on their association constants (Fitch, 1969). Fitch found that chloroquine-resistant parasites bound less drug than sensitive forms (Fitch, 1969; Fitch et al., 1974c, 1975 Kramer & Matusik (1971) suggested that one class of binding sites (high affinity) were associated with the parasite membranes whereas another class (low affinity) were freely soluble.

Fitch et al. (1974a, 1975a,b,1978) have suggested that the high affinity receptor may be present in the erythrocyte membrane of infected cells rather than in the parasites themselves. The authors propose that the parasites modify the erythrocyte surface to alter constituents which normally prevent chloroquine accumulation and that resistance to the drug arises by the way parasites modify the erythrocyte surface. There is evidence that P. berghei selectively degrades certain proteins in the erythrocyte membrane (Weidekamm et al., 1973); parasites also modify the red blood cell surface causing indentations and surface protrusions with P. berghei (Bodammer & Bahr, 1973) and with P. falciparum (Balcerzak et al., 1972).

A different theory for the mechanism of chloroquine-resistance was suggested by Howells (1970), who proposed a change to aerobic metabolism in the respiratory mechanism of chloroquine-resistant P. berghei. This theory has since been refuted due to technical difficulties in the method used for detecting functional mitochondria and because of host cell contamination (Howells & Maxwell, 1973a,b).

The exact mode of action of chloroquine, therefore, still remains unclear and the mechanisms proposed for chloroquine-resistance are still mainly speculative. However, from the work carried out so far, there is strong evidence that chloroquine-resistance may be a function of uptake. The clumping sites (Warhurst, 1973) and binding sites (Fitch, 1969) are likely candidates for such a mechanism.

1.7 Mefloquine - Mode of Action

The emergence of chloroquine-resistance in P. falciparum resulted in an intense search for new drugs. One of the most promising drugs to emerge from the antimalarial screening of drugs by the United States army in the 1960s is mefloquine. This drug is now undergoing clinical trials on its own (Canfield et al., 1973; Rieckmann et al., 1974; Trenholme et al., 1975; Hall et al., 1975c, Clyde et al., 1976b; Hall, 1976; Schmidt et al., 1978) and in combination with other drugs (Hall et al., 1977; Doberstyn et al., 1979). Mefloquine is a clinically useful quinolinemethanol with antimalarial activity. In addition, it has been shown to have prophylactic activity against drug resistant P. falciparum and might have a long term suppressive activity against asexual forms (Rieckmann et al., 1974; Clyde et al., 1976b). Clinical studies have shown that mefloquine persists for a relatively long time in man (Trenholme et al., 1975). However, there is no evidence of

activity against secondary tissue schizonts of P. vivax (Trenholme et al., 1975; Clyde et al., 1976b) and of P. cynomolgi (Schmidt et al., 1978). How mefloquine is stored and metabolized is yet to be determined.

Mefloquine does not bind significantly to DNA (Davidson et al., 1975; Peters et al., 1977a). This is the first report of an active compound in the quinoline-acridine class of antimalarials which does not strongly bind to DNA by intercalation.

Fitch and his co-workers have investigated the process of mefloquine accumulation using chloroquine-resistant and sensitive strains of P. berghei (Fitch et al., 1979). They confirm the earlier finding of Peters et al. (1977a) that chloroquine and mefloquine competitively inhibit their respective accumulation. Using P. berghei, Peters et al. (1977a) observed that mefloquine competitively inhibits pigment clumping which is induced by chloroquine, although mefloquine itself did not cause clumping. Fitch et al. (1979) found that more than half as much mefloquine was accumulated by uninfected erythrocytes as infected erythrocytes. Furthermore, mefloquine was accumulated more effectively than chloroquine by uninfected red blood cells and by cells infected with chloroquine-resistant P. berghei. Unlike chloroquine, they found that the process of mefloquine accumulation was not stimulated by glucose (substrate). In view of this, it was proposed that the two drugs shared a common group of receptors and were accumulated by the same process; for some unknown reason mefloquine may have a greater access to the receptors than chloroquine. The undiminished accumulation of mefloquine with chloroquine-resistant P. berghei infected cells provides an explanation for the activity of this drug in treating chloroquine-resistant malaria.

Toxicity tests and the study of the effect of mefloquine on the immune response in mice are being undertaken (Thong et al., 1979). These authors have found that human lymphocytes were more resistant to the drug than mouse lymphocytes. Nevertheless, the response to mitogens were equally depressed. Therefore clearly, extrapolations from mice to humans must be viewed with caution.

As mefloquine is a relatively new drug, so far there is no evidence of stable resistance to it. A report of unstable mefloquine-resistance in P. berghei has appeared (Peters et al., 1977b).

1.8 Genetics

Genetic work on malaria parasites has, until recently, been difficult due to the complex life cycle of the parasite, involving two hosts. The different stages of the parasite suggest variation in gene expression. In addition to drug resistance genetic studies would be of value to various questions concerning taxonomy. A more general application would be to confirm that malaria parasites undergo a eukaryotic life cycle, with sexual stages, rather than a prokaryotic process of genetic exchange. Haploid clones produce both male and female gametocytes which give rise to gametes which can either self or cross fertilize with gametes of different clones. It is hoped that such studies would lead to a precise location of meiotic reduction division (Walliker et al., 1975) which is thought to occur during oocyst development (Sinden & Canning, 1973).

1.8.1 Cytogenetics

The unusual pattern of chromosomal organization in malaria parasites, namely the absence of condensation throughout the division of vegetative

stages precludes the estimation of chromosome numbers by light microscopy (Wolcott, 1954, 1957; Canning & Anwar, 1968). In the absence of condensed chromosomes, Sinden (1978) proposes three methods which can be used to determine chromosome numbers. These are:

(1) To calculate the number of linkage groups by genetic analyses - there are few known markers in Plasmodia to make this type of analyses feasible in the near future.

(2) To count the number of kinetochores, each of which is attached to a diffuse chromosome.

(3) To count the number of kinetochore microtubules in serial sections of spindles.

The limited cytological data suggests a haploid number of 8 - 10 chromosomes (Schrevel et al., 1977) in murine Plasmodia. Recent studies by Sinden and Strong (1978) estimate the chromosome number as fourteen in the haploid genome of P. falciparum. As both micro-and macrogametocytes can arise from cloned haploid blood forms, conclusions about sex chromosomes are not possible; the lack of condensed chromosomes prevents positive identification of chromosomes.

Both mitosis and meiosis are thought to occur in malaria parasites. The parasites undergo mitosis during erythro-and exoerythrocytic schizogony, sporogony and microgametogenesis.

There is an absence of condensed chromatin except during telophase of the final microgametic division (Sinden 1978). Canning and Morgan (1975) suggest that meiosis is a division with no chiasmata between chromosomes. The location of meiosis remains unknown as a result of the inability to count chromosomes. None the less, the limited data available suggests that the number of kinetochores is constant throughout

the life cycle and that meiosis takes place in the zygote. If zygotic division were mitotic 2n kinetochores would be expected; however, the observed number of kinetochores found is n.

Therefore, the location of genes awaits further studies. This information could eventually come from molecular biologists; studies will probably soon be in progress to clone the Plasmodium genome.

1.8.2 Hybridization Between Strains

Investigations have mainly been restricted to laboratory animal species. Aotus monkeys in which human malarias can grow are costly and thus cannot be used for genetic work because of the large number of animals that would be required. The recent development of *in vitro* culture systems may enable genetic studies to be carried out with human malarias. The first genetic studies with Plasmodia were by Greenberg and Trembley (1954a,b; Trembley & Greenberg, 1954; Greenberg, 1956). Using the avian parasite *P. gallinaceum*, they crossed two lines which differed in their resistance to pyrimethamine and their ability to produce erythrocytic and secondary exoerythrocytic forms. A cross was conducted by allowing mosquitoes to feed on blood containing a mixture of gametocytes of the two different lines. They detected non parental parasites which indicated that recombination between the characters distinguishing the parent lines had taken place. Unfortunately, this interpretation was open to question as one of the markers (production of secondary exoerythrocytic forms) was known to be unstable (Trembley *et al.*, 1951). Furthermore, their parent lines were uncloned which adds doubt to the validity of their results.

Rodent malaria parasites have proved to be especially suitable for genetic studies. Numerous isolates have been found which have

proved to be genetically diverse (Beale et al., 1978). In addition, mice, in which the parasites can grow, are available in large numbers. Like P. gallinaceum, all stages of the life cycle can be maintained in the laboratory. Genetic recombination in rodent malaria parasites has been demonstrated by Walliker et al. (1971, 1973, 1975, 1976); Morgan (1974); Rosario (1976a,b); Oxbrow (1973) and MacLeod (1977) in this laboratory. These studies illustrate Mendelian inheritance of enzyme, drug resistance, antigenic and virulence markers. That the rodent species of malaria were polymorphic for several enzyme markers was an invaluable discovery which was developed by Carter (1970, 1973) and Carter and Walliker (1975). In these studies, two lines of P. yoelii which differed in their pyrimethamine response and had variants for the enzyme glucose phosphate isomerase (GPI) were shown to cross-fertilize resulting in recombinant markers (Walliker et al., 1971, 1973). A similar cross was conducted with another species, P. chabaudi, which was polymorphic for the enzymes 6-phospho gluconate dehydrogenase (6PGD) and lactate dehydrogenase (LDH) as well as having differences in pyrimethamine response. The results also confirmed the haploidy of the blood forms (Walliker et al., 1975). Genetic analyses with pyrimethamine-resistance in P. yoelii were conducted by Morgan (1974), with chloroquine-resistance in P. chabaudi by Rosario (1976a,b) and by MacLeod (1977) with pyrimethamine- and sulphadiazine-resistance in P. chabaudi. Walliker et al. (1976) also demonstrated that variations in virulence were due to genetic differences by crossing two lines of P. yoelii which differed in the enzyme forms of GPI, their pyrimethamine resistance and in their growth pattern (virulent or mild). Oxbrow (1973) investigated the genetic basis of cross-protection differences between subspecies of P. yoelii and found that the ability of parasites to grow in immunized

mice was controlled by genetic factors which recombine with other markers.

Yoeli et al., (1969) reported a different gene transfer mechanism between malaria parasites. They suggested that genetic exchange could take place between trophozoites of two parasite lines during their simultaneous development in the same red blood cell. They called this process synpholia. Two lines were used, a pyrimethamine-resistant P. vinckei line which was not infective to hamsters and a pyrimethamine-sensitive P. berghei line which was infective to hamsters. Both lines were mixed together and inoculated into mice; then the mixture was injected into pyrimethamine treated hamsters. In 6 out of 12 of these hamsters pyrimethamine-resistant P. berghei was detected. No confirmation of this process has been reported in subsequent work done in this laboratory or in other laboratories (for example Schoenfield et al., 1974). The results presented in this investigation are also inconsistent with synpholia as a mechanism for genetic exchange (see Sections 4.4 & 4.5). The most likely explanation for the results of Yoeli et al. (1969) is that the pyrimethamine treatment used in the drug tests had selected for a mutation to pyrimethamine-resistance in P. berghei, as the dose used would have been sufficient to select for such a change (Diggens et al., 1970).

1.9 Aims of this Investigation

The present study uses a genetic approach to investigate the basis of chloroquine- and mefloquine-resistance in the rodent malaria species P. chabaudi. This species provides a good model for genetic analyses due to the considerable amount of enzyme polymorphisms found among wild

isolates (Carter & Walliker, 1975). The aims of this study are as follows:

(1) Until recently, no genetic work on chloroquine-resistance has been carried out. Rosario (1976a,b) developed a line resistant to a low level of the drug(3mg kg^{-1} for 6 days) using a gradual increase of drug pressure over a number of passages. This line was shown to be stable and the resistance character demonstrated a Mendelian mode of inheritance. This present investigation involved selecting for a high level of chloroquine-resistance in order to determine:

(a) whether a high level of resistance was due to mutation events, and

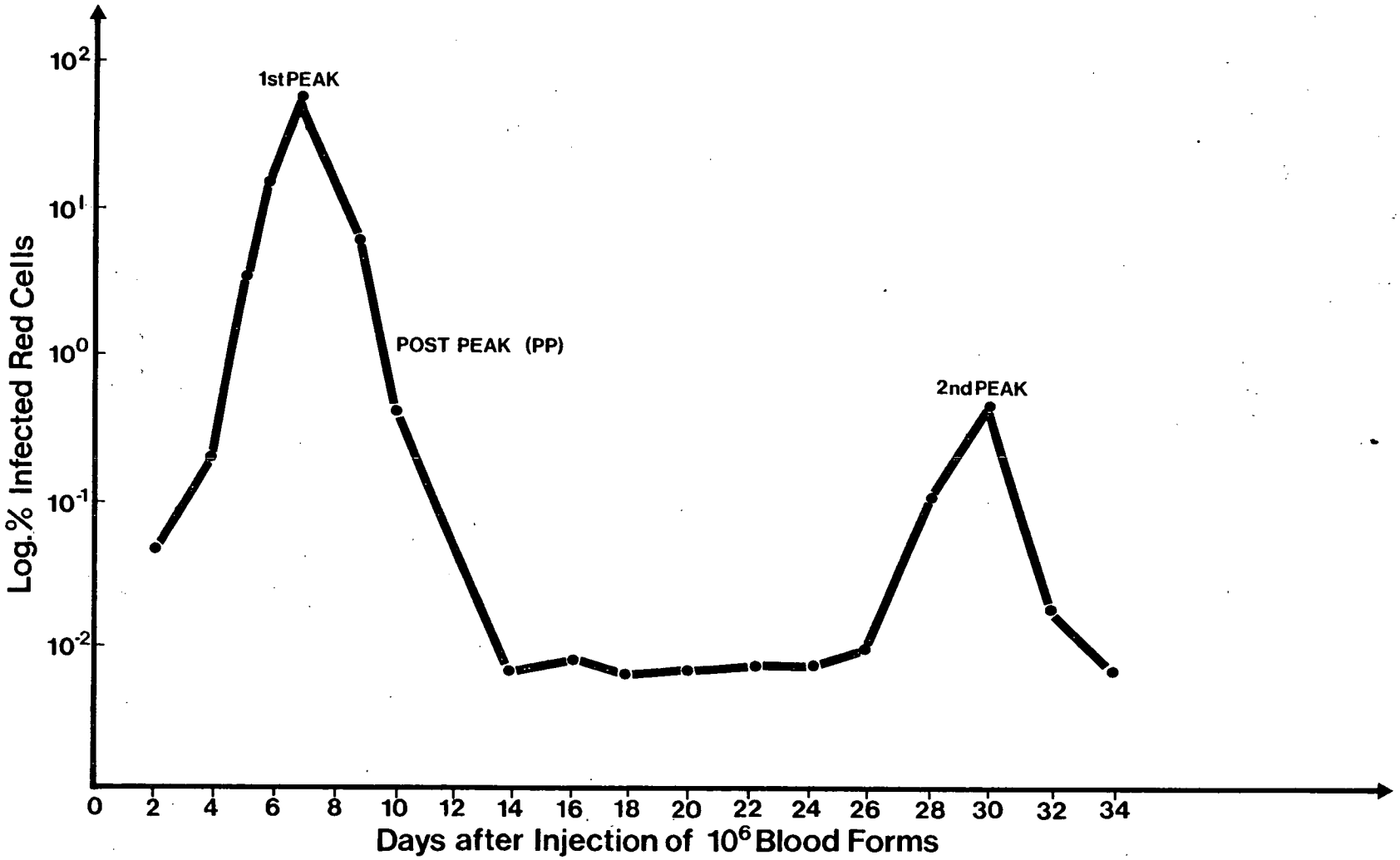
(b) if so, how many mutations were involved.

Such mutations could be at either the same locus or at several loci. The results are discussed in relation to these two possibilities.

(2) Previous studies indicated that parasites resistant to chloroquine at a low level were at a selective advantage (Rosario, 1976b; Rosario *et al.*, 1978). In this work, competition experiments are carried out with the highly chloroquine-resistant line developed. This subject is important in considering how drug resistant parasites are disseminated in the parasite population.

(3) Studies have been carried out on mefloquine to determine how easily, if at all, resistance to this drug can be obtained. In this study resistance to mefloquine was developed and the stability of this character was investigated.

Fig.2 A Typical Course of Infection of P. Chabaudi (Rosario 1976 b)



2. MATERIALS AND METHODS

2.1 Lines of P. chabaudi

2.1.1 Origins

P. chabaudi was first discovered in the Central African Republic in the thicket rat (Thamnomys rutilans)(Landau, 1965). The life cycle and basic biology of the parasite were described in detail by Landau and Killick-Kendrick (1966), Wery (1968), Landau et al. (1970) and Landau and Boulard (1978). The parasite was reduced to the rank of a subspecies (P. vinckei chabaudi) by Bafort (1968). Further studies on the morphology and enzyme patterns of the parasite has lead to its present specific name, P. chabaudi (Carter & Walliker, 1975).

2.1.2 Life cycle

P. chabaudi has a typical malarial life cycle (see Section 1.2). In the liver cells, the schizonts of this species mature in 52 - 53 hours. In the blood, the parasites undergo schizogony approximately every 24 hours producing schizonts of 4 - 8 merozoites. During the early stages of infection with P. chabaudi, the development of the parasite in the blood is synchronous. Unlike other species. where parasites at varying stages of development can be seen, P. chabaudi matures synchronously; after schizogony, for example, ring forms are predominantly seen in the peripheral blood. A typical course of infection of P. chabaudi is illustrated in Figure 2. The sexual forms are predominantly found after the peak of infection.

2.1.3 Definition of Terms

The terms isolate, line, clone and stabilate, which are used throughout the text are defined as follows:

(a) Isolate - This is a sample of parasites collected on a unique occasion from a wild rodent or mosquito and preserved either by passage in laboratory animals or as deep-frozen material. An isolate is not necessarily genetically homogeneous and may contain mixtures of more than one distinct species.

(b) Line - This is a group of parasites which has undergone a particular laboratory passage. A "line" of parasites needs not necessarily be genetically identical. By definition every laboratory manipulation of parasites results in a new "line". In this investigation, parasites are classified as belonging to a new "line" only after a special treatment such as selection for drug resistance.

(c) Clone - A "clone" is an infection derived from a single parasite by asexual reproduction. All parasites obtained from a single clone are assumed to be genetically similar.

(d) Stabilate - This refers to a collection of parasites preserved on a unique occasion. Liquid nitrogen preservation is the usual technique employed.

2.1.4 Parasite Lines

The parasite lines used in this investigation are shown in Table 1. The original drug sensitive line of AS and AJ were obtained from separate thicket rats. These lines were cloned and were characterized for two enzymes by starch gel electrophoresis; they were observed to be polymorphic for lactate dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6PGD). The AS(OCQ) line was derived from the AS(Sens) line as were all the other AS lines. This line was sensitive to chloroquine and resistant to pyrimethamine. Pyrimethamine resistance was established in a single step using a high pressure method (MacLeod,

TABLE 1. Parasite Lines of P. chabaudi

Line	Enzyme Types		Drug Response		
	LDH	6PGD	PYR (Pyrimethamine)	CQ (Chloroquine)	MF (Mefloquine)
AJ (Sens)	2	3	S	S	S
AS (Sens)	3	2	S	S	S
AS (0CQ)	3	2	R	S	S
AS (3CQ)	3	2	R	R(3)	S
AS (15CQ)	3	2	R	R(15)	S
AS (20CQ)	3	2	R	R(20)	S
AS (30CQ)	3	2	R	R(30)	S
AS (12MF)	3	2	R	R(?)	R(12)
AS (15MF)	3	2	R	R(?)	R(15)
AS (30MF)	3	2	R	R(?)	R(30)

PYR-S : Sensitive to 15 mg kg^{-1} pyrimethamine for 4 days.

PYR-R : Resistant to 15 mg kg^{-1} pyrimethamine for 4 days.

CQ-S : Sensitive to 3 mg kg^{-1} chloroquine for 6 days.

CQ-R : Resistant to dose of chloroquine indicated in brackets for 6 days (see Table 4).

MF-S : Sensitive to 10 mg kg^{-1} mefloquine for4 days.

MF-R : Resistant to the doses of mefloquine indicated in brackets for 4 days.

1977). The AS (3CQ) line was derived from the AS (0CQ) line by Rosario (1976a,b). This line was cloned and was subsequently used for selection in this present study. The selection of the other lines are described elsewhere (see Sections 2.8 & 2.9).

2.2 Maintenance of Parasites in the Laboratory

2.2.1 Mammalian Host

The mammalian hosts used were mice and white rats. The mice (Mus musculus) were predominantly inbred 4-6 week old C57 black mice from the Centre for Laboratory Animals, Edinburgh. Mixed strains of mice from the mouse house in the Department of Genetics, Edinburgh, were occasionally used for routine blood passage and cloning. White rats (Rattus norvegicus) were used for concentrating gametocytes (MacLeod & Brown, 1976); these were supplied by the Centre for Laboratory Animals, Edinburgh. All rodents were kept in polypropylene cages with sawdust as bedding, fed with animal cake (MacGregor's of Leith) and were given drinking water ad lib supplemented with 0.05% PABA (para-aminobenzoic acid), an essential parasite growth requirement (Hawking, 1953; Peters, 1970). The animal room was maintained at 18 - 22^{o}C under natural light conditions.

2.2.2. Blood passage of Parasites

For blood passage, parasitized red blood cells were taken from infected rodents and diluted in either cold citrate saline (0.9% NaCl, 1.5% Na Citrate) or in cold heparinized serum Ringer (50% calf serum & 50% mammalian Ringer). The diluted blood was then injected into uninfected animals either by intravenous or intraperitoneal routes. The day of inoculation was called Day 0 (D_0) and subsequent days Day 1

(D_1), Day 2 (D_2), etc. Infection levels were monitored by examining thin blood films made from the tail and stained with Giemsa's stain (B.D.H. Chemicals Ltd.) at pH 7.2.

2.2.3 Mosquito Transmission

The invertebrate host used for cyclical transmissions was Anopheles stephensi. The mosquitoes were kept at a temperature of about 25°C and 90% humidity. They were subjected to a 12 hour light/dark cycle, and fed with a 10% solution of glucose in a 0.05% solution of PABA in water.

Transmissions of the parasite were based on methods described by Landau and Killick-Kendrick (1966). Infected rodents in which mature gametocytes were present were exposed to mosquitoes which had been previously starved for 24 - 48 hours. The mosquitoes were allowed to feed on the rodents for about 1 - 3 hours. Seven days later, mosquitoes were dissected to check for oocysts. On days 15 and 17 after the initial blood meal, the mosquitoes, which were presumed to contain sporozoites in their salivary glands, were allowed to feed on an uninfected mouse, thus transmitting an infection. Parasites could be detected in the blood 4 - 8 days after the mosquito feed.

2.2.4 Liquid Nitrogen Preservation

Parasites were stored in liquid nitrogen using the method described by Lumsden et al. (1966). Before freezing, parasitized blood was mixed with a balanced solution of inorganic salts buffered at pH 8.0 with phosphate buffer in the proportion of 9 parts blood to 1 part salts solution. Glycerol was added to give a final concentration of 7.5%. The mixture was then dispensed into capillaries, sealed and plunged into liquid nitrogen to be stored until required. Parasites were

removed from storage by transferring a capillary from nitrogen into water at room temperature. The capillary was then opened, the contents diluted and injected intraperitoneally into a mouse. Infections became patent 7 - 14 days after inoculation.

2.3 Parasitaemia Estimations

The percentage parasitaemia was determined by counting the number of parasitized red blood cells in Giemsa-stained smears. A total of 2,000 red blood cells was counted if the infection was high (>5%) and in a total of 4,000 red blood cells if the infection was low (<5%).

$$\% \text{ Parasitaemia} = \frac{\text{No. parasitized RBCs}}{\text{Total no. RBCs}} \times 100$$

Standard Error (S.E.) estimates were calculated according to the formulae shown on Table 34 (in Appendix).

2.4 Preparation of Standard Inocula

2.4.1 Inocula Containing 10^6 Parasites

A donor rodent was either bled from the tail or sacrificed and bled from the brachial vessels. The blood was diluted with serum/Ringer so that 0.1 ml contained 10^6 parasites. The diluted blood was kept cold until required. Mice were then inoculated with 0.1 ml aliquots of the diluted blood.

2.4.2 Inocula Containing 0.5 Parasite for Cloning

Clones of blood forms were obtained by a dilution technique (Walliker, 1976). The donor animals used for cloning were those with rising infections, to ensure that the infected cells were infected by only a single parasite. Dilutions were made until a concentration of

0.5 parasite per 0.1 ml of inoculum was obtained. 0.1 ml aliquots of the diluted blood were then injected by the intraperitoneal route into a large number of mice (50 - 100). From the proportion of mice which become infected, the numbers established from one, two or more parasites could be estimated by means of the Poisson distribution. For example, by inoculating 0.5 parasite per mouse, approximately 40% of the animals injected should become infected. Of these 75% are predicted to be clones. Although these calculations are only approximate, they are adequate for the purpose of this work; with several markers, mixtures of clones can readily be detected. This technique is based on the assumption that a single parasite is capable of establishing an infection; this was originally demonstrated by Diggens (1970) using a dilution method.

2.5 Starch Gel Electrophoresis

Enzyme characterization of the blood forms of the parasite was undertaken using starch gel electrophoresis following the technique described by Carter (1973, 1978). The material was prepared for electrophoresis as described by Carter (1978), the parasites being lysed with saponin and freeze-dried until required. Variants of lactate dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6PGD) were examined using the enzyme assay solutions outlined by Carter (1973, 1978). The gel buffer used for LDH was 0.065M Tris-0.222M citrate, pH 6.5 and the electrode buffer was 0.22M Tris-0.0785M citrate, pH 6.2. The gel buffer used for 6PGD was modified; 0.01M phosphate buffer, pH 7, with the addition of NADP was used (0.02 mg/ml). The electrode buffer was 0.1M phosphate pH 7; this was also modified by adding 10 mg NADP to the cathodal

trough. The gel was run for 17 hours instead of 4 hours used by Carter (1973, 1978). All reagents were obtained from Sigma Chemical Company. The voltage gradient used was 3V/cm (42 volts and 24 mA).

2.6 Source, Preparation and Administration of Drugs

All drug doses were expressed as mg kg^{-1} body weight. The drugs were administered orally. When not in use, all of the drug solutions were kept cold.

2.6.1 Chloroquine (CQ)

Chloroquine sulphate (commercially known as Nivaquine) was obtained from May & Baker Ltd., England, in the form of a solution (40 mg CQ base per ml). This solution was diluted with distilled water to obtain the concentrations required. The drug was diluted so that for mice weighing 10g, 0.1 ml of the drug solution contained the dose of chloroquine required. The mice were individually weighed so that exact amounts of the drug were given to each animal.

2.6.2 Pyrimethamine (PYR)

Pyrimethamine (commercially known as Daraprim) was supplied by the Wellcome Research Laboratories, England, in the form of a powdered base. The drug dose was related to the mean weight of the group of mice used for the test. Dilutions were made so that a specific dose of the drug could be administered in 0.1 ml of the solution. Because pyrimethamine is insoluble in water, the powder was dissolved in a small quantity of warmed lactic acid. The solution was then diluted with distilled water to the required concentration.

2.6.3 Mefloquine (MF)

Mefloquine (WR 142, 490) was supplied by the Walter Reed U.S. Army Institute of Research in the form of a powder of the hydrochloride salt. As it was insoluble in water, the drug was dissolved in a small quantity of warmed DMSO (dimethyl sulphoxide). The solution was then diluted with distilled water to obtain the required concentration.

2.6.4 Mepacrine (MP)

Mepacrine (quinacrine dihydrochloride) was obtained from Sigma Chemical Company in the form of a powder. It was dissolved in distilled water to the concentration required.

2.6.5 Quinine (QN)

Quinine (quinine dihydrochloride) was obtained from B.D.H. Chemicals Ltd., in the form of a powder. The drug was dissolved in distilled water.

2.7 Standard Drug Tests

All of the drug tests involved inoculating intraperitoneally a group of five or seven female C57 black mice each with 10^6 blood forms of the parasite line under investigation. The mice were standardized for age (4 - 5 weeks old) and were approximately the same weight (15 - 20g). Three hours after inoculation the first dose of the drug was administered; two mice were left undrugged as controls. The drug was then administered at the same time each day thereafter. Blood smears were taken from the tail of each mouse on appropriate days after completion of drug treatment and examined for parasites.

2.7.1 Chloroquine

Several tests were conducted to evaluate the most effective drug test. 4-day and 6-day tests were compared. In these tests the drug was administered for the appropriate number of days and infections monitored on specific days after treatment was completed.

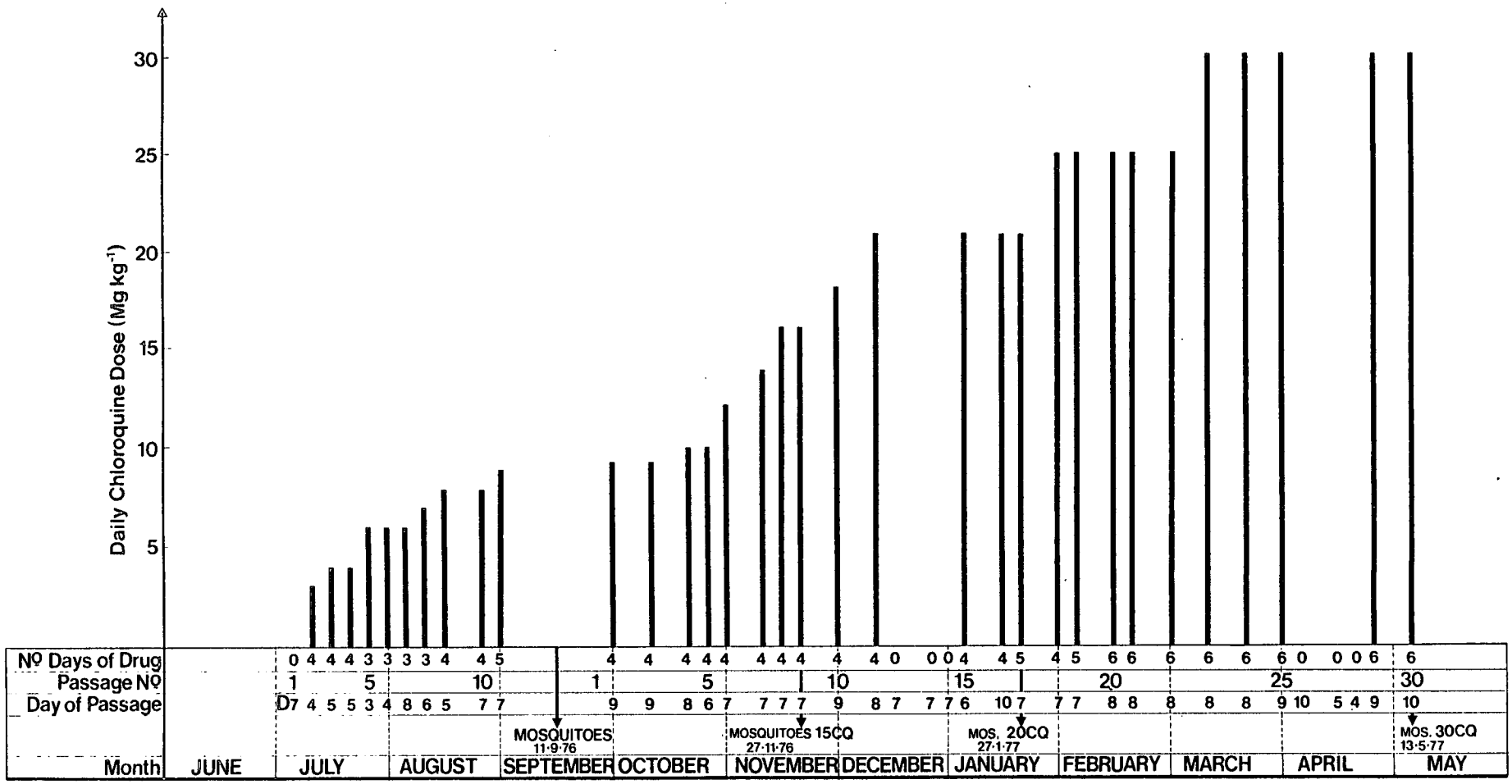
The infectivity of the parasites during drug treatment was determined. The AJ(Sens) and AS(3CQ) lines were compared. 10^6 parasites were inoculated into each of a group of mice. 2 mice of each line were left undrugged (UND) and the other mice were drugged (DG) with either 5 mg kg^{-1} or 10 mg kg^{-1} chloroquine daily for 6 days. After the initial inoculation, subinoculations into clean mice were carried out on specific days. Thereafter, blood smears were taken to monitor any resulting infections.

"Drug delay" tests were carried out in which a group of mice were inoculated each with 10^6 parasites. On Day 3, when the parasitaemia was about 1% drug treatment was started. The parasite response was monitored daily. Drug treatment was continued to determine whether there were any differences between parasite lines. The parasitaemias of the different parasite lines were compared.

2.7.2 Pyrimethamine

A four-day test was used to distinguish between pyrimethamine-resistant (PYR-R) and sensitive (PYR-S) blood forms of the parasite. 15 mg kg^{-1} pyrimethamine was administered daily for four days. Thin blood smears were examined on days 6 and 8; the parasitaemias were calculated. The parasites which survived the treatment were classified as resistant and those which failed to survive, as sensitive.

Fig.3 Development of a High Level Chloroquine-Resistance



2.7.3 Mefloquine

A '4-day' suppressive test was used to distinguish between the lines. The appropriate dose was used (7, 10, 15 & 30 mg kg^{-1}). Parasites which survived the treatment were classified as resistant and those which failed to survive as sensitive.

2.7.4 Mepacrine

Drug tests similar to those of mefloquine were conducted. Doses of 13 and 15 mg kg^{-1} mepacrine were used.

2.7.5 Quinine

Drug tests similar to those of mefloquine were conducted with the exception of the drug dose used. Much higher doses of 150, 200 and 250 mg kg^{-1} were required.

2.8 Chloroquine-resistance

2.8.1 Selection of a High Level of Chloroquine-Resistance

A continuous low pressure method was used to increase the level of resistance. Starting from a line already resistant to 3 mg kg^{-1} chloroquine (AS 3CQ)(Rosario, 1976a,b), a group of mice were each injected with 10^6 parasites and treated daily with chloroquine. The parasites which survived the treatment were then taken and 10^6 parasites were injected into each of a further group of mice. These mice were in turn treated with a slightly higher dose of chloroquine. This procedure was continued, the doses used for selection being shown in Figure 3. The dose and duration of drug treatment was chosen according to the parasite response. If the parasites were increasing in number during selection, the dose was increased. At

various stages of the selection procedure the parasites were transmitted through mosquitoes in order to investigate the stability of the resistance on mosquito transmission. The parental AS(3CQ) line was used as a control and the parasites were tested at the same time as the parasites undergoing selection, to determine whether any significant changes of resistance had occurred.

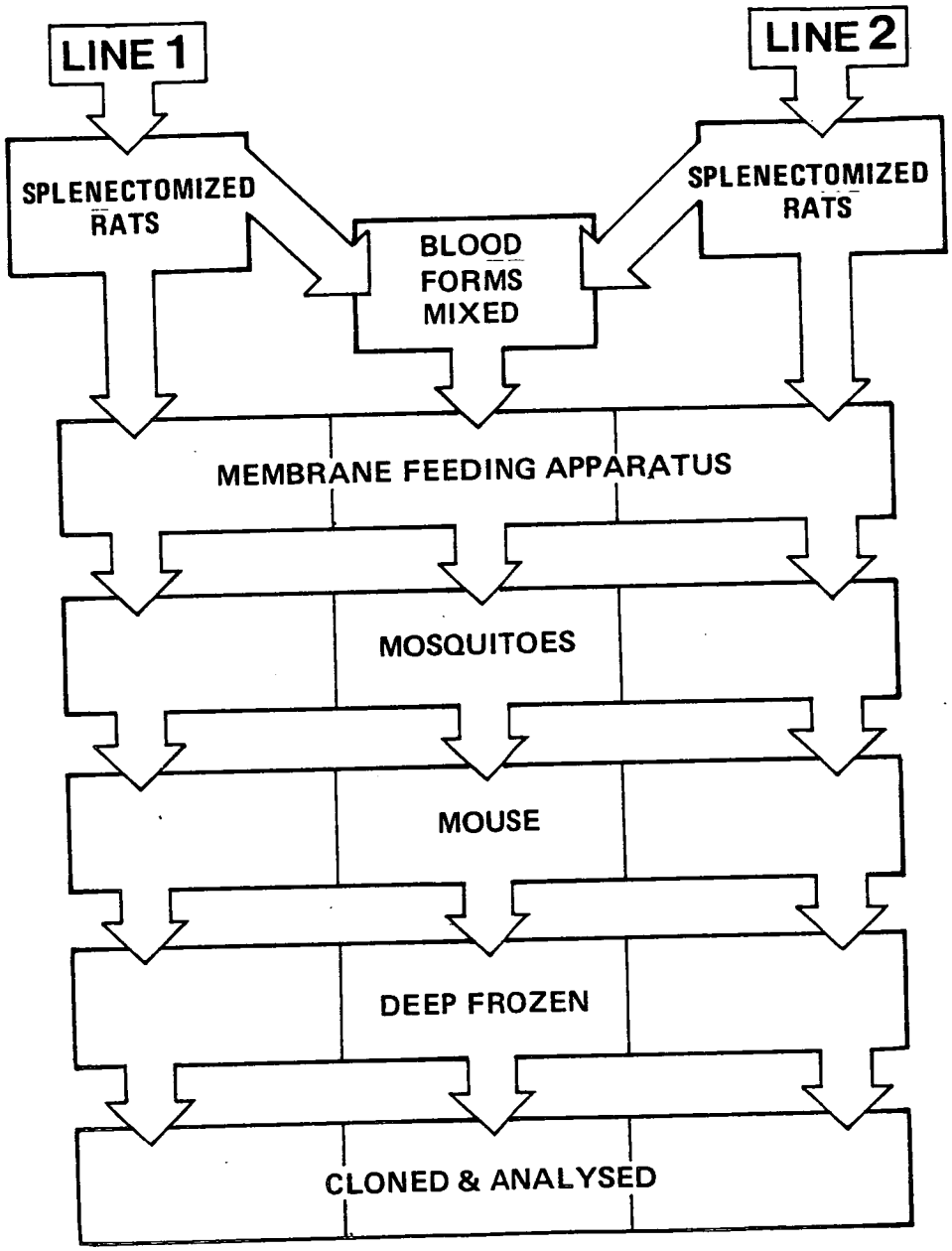
2.8.2 Tests for Different Levels of Chloroquine-Resistance

At various stages of selection the parasites were transmitted through mosquitoes to test for the stability of the resistance (Fig. 3). These included parasites which had survived 15, 20 and 30 mg kg^{-1} of the drug; these lines were denoted as AS (15CQ), AS (20CQ) and AS (30CQ) respectively. Comparative tests were devised to determine whether these lines differed from each other and could therefore be classified as demonstrating different levels of resistance. Each line was tested using a range of doses from 5 - 30 mg kg^{-1} chloroquine. In addition the parasites were tested for stability on multiple blood passage without drug pressure. The different lines were also tested for their response to mefloquine, mepacrine and quinine. The parasites were classified according to the rate at which they produced patent parasitaemias after drug treatment. The most important factor in these drug tests was the day in which the parasites could be detected in the blood.

2.8.3 In vitro Drug Tests

Short term *in vitro* drug tests, according to the micro-technique described by Rieckmann *et al.* (1978) for *P. falciparum*, were undertaken to determine whether this method could be used to differentiate between chloroquine-resistant and chloroquine-sensitive lines of *P*

Fig.4 Method for a Genetic Cross



chabaudi.

Two lines of P. chabaudi were tested:-

(a) AJ(Sens)

(b) AS(30CQ)

Because P. chabaudi undergoes synchronous development in the blood, with schizogony occurring around midnight, cultures were set up in the morning when the parasites were at an early trophozoite stage. They were incubated for various periods up to 24 hours in wells of micro-titre plates. Each well contained:

(a) 50 µl RPMI 1640 medium, supplemented with Hepes buffer and sodium bicarbonate.

(b) 5 µl parasitized blood from donor mice.

(c) Various doses of chloroquine diluted in RPMI 1640 medium.

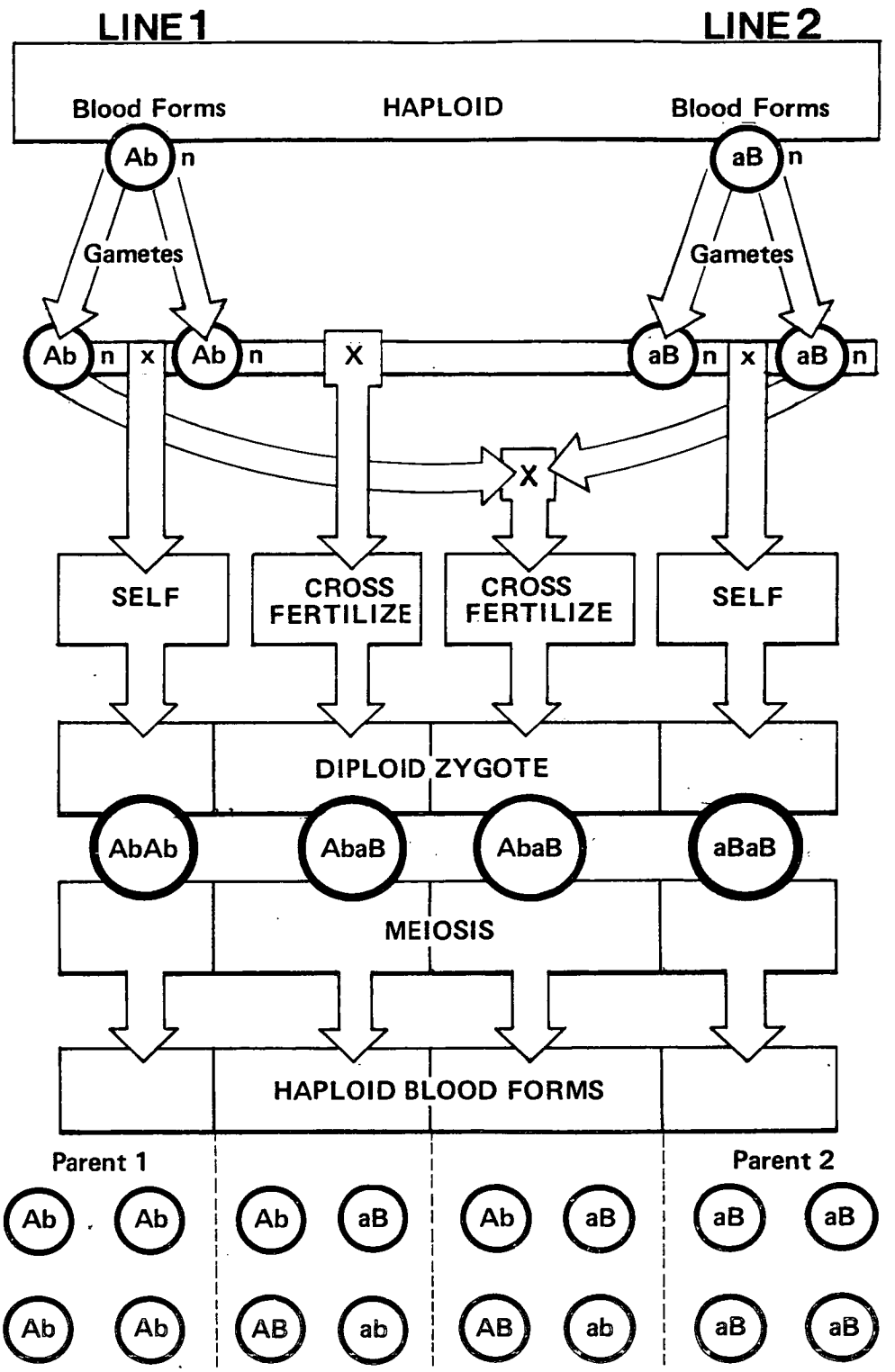
Cultures were maintained in a candle jar at 37°C. After incubation, thin blood smears were taken from each well and examined for parasite maturation. The development of the parasites was also monitored in vivo by taking thin blood films of the donor mice. This was done in order to determine when the cultures should be examined.

2.8.4 Genetic Studies

(a) Conducting a Cross

The strategy used for making crosses in this investigation is illustrated in Figure 4. This is a modified method to that described by Walliker et al. (1975). Each parent line was first injected separately into splenectomized rats; splenectomized rats were used to enhance gametocyte production (MacLeod & Brown, 1976; MacLeod, 1977). Splenectomy was carried out at least 48 hours before inoculation of parasites by the intravenous route. Four days later, when

Fig.5 Schematic Diagram of a Cross



Genotypes:	Ab	AB	ab	aB
	6	2	2	6
Ratios:	3	1	1	3

a large number of mature gametocytes were present and exflagellation of the microgametes was observed, the rats were sacrificed and the blood of each parent line mixed. The blood mixture was then placed in a membrane feeding apparatus (Rutledge et al., 1964). Minimal heparin (5 units per ml) was used to prevent clotting. Mosquitoes were then allowed to feed on the blood mixture. After 15 days the mosquitoes were permitted to feed on further animals to transmit infections. Control studies on each parent line were conducted in parallel. After mosquito transmission stabilates of infected blood were prepared and stored in liquid nitrogen and used for subsequent analyses.

During mosquito transmissions, midguts were examined (on D 7) to determine whether one parent line had produced more oocysts than the other line used for the cross. This was to give an indication whether each line had produced similar numbers of gametocytes and hence whether equal numbers of each line were present in the cross.

Assuming Mendelian inheritance, a schematic diagram can be drawn to illustrate what may happen during a cross (Fig. 5). Assuming random fertilization of gametes, the two lines differing in two unlinked markers A and B, for example, should produce progeny in the ratios shown. Reassortment of genetic markers takes place at meiosis; meiosis is thought to occur some time between zygote formation and the emergence of the blood forms.

(b) Analyses of the Products of a Cross

(i) Tests on Uncloned Products

The uncloned products of crosses between drug resistant and sensitive lines differing in enzyme forms, were examined for their enzyme types after treatment with pyrimethamine and chloroquine.

Figure 6: Reconstruction Experiments

Experiment No.	%AS	%AJ
1	100	0
2	90	10
3	80	20
4	70	30
5	60	40
6	50	50
7	40	60
8	30	70
9	20	80
10	10	90
11	0	100

This gave an indication of whether or not cross-fertilization had taken place. If the parental lines were transmitted without cross-fertilization only the enzyme types of the drug resistant parents would have been observed after drug treatment. The presence of the sensitive parental enzyme types after drug treatment, showed that cross-fertilization had occurred between the two lines.

(ii) Cloning and Characterization

Clones were obtained from the products of the cross and from the parental controls by dilution. Each clone was analysed for parental characters. In some experiments, the uncloned progeny were treated with chloroquine at a specific dose for 5 or 6 days before cloning. The clones obtained in these experiments had thus been pre-selected for resistance to chloroquine at the level given.

2.8.5 Reconstruction and Competition Experiments

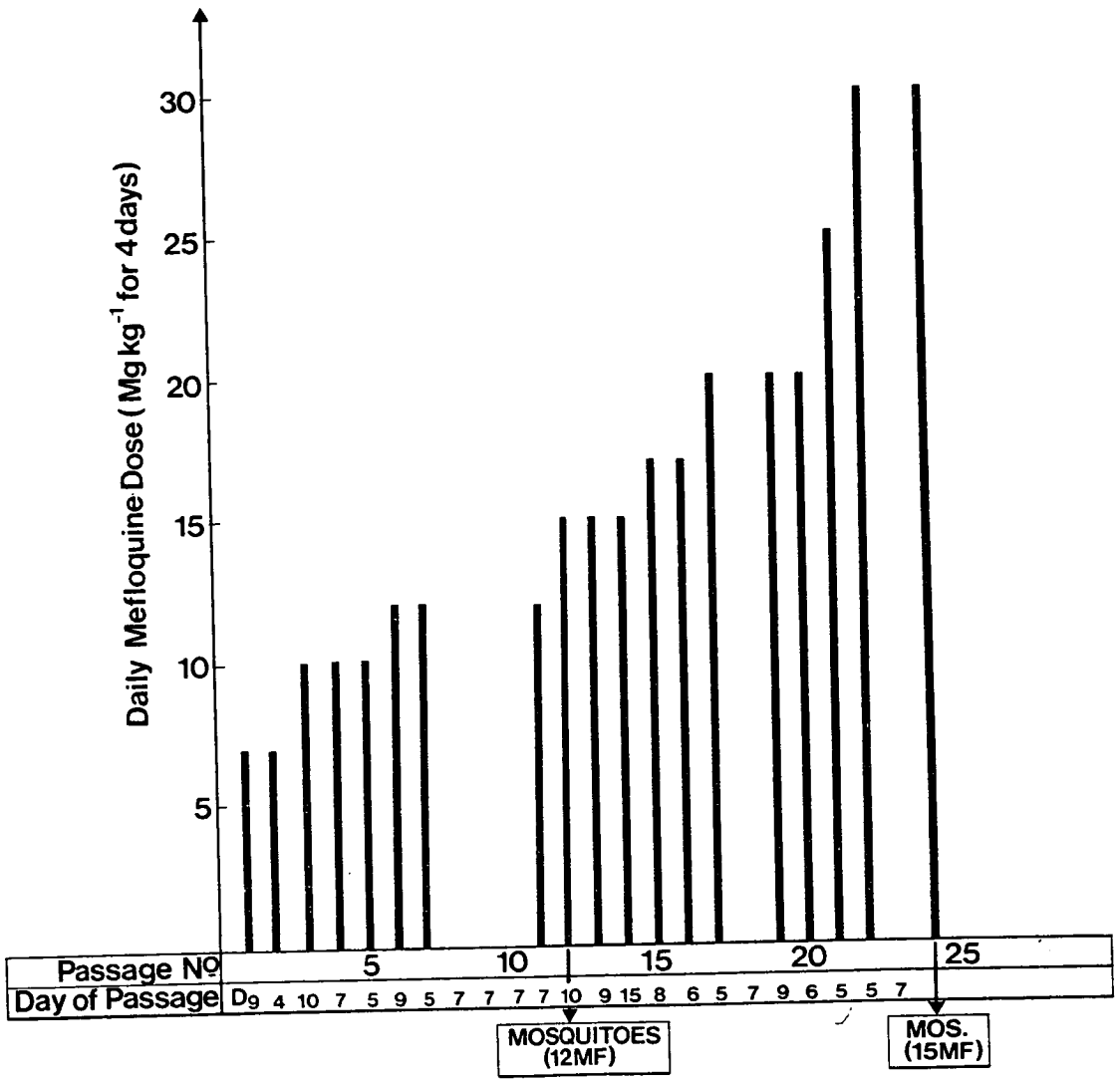
(a) Reconstruction

Reconstruction experiments were carried out in which deliberate mixtures of parasites with AS and AJ enzyme types were made in various proportions in order to establish at what level the electrophoresis system could detect quantitative differences. The lines used were AS(30CQ) and AJ(Sens) (See Table 1). In these experiments 10^6 parasites of each of the two lines were each injected into groups of 10 mice. When the infections were high (about 40%) the animals were sacrificed and the parasitized blood of each line was pooled separately. The pooled parasitaemias of each line were measured from thin blood films. Estimates of pooled red blood cell counts were made using a Coulter counter. The parasites were then mixed so that each mixture contained each line in the proportion shown on Figure 6. The parasites

Figure 7: Competition Experiments
50:50 Mixtures of Blood Forms of Different Lines

Experiment 1	AS (Sens)	Vs	AJ (Sens)
Experiment 2	AS (30CQ)	Vs	AS (0CQ)
Experiment 3	AS (30CQ)	Vs	AJ (Sens)
Experiment 4	AS (30CQ)	Vs	AJ (30CQ)

Fig.8 Development of Mefloquine-Resistance



were then prepared for starch gel electrophoresis and examined for LDH. This enzyme was more suitable for examining mixtures than 6PGD as the parasites of each line produced only a single LDH band which did not overlap in a mixture of the two types. 6PGD of the parasites had three bands. Mixtures of the two lines were not easily detectable as two of the three bands overlapped. As it was important to detect differences in the activities of the enzymes from each of the two lines, the 6PGD enzyme assay was not used.

(b) Competition

Competition experiments were carried out in order to discover whether there were differences in the growth patterns of the different lines of P. chabaudi. The competition experiments undertaken are shown in Figure 7. In each case 10^6 blood forms containing equal numbers of each line were injected intraperitoneally into mice. The infections were then passaged weekly and either cloned or prepared for enzyme analyses (for further details, see Section 3.6.2). The LDH enzyme electrophoresis bands of the parasites were examined at various intervals to determine whether their relative intensities changed with time. Cloned parasites were tested for their chloroquine response.

2.9 Mefloquine

2.9.1 Selection for Mefloquine-Resistance

Selection of a mefloquine-resistant line of P. chabaudi was undertaken by exposing parasites to gradual increases of drug pressure (Fig. 8) during blood passage through mice. This was a similar selection programme to that used for the selection of chloroquine resistance. Three lines were used for the selection of mefloquine resistance. These were:

(a) AS(0CQ)

(b) AS(3CQ)

(c) AS(15CQ)

A group of mice were each infected with 10^6 parasites of each line. Three hours after inoculation the first drug treatment was administered at a dose of 7 mg kg^{-1} for 4 days. The parasites which survived the drug treatment were taken and after dilution, 10^6 parasites were injected into further mice. These mice were treated with a slightly higher dose of mefloquine. This procedure was repeated using higher doses. A 4-day suppressive selection programme was adopted throughout the selection for mefloquine-resistance. Mefloquine-resistant lines obtained were also examined for chloroquine-response to determine whether an increase in mefloquine-resistance would affect its level of chloroquine-resistance.

2.9.2 Stability of Mefloquine-Resistance

Stability studies on the mefloquine-resistant line obtained were undertaken. The stability of the line on cyclical transmissions through mosquitoes and on multiple blood passage without drug pressure was investigated.

2.9.3 Cross-Resistance to Quinine

The possibility of cross-resistance between mefloquine and quinine was examined. Mefloquine-resistant lines were tested with varying doses of quinine.

3. RESULTS

3.1 Selection of Chloroquine-Resistant Lines

The parasites responded to selection by a gradual increase in drug pressure (Fig. 3). The parasites were transmitted through mosquitoes as shown to result in the lines AS(15CQ), AS(20CQ) and AS(30CQ).

3.2 Response of Selected Lines to Chloroquine

3.2.1 4-Day Versus 6-Day Tests

A 4-day test was compared with a 6-day test using high doses of the drug (10 mg kg^{-1} & 30 mg kg^{-1}). Three lines were compared at each dose. It was found that even at high doses a '4-day' test did not eliminate either the AJ(Sens) or AS(3CQ) lines whereas '6-day' tests were more effective (Table 2). '6-day' suppressive drug tests were therefore adopted as the standard chloroquine test. These results show that not only the dose but the duration of drug treatment is important for anti-malarial activity.

3.2.2 Infectivity During Drug Treatment

The infectivity of the parasites were examined by subinoculation into uninfected mice at various stages of the course of drug treatment (Table 3). A chloroquine sensitive AJ(Sens) and an AS(3CQ) line were compared. On D_{15} the animals were sacrificed and the blood was used to inoculate clean mice. The sensitive line was eliminated from the circulation after treatment with 5 mg kg^{-1} chloroquine for 6 days and could not be detected on days 5, 7 and 9 of infection. The subinoculations on these days confirm the absence of parasites in the peripheral blood (Table 3). By D_{15} the parasites recrudesce at this dose and this observation was consistent with the results of the subinoculations. The AS(3CQ) line

TABLE 2. Comparison between 4-Day and 6-Day Chloroquine Tests

	% Parasitaemia (± S.E.)							
	10 mg kg^{-1} for 4 days				10 mg kg^{-1} for 6 days			
Day of Infection	D_5	D_7	D_9	D_{11}	D_5	D_7	D_9	D_{11}
AJ(Sens)	-	-	-	±	-	-	-	-
AS(3CQ)	-	-	0.003 (±0.003)	0.5 (±0.4)	-	-	-	-
AS(30CQ)	0.003 (±0.003)	3.0 (±1.2)	26.0 (±5.3)	6.1 (±2.9)	0.01 (±0)	0.2 (±0.08)	5.07 (±1.3)	8.5 (±3.4)
Drug Treatment	30 mg kg^{-1} for 4 days				30 mg kg^{-1} for 6 days			
Day of Infection	D_{11}	D_{13}	D_{15}		D_{11}	D_{13}	D_{15}	
AJ(Sens)	-	-	±		-	-	-	
AS(3CQ)	-	-	±		-	-	-	
AS(30CQ)	3.0 (±0.8)	16.0 (±4.9)	3.0 (±1.3)		0.4 (±0.3)	7.9 (±4.1)	8.3 (±3.6)	

Drugged animals - mean of 4 mice.
Undrugged animals were all at Post Peak by DAY 9 of infection
± ~ 0.01%

TABLE 3. Infectivity of Parasites During Chloroquine Treatment

1. Parasitaemias of donor animals

Drug Treatment (5mg kg^{-1}/6 days) Day of Infection		D_5	D_7	D_9	D_{15}
AJ(Sens)	DG	-	-	-	0.17±0.17
	UND	10.3 (±2.1)	47 (±6.9)	PP	PP
AS(3CQ)	DG	-	-	0.01 (±.01)	11.02 (±11.0)
	UND	4.2 (±2.3)	36.7 (±13.4)	PP	PP

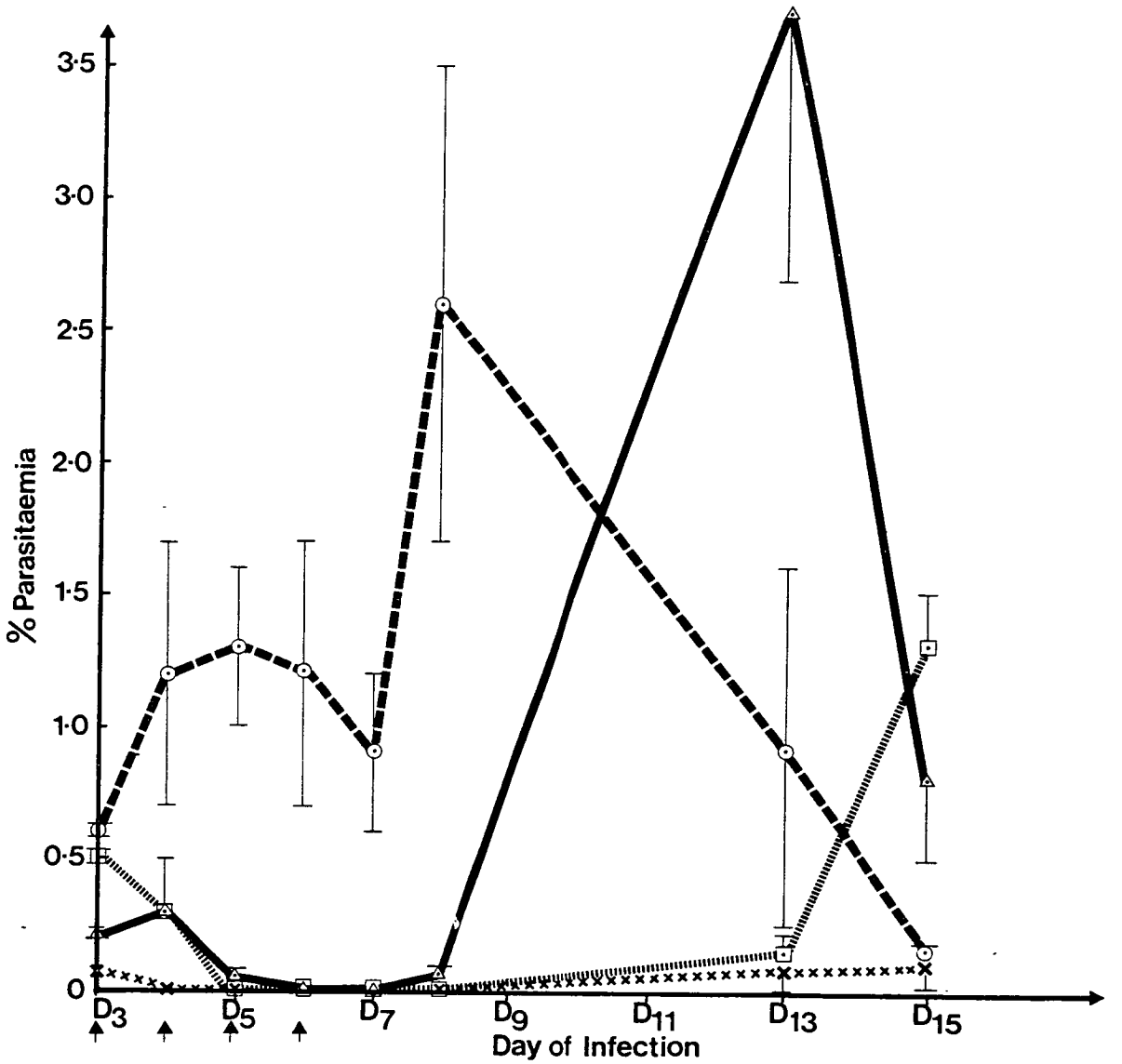
2. Parasitaemias of animals inoculated from drugged mice.

	Days of inoculation											
	Day 5			Day 7			Day 9			Day 15		
	D_6	D_{10}	D_{15}	D_6	D_{13}	D_{15}	D_5	D_{11}	D_{16}	D_5	D_{10}	D_{21}
AJ(Sens)	-	-	-	-	-	-	-	-	-	13.3 (±2.3)	+	-
AS(3CQ)	-	2.8 (±1.7)	+	-	+	+	-	+	+	43 (±5.8)	+	±

Drugged and subinoculated animals - mean of 3 mice.
Undrugged animals - mean of 2 mice.

PP - Post Peak
+ - Greater than 5%

Fig.9 Drug Delay Test Using 10mg kg^{-1} Chloroquine



KEY

▲ Treated with 10mg. kg.$^{-1}$CQ

×××× AJ(sens)

AS(3CQ)

AS(15CQ)

AS(30CQ)

Data from Table 35 (In appendix)

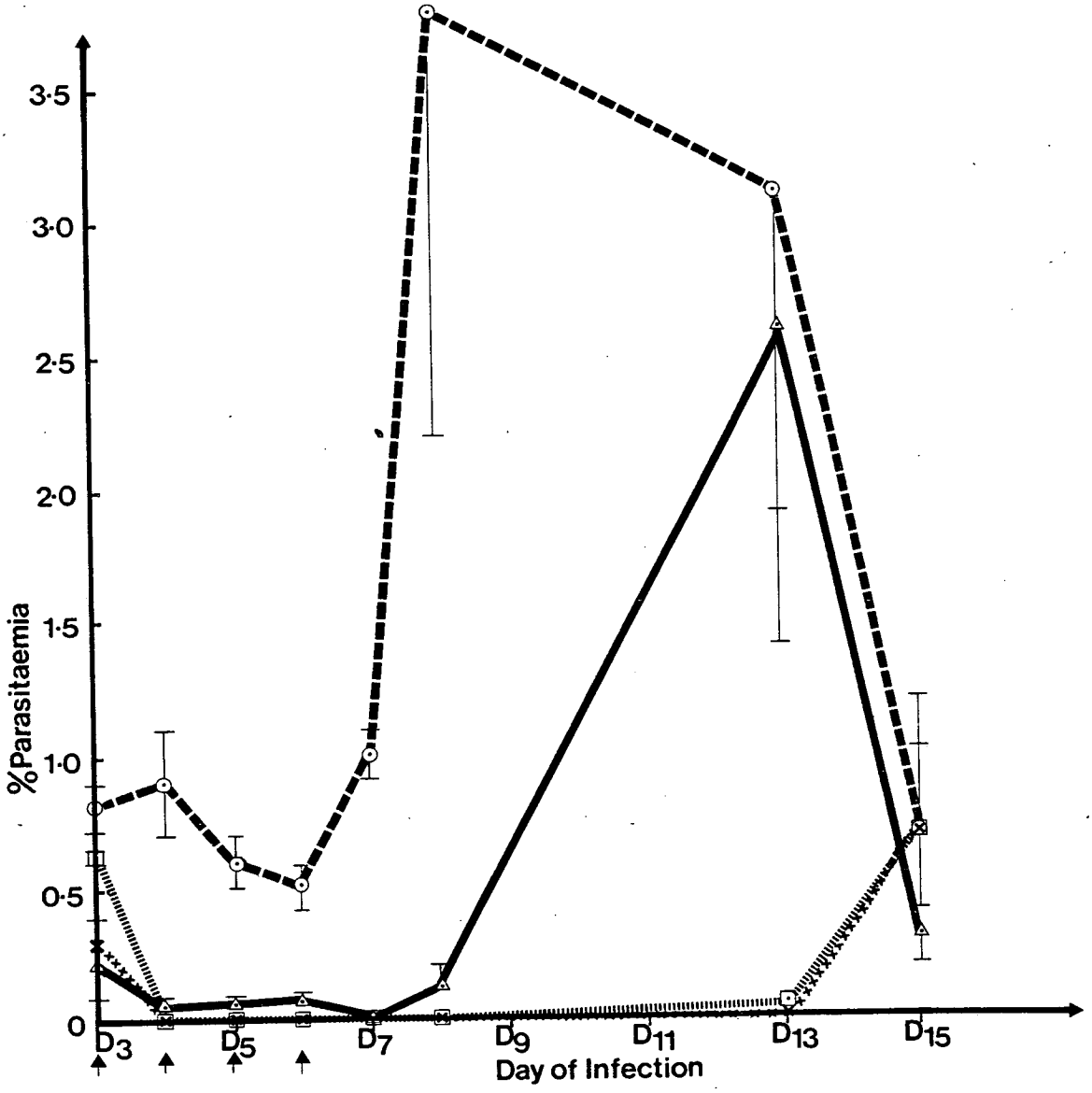
survived the drug treatment. Although parasites could not be detected until D_9, the subinoculations show that the parasites were present as all the animals subinoculated on D_5 showed positive infections (Table 3). These results confirm that at this dose (5 mg kg^{-1}) a difference can be detected between the AJ(Sens) and AS(3CQ) lines; the AS(3CQ) line remains infective whilst the AJ(Sens) line exhibits a reduced infectivity.

Both lines were treated in the same way using a higher dose of 10 mg kg^{-1} chloroquine for 6 days; none of the parasites survived the drug treatment and all the subinoculations showed negative results. The mice were examined up to D_{15} in each case. On D_{15} the mice were sacrificed and subinoculated into uninfected mice as before; these gave negative results as well. Thus, it would seem that this dose eliminates these two lines completely (see Table 4 also).

3.2.3 Drug Delay Tests

Drug delay tests were devised to observe the rate of decline of parasites of different lines during drug treatment using doses of 10 mg kg^{-1} and 20 mg kg^{-1} given for 4 days. The AJ(Sens), AS(3CQ), AS(15CQ) and AS(30CQ) lines were compared. The AJ(Sens) and AS(3CQ) lines declined rapidly after treatment at both doses. The parasites do not increase in numbers again until D_{15}. The AS(15CQ) line grew in the presence of the drug at a dose of 10 mg kg^{-1}, however on prolonged exposure the parasite numbers decreased rapidly by D_5. Once drug pressure was released the parasites grew rapidly to reach a high parasitaemia by D_{13}. Drug treatment at the higher dose of 20 mg kg^{-1} gave a similar result with the exception that the parasites declined rapidly soon after exposure to the drug. The parasites of the AS(30CQ) line grew in the presence of the drug. After continued exposure to the drug, the

Fig.10 Drug Delay Test Using 20mg kg^{-1} Chloroquine



KEY

▲ Treated with 20mg. $kg.^{-1}$ CQ

×××× AJ(sens)

□ AS(3CQ)

△ AS(15CQ)

○ AS(30CQ)

Data from Table 36 (In appendix)

parasites were suppressed. Once drug pressure was released the parasitaemia increased exponentially reaching a peak by D_8. Although the initial parasitaemias of the lines used were different, the test shows some clear differences between the lines. The AJ(Sens) and AS(3CQ) lines were indistinguishable from each other, however they were clearly different from the high levels of resistance. The AS(15CQ) and AS(30CQ) lines differed in their response. The rate of suppression of the AS(30CQ) line was slower than that of the AS(15CQ) line; the former continued to recrudesce earlier once drug pressure was removed than the latter (Figs. 9 & 10).

3.2.4 Response to Different Doses of Chloroquine

The most convincing evidence for different levels of chloroquine-resistance was that obtained from drug tests with chloroquine at varying doses (Table 4). A dose of 3 mg kg^{-1} did not always distinguish between the lines (Table 3Z, in Appendix); at a dose of 5 mg kg^{-1} the sensitive lines could be differentiated from the low level (AS(3CQ)) and higher levels. A dose of 30 mg kg^{-1} of the drug revealed differences between the higher levels of resistance. The most significant result was the rate at which the parasites emerged after drug treatment. The AS(30CQ) line almost always emerged before the other lines. At this high dose of 30 mg kg^{-1} the parasites of the AS(30CQ) line recrudesced by D_9. The parasites of AS(20CQ) line emerged by D_{11}, followed by those of AS(15CQ) line at D_{13}.

These tests were used in subsequent genetic work. Clones of unknown chloroquine response were tested at 30 mg kg^{-1}. If the parasites emerged by D_9 or D_{11} they were considered to be in the 30CQ class; those

TABLE 4. Response of Different P. chabaudi Lines to Varying Doses of Chloroquine

DRUG DOSE mg/kg⁻¹ for 6 days	% Parasitaemia (± S.E.)																																			
	5						10						15						20						25						30						UNDRUGGED	
Days of Infection	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	D_5	D_7
Lines used																																				
AJ(Sens)	-	-	-	-	0.2 ± (0.1)	2.7 ± (1.3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11 ± (2.7)	37 ± (5.3
AJ(Sens)	-	-	-	-	.08 ± (0)	6.6 ± (2.0)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15 ± (0)	47 ± (0)
AS(30Q)	-	-	0.11 ± (0.05)	2.02 ± (0.5)	0.9 ± (0.8)	0.7 ± (0.8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7 ± (1.3)	33 ± (1.8)
AS(15Q)	.006 ± (0.005)	0.08 ± (0.05)	2.5 ± (1.3)	16.0 ± (5.4)	11.6 ± (3.3)	0.6 ± (0.3)	-	-	-	0.12 ± (0.04)	3.2 ± (1.2)	14.0 ± (5.1)	-	-	-	0.09 ± (0.05)	1.7 ± (0.6)	7.8 ± (1.0)	-	-	-	0.2 ± (0.09)	2.7 ± (1.07)	15.7 ± (4.8)	-	-	-	-	0.3 ± (0.1)	5.2 ± (1.9)	-	-	-	-	0.15 ± (0.01)	5.8 ± (1.3)	2.22 ± (0.4)	22 ± (2.4)
AS(20Q)	0.04 ± (0.02)	0.2 ± (0.1)	3.1 ± (0.8)	3.3 ± (0.7)	0.11 ± (0.05)	-	-	-	0.05 ± (0.03)	.4 ± (0.1)	5.9 ± (2.1)	1.0 ± (0.3)	-	0.002 ± (0.002)	0.012 ± (0.007)	0.09 ± (0.03)	1.9 ± (0.5)	5.8 ± (1.0)	-	-	-	0.06 ± (0.03)	1.4 ± (0.2)	10.4 ± (1.8)	-	-	-	0.03 ± (0.02)	0.9 ± (0.2)	2.2 ± (0.5)	-	-	-	0.05 ± (0.03)	0.9 ± (0.3)	2.8 ± (0.9)	3.2 ± (0.5)	25 ± (3.2)
AS(30Q)	0.03 ± (0.01)	0.1 ± (0.05)	20 ± (0.5)	4.3 ± (1.3)	0.1 ± (0.07)	-	-	-	0.09 ± (0.05)	2.8 ± (0.8)	5.7 ± (1.2)	0.05 ± (0.02)	-	-	0.03 ± (0.01)	1.3 ± (0.4)	3.6 ± (0.6)	0.07 ± (0.03	-	-	0.012 ± (0.01)	0.3 ± (0.1)	5.9 ± (1.07)	0.5 ± (0.3)	-	-	0.02 ± (0.009)	0.4 ± (0.09)	1.7 ± (0.5)	0.1 ± (0.07)	-	-	0.002 ± (0.002)	0.3 ± (0.06)	4.3 ± (1.4)	2.0 ± (1.6)	13.7 ± (1.9)	38 ± (2.5)
AS(30F)	0.04 ± (0.02)	0.3 ± (0.08)	4.7 ± (0.09)	3.1 ± (0.8)	0.04 ± (0.007)	0.02 ± (.01)	-	-	0.10 ± (0.05)	1.5 ± (0.63)	0.4 ± (0.18)	0.006 ± (0.005)	-	-	0.06 ± (0.02)	1.0 ± (0.4)	2.6 ± (0.7)	0.7 ± (0.3)	-	-	0.012 ± (0.007)	0.6 ± (0.2)	4.7 ± (0.9)	2.1 ± (0.8)	-	-	0.02 ± (0.01)	0.4 ± (0.2)	2.0 ± (0.7)	2.5 ± (0.6)	-	-	-	0.05 ± (0.03	1.9 ± (0.4)	1.5 ± (0.4)	1.7 ± (0.3)	0.1 ± (1.1)

Undrugged - mean of 12 mice.
Drugged - mean of 5 mice.

which emerged later, on either D_{13} or D_{15}, were grouped in the 15CQ class. Parasites which survived treatment at 3 mg kg^{-1} chloroquine but failed to survive either 15 or 30 mg kg^{-1} of the drug were classified as 3CQ parasites.

3.3 Stability of Resistance of Selected Lines

3.3.1 Response of AS(15CQ) Line

The resistance of the AS(15CQ) line was stable following mosquito transmission (Table 5), and after multiple blood passage in the absence of the drug. The resistance appeared to increase after passage. This may be either due to experimental error or indeed an increase of virulence due to continued blood passage. However, an increase in virulence is unlikely to occur in two weekly passages; nevertheless, this possibility cannot be ruled out.

3.3.2 Response of AS(20CQ) line

The AS(20CQ) line was stable after mosquito transmission (Table 6). This line was found to be stable on multiple blood passages without drug pressure. There seemed to be an increase in virulence from passages 2 to 8. This may be due to blood passage. An alternative explanation is, as this line was uncloned at this stage, the population may consist of different levels of resistance and the high levels of resistance may be outgrowing the lower levels producing the results obtained.

3.3.3 Response of AS(30CQ) Line

Four clones were established from this line and tested for drug resistance at 30 mg kg^{-1} (Table 7). Clone numbers 1 and 3 were classified as 30CQ and clone numbers 2 and 4 as 15CQ (See 3.2.4). The AS(30CQ)

TABLE 5. Stability of AS(15CQ) line after Mosquito Transmission

	% Parasitaemia(± S.E.)			
	AS(15CQ)		AS(3CQ) CONTROL	
Day of Infection	D_{10}		D_{10}	
Passage No. after mosquitoes	DG	UND	DG	UND
5	0.17 (±0.08)	PP	-	PP
7	2.6(± 1.6)	PP	-	PP

DG : Drugged Animals, Mean of 3 Mice, Treated with 15 mg kg^{-1} chloroquine for 6 days.

UND: Undrugged Animals, Mean of 2 Mice.

PP : Post Peak.

TABLE 6. Stability of AS(20CQ) Line After Mosquito Transmission

	% Parasitaemia(± S.E.)			
	AS(15CQ)		AS(3CQ) CONTROL	
Day of Infection	D_{11}		D_{11}	
Passage No. after mosquitoes	DG	UND	DG	UND
2	0.2(±0.2)	PP	-	PP
8	3.2(±1.6)	PP	-	PP
14	2.5(±0.5)	PP	-	PP

DG : Drugged animals, mean of 5 mice treated with 20 mg kg^{-1} chloroquine for 6 days.

UND: Undrugged animals, mean of 2 mice.

PP : Post Peak.

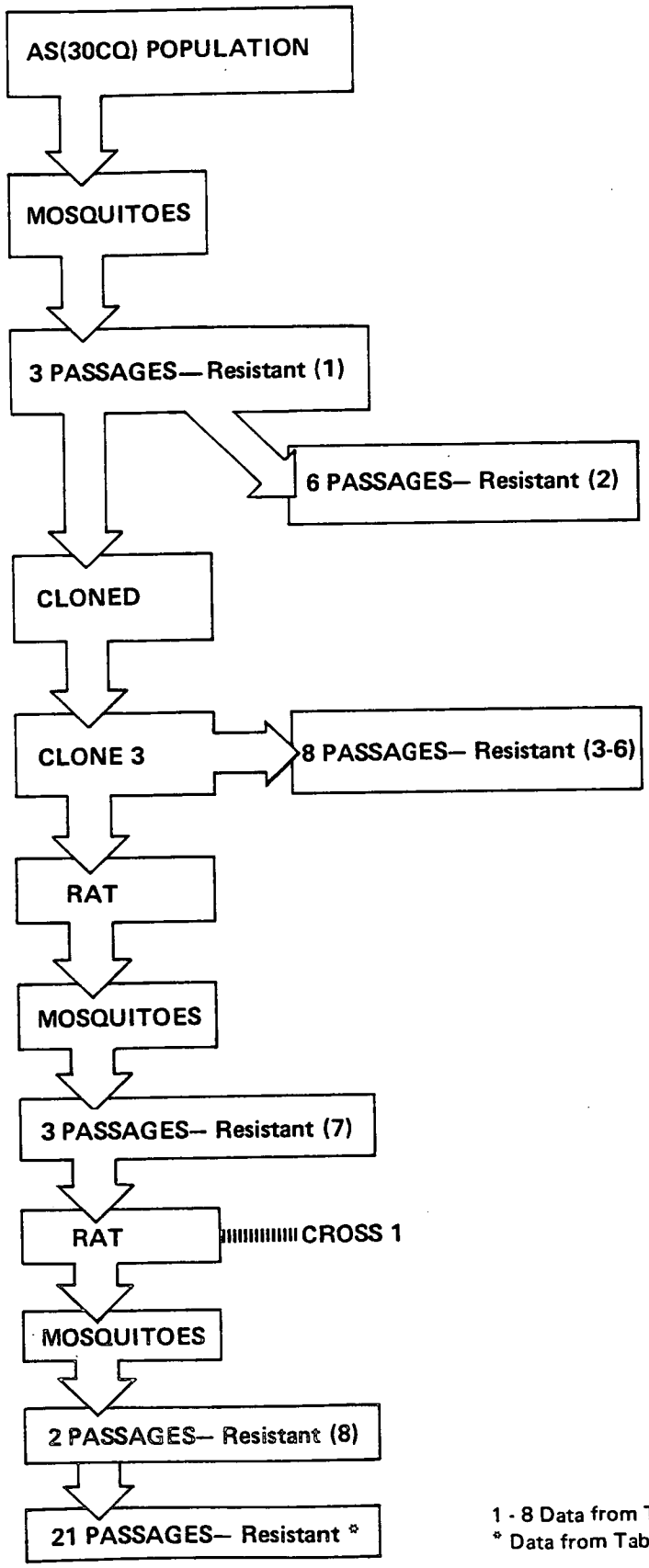
TABLE 7. Drug Response of Clones Derived from the AS(30CQ) Population

	% Parasitaemia (± S.E.)		
	DRUGGED*		UNDRUGGED+
Day of Infection	D_{11}	D_{13}	D_7
Clone No.			
1	0.17(±0.08)	1.0(±0.3)	42(±18.4)
2	-	0.05(±0.01)	50(±0)
3	0.09(±0.01)	0.8(±0.2)	22(±5.0)
4	-	0.06(±0.035)	23.5(±18.4)
AS(3CQ) Control	-	-	42(±14.9)

* Drugged Animals - mean of 3 mice, treated with 30 mg kg^{-1} for 6 days.

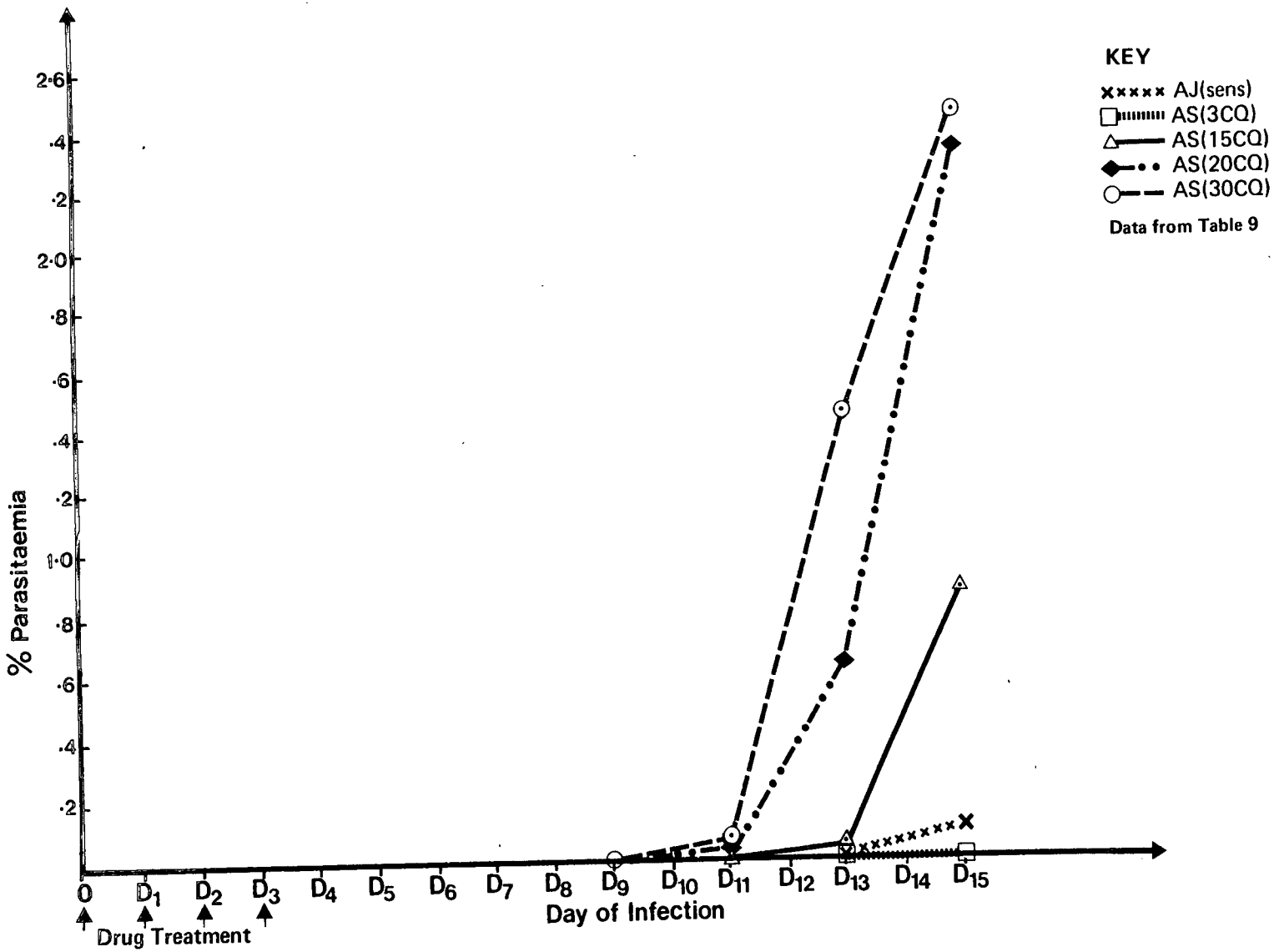
+ Undrugged Animals - mean of 2 mice.

Fig.11 Stability of the AS(30CQ) Line



1 - 8 Data from Table 38 in appendix
* Data from Table 8

Fig. 12a The Response of Different Lines of P. Chabaudi to Mefloquine (7mg kg^{-1})



population was probably a mixture of different levels of resistance which had survived the drug treatment. Clone No. 3 was used for further stability studies (Fig. 11), and was subsequently used for genetic analyses. As seen from Figure 11, this clone was stable through many blood passages in the absence of the drug (Table 8).

3.4 Response of Selected Lines to Other Drugs

3.4.1 Response to Mefloquine

Using the '4-day' suppressive drug test, the different chloroquine-resistant lines of P. chabaudi were treated at two doses of mefloquine (7 and 10 mg kg^{-1}). An AS(30MF) line was used as a control (see Section 3.8). The higher dose of 10 mg kg^{-1} mefloquine eliminated all of the parasites apart from the control. The response to 7 mg kg^{-1} mefloquine showed some degree of cross-resistance between this drug and chloroquine (Fig. 12a and Table 9). A difference could be distinguished between the low level of chloroquine-resistance AS(3CQ) and the higher levels; the AS(3CQ) line responded like the sensitive lines. The AS(15CQ) and the AS(20CQ) lines could be compared as the control values indicate that equal numbers of parasites were inoculated. The AS(20CQ) parasites emerged before those of the AS(15CQ) line. From the results, three classes can be distinguished with this test. These are:

(1) AJ(Sens), AS(Sens) and AS(3CQ).

(2) AS(15CQ).

(3) AS(20CQ) and AS(30CQ).

3.4.2 Response to Mepacrine

The response of different lines to a '4-day' suppressive test using 13 and 15 mg kg^{-1} mepacrine was investigated. A dose of 15 mg kg^{-1}

TABLE 8. Stability of the AS(30CQ) Line (Clone 3) After Multiple Blood Passage.

	% Parasitaemia(± S.E.)							
	AS(30CQ)				AJ(Sens) CONTROL			
	DG			UND	DG			UND
Day of Infection	D_9	D_{11}	D_{13}	D_9	D_9	D_{11}	D_{13}	D_9
Passage No. after mosquitoes								
10	-	0.5 ± (0.3)	3.5 ± (1.4)	7.15 ± (2.12)	-	-	-	PP
12	0.05 ± (0.035)	0.9 ± (0.6)	4.0 ± (2.7)	PP	-	-	-	PP
14	0.003 ± (0.003)	1.0 ± (1.2)	11.00 ± (5.9)	PP	-	-	-	PP
16	0.025 ± (0.003)	1.3 ± (1.5)	8.4 ± (5.3)	PP	-	-	-	PP
18	0.06 ± (0.035)	3.5 ± (1.5)	±	PP	-	-	-	PP
21	0.01 ± (0.01)	0.4 ± (0.4)		PP	-	-	-	PP

DG : Drugged Animals, mean of at least 3 mice treated with 30 mg kg^{-1} chloroquine for 6 days.

UND: Undrugged animals, mean of 2 mice.

Legend as for Figure 11.

TABLE 9. Response of Different Lines of P. chabaudi to Mefloquine

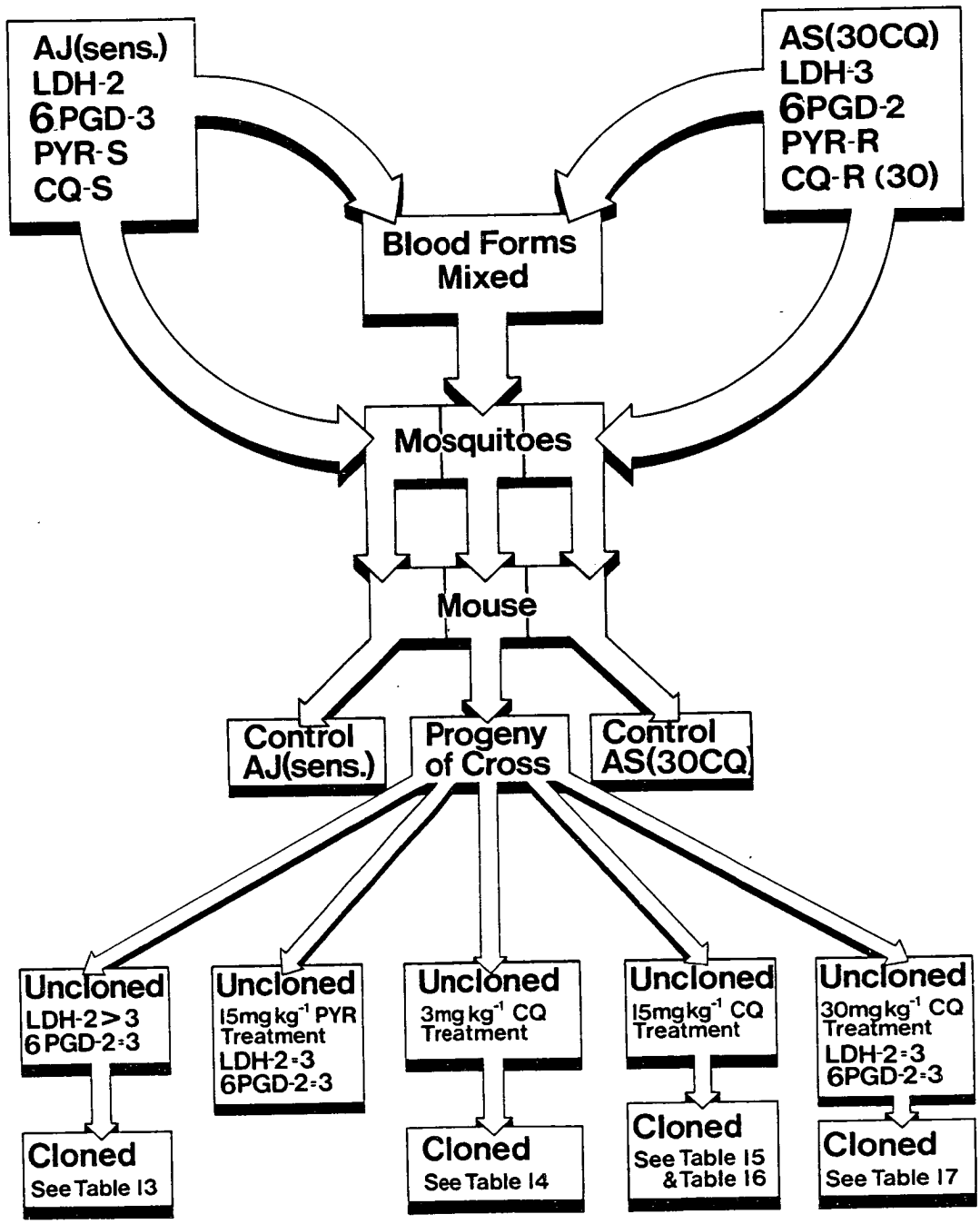
	% Parasitaemia(± S.E.)						
Day of Infection	D_5	D_7	D_9	D_{11}	D_{13}	D_{15}	UND D_7
LINES							
AJ(Sens)	-	-	-	-	-	0.02(±0)	56(±0)
AS(Sens)	-	-	-	-	-	0.1(±0.04)	40(±13)
AS(3CQ)	-	-	-	-	-	-	46(±2.1)
AS(15CQ)	-	-	-	-	0.08(±0.01)	0.9(±0.4)	27(±8.0)
AS(20CQ)	-	-	-	0.006(±0.004)	0.6(±0.3)	2.3(±0.9)	24(±2.9)
AS(30CQ)Population	-	-	-	0.07(±0.07)	1.5(±1.3)	2.5(±0.7)	51.3(±1.2)
AS(30MF)	0.2(±0.05)	0.6(±1.5)	5.0(±1.5)	7.4(±2.0)	1.5(±0.6)	0.4(±0.2)	10.5(±3.0)

Drugged Animals, mean of 5 mice treated with 7 mg kg^{-1} Mefloquine

UND: Undrugged Animals, mean of 4 mice.

Legend as for Figure 12a.

Fig.12 Crosses Between AS(30CQ) & AJ(sens) Lines



eliminated the parasites of all the different lines tested. Using a dose of 13 mg kg^{-1} the data indicated that the AS(Sens) and AS(3CQ) lines were more sensitive to the drug than those showing higher levels of chloroquine-resistance (Table 10). However, this test could not distinguish between the AS(15CQ) and AS(30CQ) lines.

3.4.3 Response to Quinine

Three lines were investigated using a '4-day' suppressive test with different doses of quinine. Although these tests could distinguish between the chloroquine-resistant and chloroquine-sensitive lines, thus demonstrating cross-resistance between the two drugs, they could not differentiate between the high levels of resistance (Table 11).

3.5 Genetic Analyses

The AS(30CQ) line was used for genetic analyses in order to determine the genetic basis of this high level of resistance. As the way in which the high level of chloroquine-resistance was obtained involved a gradual increase of drug pressure, a series of several mutations may have been involved in producing this line. This hypothesis was tested by crossing the AS(30CQ) line with chloroquine-sensitive lines.

3.5.1 Crosses 1 & 2: AJ(Sens) x AS(30CQ)

These crosses were conducted as shown in Figure 12. On D_7 after the mosquitoes had engorged, five mosquitoes of each of the crosses and control cages were dissected and examined for oocysts. All of the cages contained infected mosquitoes. The parent lines each formed approximately equal numbers of oocysts. The mosquitoes were allowed to

TABLE 10. Response of Different Lines of P. chabaudi to Mepacrine

	% Parasitaemia(± S.E.)									
	I					II				
Day of Infection	D_5	D_7	D_9	D_{11}	UND D_7	D_5	D_7	D_9	D_{11}	UND D_7
AS(Sens)	-	-	-	-	34 ± (14.1)	-	-	-	-	39 ± (3.5)
AS(3CQ)	-	0.02 ± (0.017)	2.6 ± (2.2)	22 ± (13.9)	PP	-	-	-	-	52 ± (1.5)
AS(15CQ)	0.05 ± (0.02)	0.8 ± (0.18)	20 ± (5.2)	29 ± (3.5)	43 ± (1.0)	-	0.003 ± (0.003)	0.03 ± (0)	0.2 ± (0.03)	33 ± (12.0)
AS(30CQ)	0.003 ± (0.0035)	0.22 ± (0.2)	1.35 ± (0.4)	11.7 ± (3.5)	32 ± (2.8)	-	0.05 ± (0.03)	1.23 ± (0.4)	13.3 ± (6.4)	PP

Drugged Animals - Mean of 3 Mice, Treated with 13 mg kg^{-1} for 4 days.
UND: Undrugged Animals - Mean of 2 Mice.
PP : Post Peak
I & II : Test Number.

TABLE 11. Response of Different Lines of P. chabaudi to Quinine

	% Parasitaemia (± S.E.)											
Dose (mg kg^{-1} for 4 days)	150				200				250			
Day of infection	D_6	D_8	D_{10}	UND D_6	D_6	D_8	D_{10}	UND D_6	D_6	D_8	D_{10}	UND D_6
AJ(Sens)	-	-	0.3 ± (0.2)	20 ± (7.8)	-	-	0.1 ± (0.11)	13 ± (0)	-	0.02 ± (0.02)	0.6 ± (0.6)	4.0 ± (0)
AS(15CQ)	0.8 ± (0.4)	12 ± (6.3)	PP	38 ± (2.0)	-	0.13 ± (0.07)	5.6 ± (3.6)	30 ± (8.1)	0.007 ± (0.009)	0.61 ± (0.4)		14.8 ± (5.2)
AS(30CQ)	0.7 ± (0.3)	9.4 ± (2.3)	10.7 ± (0.5)	45 ± (5.0)	-	0.33 ± (0.2)	7 ± (2.9)	4.8 ± (4.0)	-	0.04 ± (0.04)	0.11 ± (0.08)	2.2 ± (0.1)

Drugged animals - mean of 3 mice.

UND: Undrugged animals - mean of 2 mice.

reinfect mice on D_{15} and D_{17} of the mosquito infections. The blood forms emerged between days 4 - 7, were passaged into clean mice and deep frozen.

3.5.2 Tests on Uncloned Progeny

The progeny of the crosses were examined for their response to pyrimethamine and chloroquine (30 mg kg^{-1}) and the parasites surviving the drug treatments were examined for theirenzyme types (Fig. 12). Drug treatment should have eliminated all of the sensitive parasites. The presence of both enzyme types following administration of drugs indicated that exchange of genetic material had occurred; in the absence of 'recombination', only enzyme types LDH-3 and 6PGD-2 would have been present, being characteristic of the drug resistant parasites.

Parasitaemias of crosses 1 and 2 following treatment with chloroquine at 30 mg kg^{-1} are shown on Table 12.

3.5.3 Control Studies

Clones were established from the two parental lines transmitted as controls for Cross 1. Of 100 mice inoculated, 14 clones of the AS(30CQ)parent line were obtained. These clones were analysed for their enzyme types and drug resistance and were found to have the phenotypes expected of the parent AS(30CQ) line (Table 1).

Six clones of the AJ(Sens) control were established from the inoculation of 50 mice. The phenotypes of these clones were those expected of the AJ(Sens) line (Table 1).

The absence of recombinant classes in these control studies indicate that mutation events were unlikely to account for the recombinant classes obtained from crosses 1 and 2. These classes could thus be presumed to

TABLE 12. Response of the Uncloned Products of Crosses 1, 2 after Multiple Blood Passage

	% Parasitaemia (± S.E.)									
Line	Passage No.	D_9	D_{11}	D_{13}	D_{15}	Passage No.	D_9	D_{11}	D_{13}	D_{15}
Cross 1	5	0.002 ± (0.002)	0.075 ± (0.05)	0.3 ± (0.2)	-	10	-	-	-	0.2 ± (0.2)
Cross 2	6	-	-	0.04 ± (0.02)	1.1 ± (0.4)	11	-	-	-	0.09 ± (0.06)
30CQ Control	6	0.6 ± (0.2)	4.9 ± (2.3)	0.9 ± (0.7)	-	12	0.7 ± (0.4)	9.5 ± (5.5)	11.3 ± (6.4)	-
AJ Control	6	-	-	-	-	12	-	-	-	-

Drugged Animals - mean of 4 mice, treated with 30 mg kg^{-1} chloroquine for 6 days.

Undrugged Animals of each line showed post peak parasitaemias on D_9

have arisen following cross-fertilization of the two parental types.

3.5.4 Characterization of Clones Derived from Cross 1

(a) Unselected Clones

On cloning the progeny of Cross 1, 33 clones were established from a total of 350 mice inoculated. Each clone was examined for its enzyme type and drug response, the results of which are shown in Table 13. The 16 possible combinations are represented by groups A - P. Only 7 of the classes are represented among the progeny of which there is a preponderance of the AJ(Sens) parental types (Group A) compared with any other class. 10 of the 33 clones were found to be resistant to chloroquine. The chloroquine-resistant clones were tested at doses of 3 mg kg^{-1}, 15 mg kg^{-1} and 30 mg kg^{-1}. 9 of the 10 clones were resistant to a low level (3 mg kg^{-1}) whereas one of the clones was resistant to an intermediate level (15 mg kg^{-1}) (Table 13). These clones were classified according to the criteria defined in Section 3.2.4.

(b) Selected Clones

To attempt to obtain more highly resistant clones from the progeny of the cross, uncloned parasites were treated with chloroquine at various doses. Clones were then established after drug treatment and examined for the four markers, particularly their level of chloroquine-resistance. This was measured by conducting drug tests at two doses of the drug, 3 mg kg^{-1} and 30 mg kg^{-1}. The higher dose differentiated between high levels of chloroquine-resistance; parasites were classified according to the rate at which they produced patent parasitaemias after drug treatment (see Section 3.2.4) as either 15CQ or 30CQ clones.

TABLE 13. Characterization of Clones Established from the Unselected Products of Cross 1.

AJ (Sens): LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R (30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	19
B	2	3	S	R(3)	1
C	2	3	R	S	0
D	2	3	R	R	0
E	2	2	S	S	3
F	2	2	S	R(3)	1
G	2	2	R	S	0
H	2	2	R	R	0
I	3	3	S	S	0
J	3	3	S	R	0
K	3	3	R	S	0
L	3	3	R	R	0
M	3	2	S	S	0
N	3	2	S	R(3)	2
O	3	2	R	S	1
P	3	2	R	R(3)	5
	"	"	"	R(15)	1
				TOTAL NO. OF CLONES	33

R : Resistant to Drug Tests (see Section 3.2.4) and classified at the levels bracketed for CQ.

S : Sensitive to 3 mg kg^{-1} CQ for 6 days or 15 mg kg^{-1} PYR for 4 days.

LDH 2 or 3 : Variants of enzyme lactate dehydrogenase.

6PGD 3 or 2 : Variants of 6-phosphogluconate dehydrogenase.

(i) Selection at 3 mg kg^{-1} Chloroquine

After treating the progeny of the cross with 3 mg kg^{-1} chloroquine for 6 days, the surviving parasites were cloned. 15 clones were obtained from inoculating 200 mice; of these 2 were found to be chloroquine-sensitive and 13 were chloroquine-resistant (Table 14). 12 of the resistant clones were resistant to a low level (3 mg kg^{-1}) while one of the clones was resistant to an intermediate level (15CQ). The stability of the 15CQ clone obtained was examined further. This clone was presumed to be a product of cross-fertilization as it demonstrated an intermediate (15CQ) level of chloroquine-resistance. This clone was found to be stable after 6 blood passages in the absence of the drug (Table 20) and maintained its level of resistance.

(ii) Selection at 15 mg kg^{-1} Chloroquine

Two courses of treatment were given. Firstly, the drug was administered for only 5 days before cloning in the hope that a wide range of recombinant classes would be obtained; previously it had been found (unpublished) that '5-day' chloroquine drug tests were not sufficient to eliminate sensitive parasites. The phenotypes of the 12 clones (from inoculating a total of 100 mice) obtained following this treatment, are shown on Table 15. It can be seen that there is an absence of the highly resistant (30CQ) class.

Secondly, 15 mg kg^{-1} of the drug was administered for 6 days before cloning. 8 clones were derived from inoculating 50 mice. The phenotypes of the clones are shown on Table 16. 7 clones were classified as 15CQ and 1 clone was like a parental AS(30CQ) type.

TABLE 14. Characterization of Clones Established from Cross 1 Selected at a Low Level (3CQ/6 days).

AJ (Sens): LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R(30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	1
B	2	3	S	R(3)	3
C	2	3	R	S	0
D	2	3	R	R	0
E	2	2	S	S	0
F	2	2	S	R	0
G	2	2	R	S	0
H	2	2	R	R	0
I	3	3	S	S	1
J	3	3	S	R	0
K	3	3	R	S	0
L	3	3	R	R(3)	1
M	3	2	S	S	0
N	3	2	S	R(3)	2
O	3	2	R	S	0
P	3	2	R	R(3)	6
	"	"	"	R(15)	1
				TOTAL NUMBER OF CLONES	15

SEE TABLE 13 FOR KEY.

TABLE 15. Characterization of Clones Established from Cross 1 Selected at an Intermediate Level (15CQ/5 days).

AJ (Sens) : LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R (30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	2
B	2	3	S	R(3)	5
C	2	3	R	S	0
D	2	3	R	R(3)	1
	"	"	"	R(15)	1
E	2	2	S	S	0
F	2	2	S	R	0
G	2	2	R	S	0
H	2	2	R	R(15)	2
I	3	3	S	S	0
J	3	3	S	R	0
K	3	3	R	S	0
L	3	3	R	R	0
M	3	2	S	S	0
N	3	2	S	R	0
O	3	2	R	S	0
P	3	2	R	R(15)	1
				TOTAL NUMBER OF CLONES	12

SEE TABLE 13 FOR KEY.

TABLE 16. Characterization of Clones Established from Cross 1, Selected at an Intermediate Level (15CQ for 6 days).

AJ (Sens) : LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R (30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	0
B	2	3	S	R	0
C	2	3	R	S	0
D	2	3	R	R	0
E	2	2	S	S	0
F	2	2	S	R	0
G	2	2	R	S	0
H	2	2	R	R	0
I	3	3	S	S	0
J	3	3	S	R	0
K	3	3	R	S	0
L	3	3	R	R	0
M	3	2	S	S	0
N	3	2	S	R	0
O	3	2	R	S	0
P	3	2	R	R(15)	7
	"	"	"	R(30)	1
				TOTAL NUMBER OF CLONES	8

SEE TABLE 13 FOR KEY.

(iii) Selection at 30 mg kg^{-1} Chloroquine

29 clones were obtained from a total of 120 mice inoculated after drug treatment at 30 mg kg^{-1} for 6 days. The phenotypes of the clones are shown on Table 17. It can be seen that all of the clones obtained were recombinant, with enzyme types of the sensitive parent (LDH-2, 6PGD-3). 2 of the clones were resistant to an intermediate level (15CQ) and the remaining 27 clones to a high level (30CQ).

Two of the clones which were resistant to a high level (30CQ) were examined for stability on multiple blood passage without drug pressure. These clones were found to be stable in their chloroquine resistance at the high level (Table 20).

Tables 18 and 19 summarize the chloroquine response and enzyme types of all the clones derived from Cross 1.

3.5.5 Characterization of Clones from Cross 2

Cross 2 was a repetition of Cross 1. 21 clones were obtained from the progeny of the cross, each of which was examined for the characteristics involved. Their phenotypes are shown in Table 21. It can be seen that while the enzyme markers segregated randomly, all of the clones were pyrimethamine-sensitive. 14 of the clones were chloroquine-resistant. A range of levels of chloroquine-resistance was obtained.

3.6 Reconstruction and Competition Experiments

3.6.1 Reconstruction

In order to determine how sensitive the electrophoretic system is at determining quantitative differences, reconstruction experiments were carried out. Known mixtures of the two LDH enzyme types (2 & 3) were made and examined on gels (Table 22). Both forms of LDH could be

TABLE 17. Characterization of Clones Established from Cross 1, Selected at a High Level (30CQ for 6 days).

AJ (Sens) : LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R (30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	0
B	2	3	S	R(30)	1
C	2	3	R	S	0
D	2	3	R	R(15)	2
	"	"	"	R(30)	26
E	2	2	S	S	0
F	2	2	S	R	0
G	2	2	R	S	0
H	2	2	R	R	0
I	3	3	S	S	0
J	3	3	S	R	0
K	3	3	R	S	0
L	3	3	R	R	0
M	3	2	S	S	0
N	3	2	S	R	0
O	3	2	R	S	0
P	3	2	R	R	0
TOTAL NUMBER OF CLONES					29

SEE TABLE 13 FOR KEY.

TABLE 18. Chloroquine Response of Clones Established from Cross 1.

Method of Selection	No. of Clones				
	Sens	3CQ	15CQ	30CQ	Total
Unselected	23	9	1	0	33
Selected at 3CQ/6 days	2	12	1	0	15
Selected at 15CQ/5 days	2	6	4	0	12
Selected at 15CQ/6 days	0	0	7	1	8
Selected at 30CQ/6 days	0	0	2	27	29
Total No. of Clones	27	27	15	28	97

SENS: Sensitive to 3 mg kg^{-1} CQ for 6 days.

3CQ : Resistant to 3 mg kg^{-1} CQ for 6 days, and sensitive to 30 mg kg^{-1} for 6 days.

15CQ: Resistant to 30 mg kg^{-1} CQ for 6 days, positive infections on D_{13} or D_{15}.

30CQ: Resistant to 30 mg kg^{-1} CQ for 6 days, positive infections on D_{9} or D_{11}.

TABLE 19. Enzyme Types of Clones Obtained from Cross 1.

Enzyme Types	No. of Clones
LDH-2 6PGD-3 *	62
LDH-2 6PGD-2	6
LDH-3 6PGD-3	2
LDH-3 6PGD-2 *	27
Total number of clones	97

* — Parental types

TABLE 20. Stability of Progeny of Cross 1.

	% Parasitaemia(± S.E.)*									
Passage No.	6					11				
Day of Infection	D_9	D_{11}	D_{13}	D_{15}	UND D_9	D_9	D_{11}	D_{13}	D_{15}	UND D_9
AJ(Sens) Control	-	-	-	-	PP	-	-	-	-	PP
AS(30CQ) Control	0.6 ± (0.2)	4.9 ± (2.3)	0.9 ± (0.7)		PP	0.7 ± (0.4)	14.5 ± (5.5)	11.3 ± (6.4)		PP
15CQ (selected at 3CQ) Clone from Group P (Table 14)	-	-	0.6 ± (0.037)	3.2 ± (2.3)	PP					
30CQ (selected at 30CQ) Clone from Group D (Table 17)	0.07 ± (0.01)	7.6 ± (1.4)	PP		PP	0.03 ± (0.01)	2.3 ± (0.8)	26 ± (18)		PPP
30CQ (selected at 30CQ) Clone from Group D (Table 17)	0.025 ± (0.025)	1.5 ± (0.4)	8.8 ± (1.3)		PP	-	0.03 ± (0.03)	1.7 ± (0.6)		PP

* Drugged animals - mean of 3 mice, treated with 30 mg kg^{-1} for 6 days.

UND: Undrugged animals - mean of 2 mice.

PP : Post peak.

TABLE 21. Characterization of Clones Established from the unselected products of Cross 2.

AJ (Sens) : LDH-2, 6PGD-3, PYR-S, CQ-S

X AS (30CQ) : LDH-3, 6PGD-2, PYR-R, CQ-R (30).

	LDH	6PGD	PYR	CQ	No. of Clones
A	2	3	S	S	5
	"	"	"	R(3)	2
B	2	3	S	R(15)	3
	"	"	"	R(30)	1
C	2	3	R	S	0
D	2	3	R	R	0
E	2	2	S	S	0
F	2	2	S	R	0
G	2	2	S	R	0
H	2	2	R	R	0
I	3	3	S	S	0
J	3	3	S	R(3)	1
	"	"	"	R(15)	1
K	3	3	R	S	0
L	3	3	R	R	0
M	3	2	S	S	2
	"	"	"	R(3)	4
N	3	2	S	R(15)	0
	"	"	"	R(30)	2
O	3	2	R	S	0
P	3	2	R	R	0
				TOTAL NUMBER OF CLONES	21

R : Resistant to Drug Tests (see Section 3.2.4) and classified at the levels bracketed for CQ.

S : Sensitive to Drug Tests.

LDH 2 or 3 - variants of lactate dehydrogenase.

6PGD 3 or 2 - variants of 6 phosphogluconate dehydrogenase.

TABLE 22. Reconstruction Experiments to Determine the Sensitivity of the Electrophoretic System

Experiment No.	% AS	% AJ	LDH RUN I	LDH RUN II
1	100	0	3	3
2	90	10	3 >> 2	3 >> 2
3	80	20	3 > 2	3 > 2
4	70	30	3 = 2	3 > 2
5	60	40	3 = 2	3 = 2
6	50	50	2 = 3	2 = 3
7	40	60	2 = 3	2 = 3
8	30	70	2 = 3	2 = 3
9	20	80	2 > 3	2 > 3
10	10	90	2 >> 3	2 >> 3
11	0	100	2	2

LDH 2 & 3 - Electrophoretic variants of lactate dehydrogenase.

I, II - Duplicate gel runs on the same samples.

= : Denotes no detectable differences between the intensities of the bands.

> : Means that differences in intensities can just be detected.

>>: Means that differences in intensities of the bands can clearly be detected.

detected in the mixtures even when the proportion of one line was as low as 10%. Nc difference in intensity was apparent at 60:40. Proportions of 80:20 were readily detectable. A mixture in the proportion 70:30 gave fluctuating results; in some cases no differences could be detected. This level of difference may be the critical level at which the system can detect differences of varying quantities of mixtures.

3.6.2 Competition

Competition experiments were carried out to determine whether there were any differences in growth between the parasite lines used in this work which might account for some of the results obtained. Competition experiments were conducted between several lines as follows:

(a) Experiment 1: - AJ(Sens) Vs AS(Sens)

Dilutions of parasites of each line were made so that 0.1 ml of inoculum contained 10^6 parasites. The parasites were then mixed in equal volumes and 0.1 ml amounts were injected into a group of mice. Parasites from the infections were passaged weekly; on each occasion 10^6 parasites were inoculated into each uninfected mouse. At various weekly intervals shown on Table 23 (passage no.), the parasites were prepared for electrophoresis and examined for their LDH types. After about 9 weekly passages the AJ enzyme type (LDH-2) appeared to outgrow the AS enzyme type (LDH-3). This suggested that the AJ(Sens)parasites were at a selective advantage in competition with AS(Sens) forms.

(b) Experiment 2: - AS(0CQ) Vs AS(30CQ)

These two lines were identical in their enzyme types and pyrimethamine sensitivity but differed in their resistance to chloroquine. As in the previous experiment,equal numbers of parasites were mixed so

TABLE 23. Experiment 1 - Competition studies between Blood Forms of a 50:50 Mixture AJ(Sens) and AS(Sens) Lines.

Passage No.	LDH		
	RUN I	RUN II	RUN III
1	2 = 3	2 = 3	
3	2 = 3	2 = 3	
9	2 > 3	2 > 3	
11	2 > 3	2 > 3	2 > 3
12	2 >> 3	2 >> 3	2 >> 3

SEE TABLE 22 FOR KEY

I, II, III: Replicate gel runs on the same samples.

that an inoculum of 10^6 contained 0.5 x 10^6 parasites of each type. Four mice were injected with a mixture of 10^6 parasites. The parasites from resulting infections were passaged at weekly intervals. At various periods shown on Table 24, the mixture was cloned and analysed for chloroquine-resistance by using '6-day' drug tests at doses of 3 mg kg^{-1} and 30 mg kg^{-1}. After 17 weekly passages a total of 18 resistant (30CQ) clones were obtained compared to 4 sensitive clones. On passage numbers 4 and 9, the mixture was treated with 4 mg kg^{-1} chloroquine for 6 days. The parasites which survived the treatment were cloned; all of the 7 clones established were resistant to a high level (30 mg kg^{-1}) of the drug (Table 24).

(c) Experiment 3: - AS(30CQ) Vs AJ(Sens)

As in Experiment 1, a 50:50 mixture of blood forms of the two lines was made so that 0.1 ml of inoculum contained 0.5 x 10^6 parasites of each line. 0.1 ml of the mixture was then inoculated into each of 4 mice. The parasites were passaged at weekly intervals thereafter. At various periods shown on Table 25, the parasites were prepared for starch gel electrophoresis and examined for their LDH types. On passage number 12, the parasites were tested at 30 mg kg^{-1} chloroquine for 6 days and the surviving parasites were examined for their LDH enzyme type. Only the parasites of the highly resistant line (LDH-3) survived the treatment, showing that the two lines had remained separate. Had any exchange of characters taken place both types might have been detected following drug treatment. In competition these two lines demonstrated the same selective advantage.

(d) Experiment 4: - AJ(30CQ) Vs AS(30CQ)

The AJ(30CQ) line was a product of Cross 1, therefore its characteristics apart from the four markers (Group D in Table 17) were unknown.

TABLE 24. Experiment 2 - Competition Studies Between Blood Forms of a 50:50 mixture of AS(0CQ) and AS(30CQ) Lines

Passage No.	No. of mice inoculated	No. of sensitive Clones[+]	No. of resistant Clones[‡]
4*	30	0	1
7	50	1	2
9*	91	0	6
15	75	3	4
17	53	0	12

* Treated with 3 mg kg^{-1} prior to cloning.

+ Sensitive to 3 mg kg^{-1} CQ for 6 days.

‡ Resistant to 30 mg kg^{-1} CQ for 6 days.

TABLE 25. Experiment 3 - Competition Studies Between Blood Forms of a 50:50 mixture of AJ(Sens) and AS(30CQ) Lines

Passage No.	LDH		
	RUN I	RUN II	RUN III
1	2 = 3	2 = 3	
3	2 = 3	2 = 3	
7	2 = 3	2 = 3	
11	2 = 3	2 = 3	
12*	3	3	
17	3 > 2	3 > 2	
19	2 = 3	2 = 3	2 = 3
20	3 > 2	2 = 3	2 = 3

SEE TABLE 22 FOR KEY

I, II, III: Replicate gel runs on the same samples.

* After treatment with 30 mg kg^{-1} CQ for 6 days.

As all of the highly chloroquine-resistant clones (30CQ) obtained from Crosses 1 and 2 apart from one were recombinant, the question of whether recombinants were more 'vigorous' was investigated. As in Experiment 1 a 50:50 mixture of the blood forms of the two lines was made so that each mouse was inoculated with 0.5×10^6 parasites of each line. This mixture was passaged weekly and its LDH enzyme types examined at intervals (Table 26). The results suggest that the recombinant line was at a selective advantage over the parental AS(30CQ) line; the LDH-2 (AJ) enzyme type seems to be demonstrating greater activity than the LDH-3 (AS) enzyme type as passaging continued.

3.7 *In vitro* Chloroquine Drug Tests

Drug tests were conducted using the microtechnique and a range of doses comparable to those used by Rieckmann *et al*. (1978); These doses were found to be too low to significantly affect the development of the parasites. A higher range of doses (1.25 - 10 µg per well) was more effective (Table 27). Cultures were set up in the morning (10.00 hours) when the parasites were at an early trophozoite stage with parasitaemias of not greater than 5%. The cultures were harvested when ring forms were predominantly visible *in vivo*, following schizogony; *in vivo* schizogony occured around midnight or early morning. The parasite maturation *in vitro* was slower than that observed *in vivo* (Table 27). Schizonts were not visible in the peripheral blood so that evidence for schizogony *in vivo* was the presence of the ring forms accompanied by an increase in parasitaemia.

From the results it can be seen that morphological differences were apparent between the AS(30CQ) and the AJ(Sens) lines. The undrugged controls formed schizonts around midnight. At a dose of 1.25µg the

TABLE 26. Experiment 4 - Competition Studies Between Blood Forms of AJ(30CQ) and AS(30CQ) Lines

Passage No.	LDH		
	RUN I	RUN II	RUN III
1	2 = 3	2 = 3	
3	2 = 3	2 = 3	
9	2 > 3	2 >> 3	2 >> 3
11	2 > 3	2 >> 3	
12	2 > 3	2 >> 3	2 >> 3

SEE TABLE 22 FOR KEY.

I, II, III - Replicate gel runs on the same samples.

TABLE 27. Effects of Chloroquine on Two Lines of P. chabaudi

Time Examined	2000	2300	10.30 (Next Day)
No. of hours from start of culture	10	13	24½
SENSITIVE LINE - AJ(Sens)			
In vivo	4.5% Rings/few Trophozoites	6.9% Rings	10% Trophozoites
In vitro Chloroquine Dose (µg per well)			
0	Schizonts/Trophozoites	Schizonts/few Trophozoites	Rings/few Schizonts
1.25	Trophozoites/few abnormal Schizonts	Trophozoites/abnormal Schizonts	Abnormal Schizonts/ Trophozoites
2.5	Abnormal Trophozoites	Abnormal Trophozoites	Abnormal Trophozoites
5.0	Dying Parasites	Dying Parasites	Dead Parasites
10.0	Dying Parasites	Dying Parasites	Dead Parasites
RESISTANT LINE - AS30CQ			
In vivo	4.6% Rings/few Trophozoites	7.9% Rings	12% Trophozoites
In vitro Chloroquine Dose (µg per well)			
0	Schizonts/Trophozoites	Schizonts/few Trophozoites	Schizonts/rings
1.25	Trophozoites with vacuoles	Trophozoites with vacuoles	Trophozoites with vacuoles
2.5	Trophozoites with vacuoles	Trophozoites with vacuoles	Trophozoites with vacuoles
5.0	Rings	Rings	Rings
10.0	Rings	Rings	Dying Parasites.

sensitive parasites formed schizonts but to a lesser extent than the undrugged parasites. Furthermore, the schizonts formed in the presence of the drug appeared abnormal. At the same dose, the resistant line became arrested in its development at a vacuolated trophozoite stage which at times gave the parasites a foamy appearance. In the presence of a higher dose of 2.5 µg the sensitive parasites developed as they did at 1.25 µg, however, they seemed to be delayed in their development and do not form schizonts. The resistant parasites gave the same response at this dose as the dose of 1.25 µg. At higher doses, the sensitive parasites were eliminated whilst those of the resistant line were retarded; they seemed to contract to form rings. The cultures were examined later and it was found that the undrugged parasites continued to develop to form rings which reinvaded blood cells.

The contents of the wells were inoculated into clean mice after the drug tests. The infections resulting from the inoculation of the cultures revealed differences in the viability of the parasites (Table 28). The resistant parasites were more viable than the sensitive forms following drug treatment.

3.8 Selection of Mefloquine-Resistance

It has been previously shown that 10 mg kg^{-1} mefloquine eradicates different lines of P. chabaudi (see Section 3.4.1), and that a graded response is obtained with 7 mg kg^{-1} of the drug depending on the line used (see Table 9 in Section 3.4.1). The three lines used for selection were AS(0CQ), AS(3CQ) and AS(15CQ)(Table 1). Attempts to establish resistance in AS(0CQ) and AS(3CQ) lines were unsuccessful (Table 29). The AS(15CQ) line responded to a gradual increase in drug pressure until a line resistant to 30 mg kg^{-1} mefloquine was obtained (Fig. 8). During

TABLE 28. Parasitaemias in Mice Inoculated from Cultures

Chloroquine Dose In Culture (µg per well)	% Parasitaemia			
	Sensitive Line		Resistant Line	
	D_4	D_6	D_4	D_6
0	3.9	20	1.0	22
1.25	-	-	0.1	2.7
2.5	-	-	0.06	2.3
5.0	-	-	-	0.03
10.0	-	-	-	-

TABLE 29. Selection for Mefloquine Resistance in AS(OCQ) and AS(3CQ) Lines

	AS (OCQ)		AS(3CQ)	
Passage No.	DOSE (mg kg^{-1})	DAY OF INFECTION showing a positive response.	DOSE (mg kg^{-1})	DAY OF INFECTION showing a positive response
1	7	-	5	D_4
2	5	D_6	8	D_6
3	8	D_8	8	D_{14}
4	8	D_{12}	8	D_{11}
5	8	D_5	10	-
6	10	-	10	-
7	10	-		

- : Denotes a negative response up to D_{15}.

the selection procedure the parasites were transmitted through mosquitoes as shown to generate the lines AS(12MF) and AS(15MF).

3.9 Response of Selected Lines to Chloroquine

3.9.1 Response of AS(12MF) Line

The AS(12MF) line was tested with 15 and 30 mg kg^{-1} chloroquine; at these doses the mefloquine-resistant line appeared to demonstrate a level of resistance equal to the AS(20CQ) line (Tables 4 & 30). As the parasites produced patent parasitaemias at D_{11} following treatment of 30 mg kg^{-1} chloroquine, by definition (see Section 3.2.4) this line can be considered as resistant to chloroquine at a high level (30CQ).

3.9.2 Response of AS(30MF) Line

The AS(30MF) line was tested at various doses of chloroquine from 5 - 30 mg kg^{-1}. The level of resistance of this line seemed to be increased from 15CQ to 20CQ (Table 4). By definition this line can also be classified as a 30CQ line (see Section 3.2.4).

3.10 Stability of Resistance of Selected Lines

3.10.1 Response of AS(12MF) Line

The resistance of this line was stable after mosquito transmission when tested with 10 mg kg^{-1} mefloquine for 4 days (Table 30).

3.10.2 Response of AS(30MF) Line

On multiple blood passage, this line seemed to be only moderately stable, emerging progressively later after drug treatment (Table 31). During the course of selection, this line proved to have reduced infectivity to mosquitoes, probably as a result of multiple blood passage. However, after several attempts the line was eventually transmitted.

TABLE 30. Comparison Between AS(12MF), AS(3CQ) and AS(30CQ) Lines

	% Parasitaemia (± S.E.)												
DRUG TREATMENT (mg kg^{-1}/days)	10MF/4				15CQ/6				30CQ/6				
DAY OF INFECTION	D_9	D_{11}	D_{13}	D_{15}	D_9	D_{11}	D_{13}	D_{15}	D_9	D_{11}	D_{13}	D_{15}	UND D_9
AS(3CQ)	-	-	-	-	-	-	-	-	-	-	-	-	PP
AS(30CQ)	-	-	-	-	0.13 ± (0.09)	1.44 ± (0.75)	2.63 ± (0.61)		-	0.13 ± (0.09)	2.35 ± (1.2)		PP
AS(12MF)	-	0.003 ± (0.003)	0.4 ± (0.18)	4.1 ± (2.3)	-	0.16 ± (0.09)	2.8 ± (1.1)		-	0.03 ± (0)	0.47 ± (0.03)		PP

Drugged Animals - mean of 3 mice.

UND : Undrugged animals - mean of 2 mice.

PP : Post Peak.

TABLE 31. Stability of AS(30MF) Before Mosquito Transmission

	% Parasitaemia(± S.E.) on first day of Positive Infection.	
Drug Dose (mg kg^{-1}/4 days)	15	30
Passage No.		
3		D_4 0.03(±0.017)
7	D_8 0.01(±0.005)	D_{10} 0.015(±0.009)
16	D_{14} 0.35(±0.23)	D_{19} 2.1(±2.1)

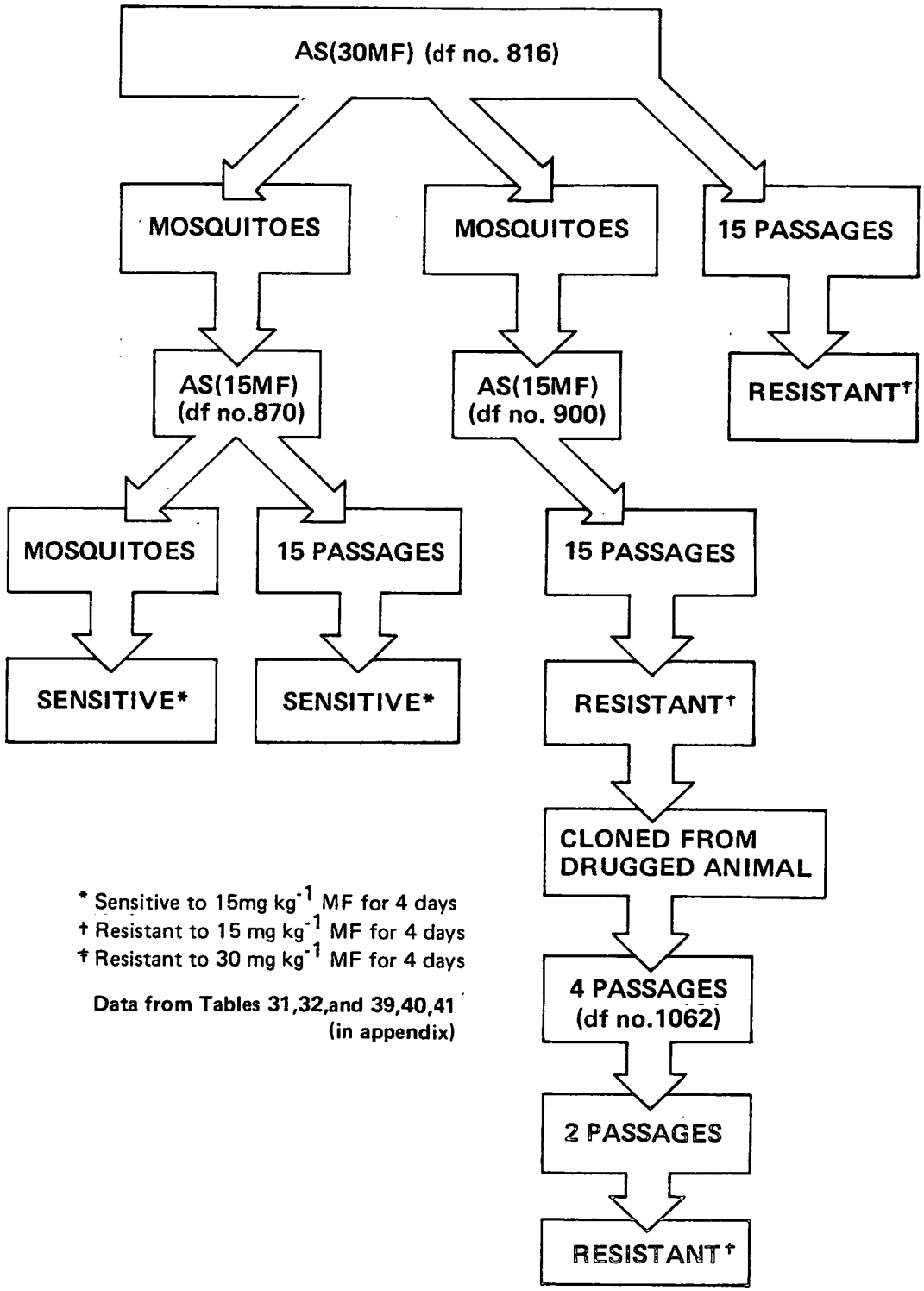
LEGEND AS FOR FIGURE 13.

Drugged Animals - mean of 3 mice.

Undrugged animals - all post peak infections on D_9

300Q controls negative in all tests.

Fig.13 Stability of the AS(30MF) Line



* Sensitive to 15mg kg^{-1} MF for 4 days
† Resistant to 15 mg kg^{-1} MF for 4 days
‡ Resistant to 30 mg kg^{-1} MF for 4 days

Data from Tables 31,32,and 39,40,41 (in appendix)

Following transmission, this line demonstrated a lower level of mefloquine-resistance (AS(15MF))(Fig. 13 and Tables 39 & 40, in Appendix). After a second mosquito transmission, the resistance was lost (Fig. 13 and Table 40 in Appendix). The AS(15MF) line had a variable stability on multiple blood passage in the absence of the drug; following one mosquito transmission (d.f. no.870) it appeared to be unstable (Fig. 13 and Table 41 in Appendix) and after another separate transmission (d.f. no.900) it was stable (Fig. 13 and Tables 32 & 41 in Appendix). The parasites which survived the drug test on passage no. 16 were cloned. A single clone was obtained after inoculating 50 mice. This line seemed to be stable after 8 blood passages without drug pressure and emerged earlier following drug treatment than the uncloned population (Table 32). The stability of this clone on further blood passage is yet to be determined. Two unsuccessful attempts were made to transmit this clone through mosquitoes.

3.11 Cross-Resistance to Quinine

As mefloquine and quinine are structurally similar, the possibility of cross-resistance between the two drugs was investigated. Two lines were tested; these were:

(a) AS(30MF) before mosquitoes.

(b) AS(15MF). This line was the AS(30MF) line transmitted through mosquitoes.

The AS(15CQ) line was used as a control. The mefloquine-resistant lines were found to be cross-resistant to quinine (Table 33). However, the degree of cross-resistance in AS(15MF) parasites did not appear to differ significantly from that of chloroquine as the AS(15CQ) control values were as high as those of the AS(15MF) line. The AS(30MF) values were moderately higher than the control values.

TABLE 32: Stability of the AS(15MF) Line

		% Parasitaemia(± S.E.)						
	Day of Infection	D_6	D_8	D_{10}	D_{12}	D_{13}	D_{14}	D_{15}
AS(15MF) POPULATION	PASSAGE NO. after Mosquitoes							
	2	-	-	-	-		-	0.12(±0.06)
	6	-	-	-		-		0.08(±0.025)
	16	-	-	-		-		0.015(±0.009)
AS(15MF) CLONE	PASSAGE NO. after DRUG TREATMENT							
	8		-	0.04 (±0.035)	0.3 (±0.1)			

Drugged animals - Mean of 3 mice, treated with 15 mg kg^{-1} MF for 4 days

Undrugged animals - POST PEAK on D_{10}

AS(30CQ) controls negative in all tests

Legend as for Figure 13

TABLE 33. Response of Mefloquine-Resistant Lines to Quinine

	% Parasitaemia(± S.E.)											
DOSE (Mg Kg^{-1} for 4 days)	150				200				250			
Day of Infection	D_6	D_8	D_{10}	UND D_6	D_6	D_8	D_{10}	UND D_6	D_6	D_8	D_{10}	UND D_6
AS(30MF)	9.3 (±1.2)	23.0 (±2.9)	PP	32.0 (±5.0)	0.06 (±0.04)	5.2 (±4.2)	25 (±10.4)	13 (±10.0)	0.03 (±0.03)	0.9 (±0.5)	5.8 (±1.5)	14.0 (±11.3)
AS(15MF) POPULATION	0.12 (±0.07)	1.5 (±0.6)	3.1 (±0.2)	22.0 (±14.1)	-	0.9 (±0.6)	8.8 (±4.2)	20(±0)	-	0.11 (±0.03)		13.3 (±7.0)
AS(15CQ) CONTROL	0.8 (±0.4)	12 (±6.3)	PP	38 (±2.0)	-	0.13 (±0.07)	5.6 (±3.6)	30 (±8.1)	0.007 (±0.009)	0.61 (±0.4)		14.8 (±5.2)

Drugged animals, mean of 3 mice

UND - Undrugged animals, mean of 2 mice

4. DISCUSSION

4.1 General Mechanisms of Drug Resistance

The development of resistance of malaria parasites to chemotherapy is a growing problem. The study of different types of mechanisms involved in the emergence of drug resistance would lead to better evaluations of the use of the present drugs available. There are several theoretical ways in which drug resistance can occur. These include:

(1) Adaptive mechanisms. Hinshelwood (1946) called this a theory of direct induction in bacterial systems. Here, the organism has the ability to switch on alternative pathways to thosewhich may have been blocked. These physiological changes are non-genetic, are likely to be unstable and would only be induced in the presence of the drug.

(2) Selection of existing resistant forms under drug pressure from mixed populations of sensitive and resistant parasites. The difficulty with this mechanism is that it does not account satisfactorily for the different levels of resistance observed; it would have to postulate the pre-existence of several different resistant parasites. It also does not account for the specificity of some of the drug resistance characters.

(3) Spontaneous mutation of nuclear genes followed by selection and subsequent multiplication of mutants. This is the most likely mechanism which is operating in Plasmodia. Here, a permanent change occurs which can recombine autonomously with other characters (for example, pyrimethamine-resistance in *P. gallinaceum* developed by Bishop, 1958; pyrimethamine-resistance in *P. yoelii* by Morgan, 1974 and in *P. chabaudi* by MacLeod, 1977).

(4) Spontaneous mutations of extranuclear (for example, mitochondrial) genes followed by selection (see review by Jinks, 1964). Examples of extrachromosomal inheritance in Protczoa areprovided by Beale & Knowles (1978) in P. aurelia where drug resistance could only be accounted for by such a mechanism; Sager (1954) in alga, Chlamydomonas reinhardi, where streptomycin-resistance was shown to develop by typical Mendelian and extrachromosomal mutations.

(5) Gene mutation induced by mutagens. These would be non-specific. For example, in the selection for pyrimethamine-resistance (Morgan, 1974) UV was used to induce a greater number of mutations; a mutation confering an increase in virulence was obtained. However, there is no evidence of mutagens inducing specific mutations to drug resistance in Plasmodia. In the work of Morgan (1974) the drug itself probably selected for the emergence of pyrimethamine-resistance.

(6) Switches in gene expression caused by changes in cytoplasmic or environmental factors (for example, altered membrane structure and permeability).

(7) Transfer of drug resistance factors in plasmids from one cell to another such as those found in bacterial systems (see reviews by Demerec, 1957; Watanabe, 1963; Watanabe & Fukasawa, 1966; Hayes, 1967). So far there is no evidence for parallel mechanisms of R (resistance) factors in Plasmodia although transformation of pararosaniline resistance has been claimed in another parasite Trypanosoma gambiense in vitro (Inoki & Matsushiro, 1960); virus particles have also been observed in Plasmodia (Terzakis, 1969). Nonetheless, there is still no convincing evidence of this type of gene transfer in Plasmodia. A

mechanism of transfer of drug resistance cytoplasmically, called synpholia, has been proposed by Yoeli et al. (1969). This is discussed elsewhere (see Section 4.5).

(8) A mechanism was proposed by Yudkin (1953) called the theory of clonal variation. This theory tries to account for features characteristic of bacterial drug resistance which are also observed in Plasmodia. These are:

(a) The training of organisms to grow in otherwise lethal concentrations of the drug.

(b) Reversion of resistance to sensitivity in the absence of the drug.

The basis of this theory is that a resistant bacterial cell may give rise to 2 cells with different and unequal degrees of resistance. Under gradually increasing drug pressure a continuous selection of daughter cells with high levels of resistance will occur. This resistance can arise without any need for conventional genetic mutation. However, there is no evidence for this mechanism from malaria studies. As Pollock (1960) pointed out, selection pressure at a given drug concentration would not necessarily favour a highly resistant gene combination when a less resistant mutantwould grow as well. This theory does not explain cases of high mutation rates when a high level of resistance is obtained very rapidly such as with pyrimethamine-resistance in Plasmodia.

The likely mechanisms involved in Plasmodia are discussed by Schnitzer and Grunberg (1957) where the authors stress the genetic origins of drug resistance. The development of drug resistance in P. chabaudi is likely to have a genetic mutational basis. Adaptive mechanisms may also be in operation which would account for the unstable types of resistance reported in the literature (see Introduction). The

development of chloroquine- and mefloquine-resistance are discussed in relation to these mechanisms.

4.2 Variation Due to Host Effects

The results of the drug tests used in this investigation were highly variable. Variation occured from one drug test to another both within lines and between supposedly parallel lines. The most important factor responsible for this variation is that of the response in experimental animals. It has been found that mice of the C57 inbred strain, which was the strain used in the drug tests of this investigation, were more variable in their response to the drug nembutal than F_1 hybrids from crosses between two inbred lines (McLaren & Michie, 1956; Biggers et al., 1958; Falconer, 1960). The rate at which a drug is concentrated and maintained in red blood cells depends on the host which controls the absorption of drugs from the alimentary tract (Vessel et al., 1971). The sex and age of the host may be contributary factors to the variation in drug response observed during drug tests. Work carried out by Konopka et al. (1966) on P. berghei suggests that female rodents were less protected by quinine than males, although the opposite effect was found with other drugs. Other workers have found that although female and male rats were equally susceptible to P. berghei infections, females had more effective natural immunity than males (Zuckerman & Yoeli, 1954). It is also thought that the route of infection would also be important in initiating uniform infections; the intravenous route is thought to be more reliable than the intraperitoneal route (Ott, 1969; Wellde et al., 1966).

During the drug tests undertaken in this investigation, only young female C57 mice were used throughout to eliminate variation due to sex.

The animals were weighed individually in the chloroquine drug tests to determine the exact amount of drug required for each mouse so that the same concentration of drug was administered to each mouse. Mean weights of mice were taken for other drug tests. As it would take a comparatively long time to inoculate each animal intravenously which would then complicate the exact interval between inoculation and administration of the drug for each mouse, the infections were started intraperitoneally.

As seen from the results of the drug tests, in spite of the precautions taken, the results were still variable. Standard errors of over 50% were common (For example, Table 4). However, the most reliable criterion for determining whether parasites were resistant was either presence or absence of healthy parasites after drug treatment, rather than the actual levels of parasitaemias.

The time of schizogony was also variable. This had particular relevance to in vitro drug tests where the infections were monitored in the donor mice to determine at what time parasites should be examined from cultures. Although mice were standardized for age and weight, the time of schizogony varied by about 2 hours from day to day and by as much as 8 hours from one season to another according to the natural light/dark cycle. Thus, the conditions of the host affected the time at which parasites underwent schizogony.

4.3 Chloroquine-Resistance in Line AS

The method with which chloroquine-resistance was developed in AS can lead to some deductions of the genetic events which may have occured. As the parasites only responded to a selection programme which involved

a gradual increase in drug pressure it would seem that several steps or mutations may have been involved in the development of the highly resistant line AS(30CQ). These mutations may be discrete changes at several loci which have an additive effect or may be a series of mutations at one locus. It was shown that the highly resistant line was stable through several blood passages and mosquito transmissions (Fig. 11). On cloning, the line remained highly resistant. It would seem, therefore, that a stable and permanent change or changes had occured in the development of this line.

The chloroquine tests carried out during selection provide evidence of different levels of resistance. The drug tests with varying doses of chloroquine suggest that there are at least four classes of resistant parasites (Table 4). The drug delay experiments reveal differences between the AS(3CQ), AS(15CQ) and AS(30CQ) lines (Figs. 9 & 10). The data on cross-resistance between chloroquine and the drugs mefloquine, mepacrine and quinine also imply that there are different levels of chloroquine-resistance (Fig. 12a, Tables 9, 10 & 11). Cross-resistance of chloroquine-resistant strains to these drugs and other 4-amino quinolines has been reviewed by Peters (1970). This author also pointed out (1967) that cross-resistance between different compounds may not be an all-or-nothing phenomenon but dependent on the level of resistance in each case (Greenberg & Bond, 1954 with pyrimethamine-resistance in *P. gallinaceum*; Contacos *et al.*, 1963 with multiple resistance in *P. falciparum* in the Far East). The data on cross-resistance between chloroquine and mefloquine indicates that there are different classes of chloroquine-resistance although the exact number of classes is unclear (Fig. 12a). The drug tests with

mepacrine clearly show differences between the low and high levels of resistance (Table 10). The results with quinine are less striking; they show differences between the sensitive and resistant lines (Table 11). It can be seen that very high doses of quinine are needed for the tests and the highest dose of 250 mg kg^{-1} is already higher than the reported maximum tolerated dose of the mouse which is 150 mg kg^{-1} (Hawking, 1966). The significance of the results obtained with the mefloquine-resistant line will be discussed elsewhere (see Section 4.8). If there are indeed different levels of chloroquine-resistance and the evidence presented here supports this view, this provides additional evidence that several steps are involved in the acquisition of the AS(30CQ) line.

4.4 Genetic Analyses

The results of the genetic crosses confirm that the AS(30CQ) line is due to several mutations, as parasites with intermediate levels of resistance were observed among the progeny. The exact number of mutations is unclear. Although three levels of chloroquine-resistance were classified there may well be more levels than this between 3 and 30CQ; the drug test may not have been sensitive enough to distinguish between them. For the purposes of this investigation, the resistance of the progeny clones was classified according to the rate at which the parasites emerged after drug treatment. The test used to classify the clones allowed for a range of different levels of resistance. Parasites were grouped into the 15CQ category if they emerged by D_{13} or D_{15}, and into the 30CQ class when they emerged by D_9 or D_{11}, following a drug test of 30 mg kg^{-1} chloroquine for 6 days. Thus, within these groups there is a range of rates of growth. These classifications may

indeed be artificial and chloroquine-resistance may be phenotypically a continuous variable character.

The question of whether discrete mutations at different loci were involved in the expression of the 30CQ resistance could be resolved by conducting a cross between a 30CQ and a 3CQ line. Should sensitive parasites emerge, then clearly the mutations are at different loci. However, should only a range of different levels of chloroquine resistance arise, then this phenomenon may be due to several mutations at a single locus. All these arguments are based on the assumption that chloroquine-resistance *per se* is genetically inherited and due to mutations of nuclear genes. The evidence from the crosses here and from previous work (Rosario, 1976a,b) suggest that this assumption is most probable as the character recombines independently of other markers. In addition, intermediate levels of resistance can only be obtained after conducting a cross. Competition experiments (see Section 4.4.5) imply that cytoplasmic inheritance is not operating in this case.

On conducting a cross, assuming random mating and no selection confering advantage on a particular character, theoretically the numbers of each class should be equal (Fig. 5). There is a preponderance of parental types due to self-fertilization. This clearly has not happened in the crosses conducted; several anomalies appeared.

4.4.1 Cross 1

The results of the drug tests on the uncloned progeny of the Crosses 1 and 2, and the electrophoresis data, indicated that both parental and recombinant types were present (Fig. 12). However, in Cross 1 in which the AS(30CQ) line was crossed with the AJ(Sens line, there was clearly an excess of the AJ parental type in the unselected progeny of the cross (Table 13). This phenomenon was also

observed by Rosario (1976a,b), where following a cross between the AS(3CQ) and AJ(Sens) lines, he obtained 32 parental AJ(Sens) clones from a total of 70 clones compared with 4 parental AS(3CQ) clones. In this work, Cross 1 yielded 10 chloroquine-resistant clones in a total of 33 clones in the unselected progeny of the cross (Table 13); on investigating the level of chloroquine-resistance in greater detail there was an absence of the high level of resistance (Table 18). This is unexpected as a quarter of the total progeny of the mating should have yielded parental highly resistant parasites due to selfing (Fig. 5). From the numbers of oocysts found in mosquitoes infected with each line separately (Control cages), both lines were apparently equally fertile.

After the progeny were selected using different levels of the drug, from a total of 27 highly resistant clones, only one was of the AS(30CQ) parental type. All of the clones established after selection at 30 mg kg^{-1} had AJ enzyme types and were therefore recombinant. It is possible that the results were due to chance and that on cloning most of the clones were derived from one class of parasites; as the cloning procedure requires low inocula, a more virulent parasite would stand a better chance of being selected. However, of the 97 clones established from Cross 1, 64 were obtained after drug treatment which would favour the emergence of resistant and hence AS parental types. There is strong evidence, therefore, that the AS(30CQ) parental types are at a selective disadvantage.

Table 19 summarizes the enzyme types of the cloned progeny of the cross; it can be seen that the AJ enzyme types are predominant. The significance of this will be

discussed elsewhere (see Section 4.5).

4.4.2 Cross 2

In Cross 1, clones exhibiting high levels of chloroquine-resistance could only be obtained by selection (Table 18). It is possible that if more clones had been obtained without selection, some clones with a high level of resistance would have been found. However, even after selection only one clone from a total of 60 resistant clones was of the AS(30CQ) parental type. Cross 2 which is a repetition of Cross 1, was therefore conducted to determine whether the results obtained in Cross 1 were unique to the cross or typical of such a cross. 21 clones were established from the cloned progeny of this cross. In this cross the enzyme markers seemed to be segregating equally (Table 21) as expected of Mendelian genetics. The chloroquine response of the clones indicate that a variety of sensitive and resistant forms were obtained. Although in this cross highly resistant 30CQ clones were obtained, unfortunately all of the clones were pyrimethamine-sensitive, and therefore the chloroquine-resistant clones were recombinants; so again, no highly resistant parental types were obtained.

Clearly, several factors are influencing the results of the crosses conducted. Because of the anomalous frequencies of parental types, an accurate assessment of recombination frequencies of the markers used was not possible. Ideally, the method of hybridization employed would be to fertilize isolated macrogametes of one line with microgametes of the other. The resulting zygotes would then develop into oocysts from which sporozoites arise; single sporozoites should then be inoculated into individual mice. This procedure is not yet technically feasible. Attempts to initiate infections from sporozoites

of single oocysts have been reported (Trembley et al., 1951; Walliker, 1972). Unfortunately, these efforts have been met with limited success and would be impractical for the large numbers required for genetic analyses.

An improvement of the present method of conducting a cross which would confirm the genetic basis of drug resistance markers would be by making use of microgamete preparations. For example, if microgametes of the AS line with the drug resistance markers were prepared and mixed with micro- and macrogametes of the AJ sensitive line, all the drug resistant parasites could only be products of cross-fertilization. In addition, this would confirm the nuclear inheritance of these markers as the structure of microgametes mainly consists of nuclei with almost no cytoplasm. Attempts to develop this technique in this laboratory have been mainly unsuccessful (Brown, unpublished).

A further refinement which would eliminate sampling errors and which would allow sampling at different stages of infection is by cloning using micromanipulation. This has been achieved with avian malaria parasites by Demidowa (1934), Coulston and Manwell (1941), Downs (1947) and Bishop (1958); and with rodent parsites by Diggens (1970). Due to the large numbers of clones required in a short time, this method of cloning had not been adopted in genetic work.

4.5 Reconstruction and Competition Experiments

The object of these experiments was to determine whether the absence of certain progeny classes was due to their selective disadvantage compared with other forms. The results of the competition experiments indicate that the AJ(Sens) line appears to be at an advantage over the AS(Sens) line (Table 23). The recombinant AJ(30CQ) line also seems to

be favoured over the AS(30CQ) lines (Table 26). In competition the parents of Crosses 1 and 2 appear to be neutral (Table 25). However, these experiments were conducted using the electrophoresis system to monitor any differences which might arise. The data of reconstruction experiments indicate that the system is not sensitive in detecting large differences; proportions of 70:30 and less are difficult to detect (Table 22).

The highly chloroquine-resistant (AS(30CQ)) character *per se* has been shown to be at an advantage over the parental sensitive AS(0CQ) character (Table 24). A low level of resistance (AS(3CQ)) was also found by Rosario *et al.* (1978) to possess a selective advantage. In addition, there is the influences of the pyrimethamine-resistance marker. This marker has been shown to be at an apparent disadvantage compared with the 'wild type' sensitive forms (Rosario *et al.*, 1978). If the pyrimethamine-sensitive forms are indeed at a selective advantage then it can be seen that as far as the drug resistance markers are concerned the selective forces would be acting in opposite directions in the competition experiment between the AS(30CQ) and the AJ(Sens) lines. This could account for the neutral results of the two lines when placed in competition with each other.

However, the mechanism involved in the chloroquine-resistant parasites outgrowing the sensitive forms is unclear. It is thought that there is a reduction of virulence with chloroquine-resistance in *P. berghei* (Sergent & Poncet, 1959; Peters, 1964; Jacobs, 1965). It has been observed that there is a tendency for resistant parasites to invade immature cells in *P. berghei* (Peters, 1968b); the diminished virulence in chloroquine-resistant forms may be due to a delay before reticulocytes are formed and consequently, parasites which can only live in young

erythrocytes may have to wait to invade the new cells. Chloroquine-resistant P. chabaudi invades both immature and mature erythrocytes so that there is no reason for such a decrease in virulence of resistant forms. The competition experiments with chloroquine-resistant lines of this species certainly suggest that the resistant parasites outgrow the sensitive forms (Table 24 & Rosario et al., 1978).

The results of the competition experiments between the drug sensitive AS and AJ lines, the AJ(30CQ) and AS(30CQ) lines are striking (Tables 23 & 26). The fact that differences can be detected means that the proportional differences are probably greater than 70:30 (Table 22). The apparent advantage of the AJ background could partly account for the results obtained both by Rosario (1976a,b) and the results of Cross 1 presented in this investigation. The apparent selective advantage of the recombinant AJ(30CQ) line could be explained in terms of 'vigour'. As the parasites are haploid and the term 'hybrid' has been mainly attributed to diploid organisms, this phenomenon should perhaps be referred to as 'recombinant vigour' in this case.

Examining the enzyme types of the clones obtained from Cross 1 reveal that 62 out of a total of 97 clones were of the AJ parental type whilst only 2.7 were of the AS parental type (Table 19). In Cross 2 the enzymes seem to segregate equally, however, all of the clones were recombinants (Table 21) apart from 5 of the clones which were of the parental AJ type. The majority of the clones were therefore recombinants which seem to have a selective advantage certainly over the highly resistant parental type. Only a small number of clones were isolated from this cross; had more clones been obtained the AJ enzyme types may have been found in excess as they were in Cross 1 and the cross conducted by Rosario (1976a,b).

The influence of these various selective factors could also account for the apparent failure of the highly resistant parasites to outgrow the sensitive forms in the crosses (Table 12); the chloroquine-resistant phenotype is expected to outgrow the sensitive forms if they are at a selective advantage.

In Experiment 2 of the competition work (AS(30CQ) vs AS(0CQ)) only highly resistant or sensitive clones were established (Table 24); no intermediate levels of resistances were obtained. After drug treatment at a low level before cloning, only highly resistant clones were obtained (Table 24). In Experiment 3 (AS(30CQ) vs AJ(Sens)), after drug treatment at a high level, only the AS enzyme type was observed (Table 25). These results indicate that the sexual mosquito stages of the life cycle of the parasites is required for exchange of genetic material to occur resulting in parasites showing intermediate levels of resistance. This gives no support to the hypothesis of Yoeli *et al.* (1969) that some form of genetic transfer or infection might occur between different parasites in the same blood cell.

However, as mentioned in the Introduction (see Section 1.8.2) the results obtained by these authors have not been repeated subsequently.

These results obtained here are interesting in the light of the variation found between species of malaria in man. The different levels of resistance developed in *P. chabaudi* may reflect the variation found in *P. falciparum* in man; RI, RII and RIII levels of chloroquine-resistance have been observed (WHO, 1973). These varying levels of

resistance in P. falciparum may have arisen by a slow increase of resistance due to the continued use of the drug as a prophylactic over a long period of time.

The competition experiments suggest that there is natural variation within species. The difference between AS(Sens) and AJ(Sens) may be in rates of growth, and such differences may exist in other species also.

The idea of cross products being more 'vigorous' and hence more of a threat than parental lines is alarming. In addition to the apparent selective advantage of the chloroquine-resistance character the increased growth rate of the cross products would compound the selection in favour of any chloroquine-resistant parasites arising from the cross. Both of these factors may have been contributary to the increase in numbers of chloroquine-resistant P. falciparum parasites. Furthermore, as the selective advantage of the resistant forms continues to persist in the absence of the drug, this would account for resistant parasites being maintained in the population even in areas where the drug has been discontinued.

4.6 In vitro Drug Tests

In vitro drug tests were attempted in order to standarize the drug test by eliminating host effects. It was hoped that these tests would provide a more accurate method of testing parasites for chloroquine response. In P. falciparum, Rieckmann et al. (1978) found that following in vitro culture, parasites of a chloroquine-resistant line formed schizonts in the presence of the drug whereas sensitive parasites did not. This was not observed with the P. chabaudi lines examined here. In the absence of the drug, parasites matured to schizonts (Table 27). Much

higher doses of the drug than those used by Rieckmann et al. (1978) were required for antimalarial activity against P. chabaudi in vitro. In the presence of the drug a morphological difference was observed between the two lines. At low doses of chloroquine (1.2 & 2.5 µg) the sensitive line had a delayed development; the parasites formed schizonts much later than the undrugged controls. Nevertheless, the sensitive parasites continued to develop, forming parasites with abnormal appearance. With the resistant line, low doses of chloroquine initiated vacuole formation and the development of the parasite appeared to be arrested at the trophozoite stage, these trophozoites contained vacuoles. At higher doses of the drug (5.0 & 10.0 µg), the sensitive line generally did not survive whereas the resistant forms were retarded to produce rings.

Inoculations from the cultures into clean mice reveal that the sensitive line is less viable after drug treatment than the resistant line (Table 28). The resistant parasites survive the dose of 5 µg whereas the sensitive parasites did not survive a dose of 5 µg. The in vitro drug tests thus revealed morphological differences between the AJ(Sens) and the AS(30CQ) lines. However, this observation is qualitative and the test did not yield quantitative results. This method was, therefore, not adopted as a drug test to distinguish between different levels of resistance. Although the inoculations from the cultures demonstrated variation in the viability of the two lines it is doubtful whether this test would be sensitive enough to show the differences between the chloroquine-resistant lines. However, the morphological differences between the lines were of considerable interest because pigment clumping and vacuolation in association with chloroquine-resistance has been found by various workers (see Section 1.6 and Peters et al., 1965; Peters and Fletcher, 1966; Rabinovich, 1968).

4.7 Mefloquine-Resistance

One of the reasons for developing a new antimalarial drug is because of the emergence of multiply resistant human malaria parasites. It is therefore important to examine the potential of parasites to develop resistance to the new drug mefloquine. By studying the patterns in which mefloquine-resistance arises, as with any other drug, means of minimising or preventing the development of drug resistance might be revealed. As the mode of action of mefloquine is not known, if resistance to the drug does arise the mechanisms involved can at this stage only be speculative.

There is evidence that higher doses of mefloquine are needed to kill chloroquine-resistant strains than sensitive strains of P. falciparum. This has been shown in Aotus monkeys (Schmidt, 1973) and in man (Trenholme et al., 1975). It has also been found that in P. berghei mefloquine is less active against chloroquine-resistant strains compared with drug sensitive strains (Peters et al., 1977a). Unstable mefloquine-resistance has been established by the latter authors in P. berghei (Peters et al., 1977b). In this investigation attempts to establish mefloquine-resistance in P. chabaudi have been undertaken.

In view of the decrease of activity against chloroquine-resistant lines of P. berghei it was not unexpected to find that mefloquine was cross-resistant to chloroquine to a limited extent (Fig. 12a). It was also relatively easy to develop mefloquine-resistance in a line already resistant to a high level of chloroquine (150Q) than in lines which were either resistant to a low level of chloroquine (30Q) or sensitive to this drug (Fig. 8 & Table 29). The level of chloroquine-resistance was raised marginally in the development of mefloquine-resistance which

confirms that the two drugs are in some way cross-resistant (Tables 4 & 30).

As with chloroquine, the strategy used for the development of mefloquine-resistance was a gradual increase in drug pressure. Mefloquine-resistance could not be obtained in a single step using the high pressure method of selection (Walliker, unpublished). This method involves inoculating a large number of mice and treating them with a high dose of the drug. Any parasites which survived this treatment are assumed to carry mutations for drug resistance. This strategy was highly successful in the selection for pyrimethamine-resistance (Morgan, 1974; MacLeod, 1977).

The mefloquine-resistant line developed was resistant to 30 mg kg^{-1} mefloquine for 4 days. This line appeared to be moderately stable on multiple blood passage in the absence of the drug (Table 31), although the parasites survived the drug treatment it took progressively longer for the parasites to emerge. This line proved to be unstable on mosquito transmission (Fig. 13). On transmitting the line through mosquitoes once, the level of resistance was reduced to 15 mg kg^{-1}; in one case this resistance seemed to be unstable on multiple blood passage (Fig. 13 & Table 41 in appendix). On another occasion, the resistance seemed to be stable (Tables 32 & 41, in appendix). On transmitting this line a second time, the parasites appeared to be sensitive (Fig. 13 & Table 40, in appendix). It must be stressed that the mefloquine-resistant line was uncloned at this stage.

Although the mefloquine-resistant line (AS(30MF)) appeared to be losing its resistance on multiple blood passage without drug pressure (Table 31), on cloning the resistant line after mosquito transmission (AS(15MF)), the resistance appeared to be stable and the parasites

emerged earlier than the original uncloned parasites after drug treatment (Table 32). As this clone was derived from parasites surviving a drug test it could be presumed that any lower levels of resistance or unstable resistant parasites would have been screened out. The presence of such unstable resistant parasites or lower levels of resistance could account for the delayed emergence of the parasites following a drug test of the uncloned mefloquine-resistant line and the loss of resistance following a second mosquito transmission.

Prior to cloning, the mefloquine-resistant AS(15MF) line had undergone 15 blood passages and had maintained its resistance to the drug. It is unlikely that a single drug test would have given rise to a stable mutation conferring resistance as the method with which mefloquine-resistance was developed involved a gradual increase of drug pressure (Fig. 8), and that mefloquine-resistance could not be obtained in a single step (Walliker, unpublished). Therefore the clone obtained from the parasites surviving a single drug test on Passage 15, was likely to have already been resistant to the drug and its apparent increased survival following a drug test compared with the uncloned population, may have been due to the "purification" of the parasites. Clearly, more work is needed to fully characterize this clone. The clone should be transmitted through mosquitoes, although previous attempts to do so have failed, probably due to the reduced infectivity to mosquitoes of the parasites following multiple blood passages. The stability of this line should be investigated further. Once this line has been fully characterized it would be important to investigate the basis of this resistance using genetic techniques.

4.8 Cross Resistance to Quinine

Mefloquine is structurally similar to quinine. So far there is no widespread quinine-resistance. There are some reports of quinine-resistance in P. falciparum (McNamara et al., 1967; Clyde et al., 1970). Glew et al. (1978) have selected for quinine-resistance in P. falciparum in Aotus monkeys by serial passage under repeated drug pressure. Therefore, clearly P. falciparum has the ability to develop resistance to this drug. However, to date, quinine is still active against most strains of P. falciparum including chloroquine-resistant parasites (Smithurst, 1971/, although there are reports that increasingly higher doses are required for radical cure (Harinasuta et al., 1965) and one Vietnamese (Smith) strain is relatively resistant to quinine (Clyde et al., 1970). However, Thai patients from Central Thailand were found to respond readily to quinine (Colwell et al., 1972). In spite of the doubts as to the reliability of quinine as an emergency measure when other drugs fail, it is still one of the few drugs available where resistance is not widespread. However it is possible that by the development of mefloquine-resistance the development of resistance to quinine could be accelerated.

Hall et al., 1976b,c)

The mefloquine-resistant line established here was tested for its cross-resistance to quinine. Although at this stage the mefloquine-resistant line was not fully characterized there is evidence that the uncloned mefloquine-resistant line, AS(30MF), is cross-resistant to quinine (Table 33). It would also be of value to know if the cloned mefloquine-resistant line (AS(15MF)), also demonstrated cross-resistance to quinine.

Mefloquine seems to be a drug of great potential value for the

suppression or the treatment of chloroquine-resistant P. falciparum. From this study it is clear that resistance to this drug can be readily developed. In addition, resistance to mefloquine may be a route towards the development of quinine-resistance. Efforts should be made to evaluate how best to use this drug. It has been proposed by several authors that studies of combinations of mefloquine with other drugs, both to search for true potentiating properties and to find a safe combination with other antimalarials that will extend its useful life, should be undertaken (Peters et al., 1977b). Such drug combination studies are already under way (Hall et al., 1977). Therefore, the continuing spread of drug resistance in malaria emphasises the need to use the existing antimalarial drugs wisely.

4.9 General Discussion

The present study uses a genetic approach to investigate chloroquine- and mefloquine-resistance in the rodent malaria, P. chabaudi. Reports of unstable chloroquine- and mefloquine-resistance have already been reviewed (see Introduction). The first case of laboratory-induced stable chloroquine-resistance in rodent malaria was reported by Rosario (1976a,b). As mefloquine is a relatively recent drug, as yet, there are no reported cases of stable resistance to this drug. In this study a high level of chloroquine-resistance was selected in order to determine how many mutations are responsible for this high level of resistance. In addition, mefloquine was investigated to determine the feasibility of developing resistance to this drug.

The highly resistant, AS(30CQ), line proved to be stable; this implies that permanent changes which presumably had a genetic basis had occured. The results of the crosses provided evidence for the

multigenic nature of this chloroquine-resistance. The competition experiments provided support for the nuclear origins of the mutation involved as intermediate levels of resistance could only be obtained following a cross and not from blood mixtures. This is the first case where drug resistance in Plasmodia has been shown to be determined by more than one mutation.

The possible mechanism by which chloroquine-resistance arises is still unclear. The theories so far put forward include the modification of clumping (Warhurst, 1973) and binding (Fitch, 1969) sites. These sites accumulate the drug; once inside the parasite, the drug probably intercalates with DNA and inhibits vital processes such as RNA and protein synthesis. Mutations affecting clumping sites may be specific to chloroquine as they are structure specific (Warhurst & Thomas, 1975a), whereas mutations affecting binding sites may serve as the basis of cross-resistance observed between the 4-aminoquinolines and related drugs as the sites are not highly structure specific(Fitch, 1972; Fitch et al., 1974c). If these mechanisms are correct, then it is likely that the high level of chloroquine-resistance may be due to several mutations of the genes coding for clumping and binding sites. These are likely to be discrete mutations at several loci as it would be unlikely that the same genes would code for both of the different sites.

Even if phenotypically chloroquine-resistance demonstrates continuous variation, at the gene level it may well be determined by several discrete genes at different loci which have additive effects confering a high level of resistance. The different classifications of levels of chloroquine-resistance in this investigation may be

arbitrary as it is based on the rate of emergence of the parasites after drug treatment; this criterion is not absolute and, as seen from the results, is subject to variation.

The competition experiments reveal some unexpected consequences of the development of chloroquine-resistance. Previous work (Rosario, 1976b; Rosario et al., 1978) and this present study has shown that not only do various lines differ in their selective advantage which may reflect the natural variability in wild populations, but that by changing their drug resistance properties, other features may be altered. The mutations to drug resistance may have pleiotropic effects, or in the course of selection for resistance, mutations altering growth patterns may have occurred.

From studies with cross-resistance it is possible to evaluate how best to use the present antimalarial drugs. Previous work has shown that although pyrimethamine and chloroquine are drugs of different groups which do not demonstrate cross-resistance, it was easier to develop chloroquine-resistance on a pyrimethamine-resistant background than on a drug sensitive background (Powers, 1969; Rosario, 1976b). The basis of this phenomenon is unknown. It can be envisaged that in the course of selecting for pyrimethamine-resistance some general mechanism may have been altered, such as permeability of parasite membranes, which would render the parasites inaccessible to other drugs; this could involve modifications of the binding sites (Fitch, 1969). This present study has shown that mefloquine and chloroquine demonstrate a small degree of cross-resistance which may have facilitated the development of mefloquine-resistance. In addition, there are indications that mefloquine and quinine are cross-resistant. Although

the reliability of using quinine when other drugs fail is now in question, it is still one of the drugs left where resistance to it is not widespread. It would be unfortunate if by the development of mefloquine-resistance the development of quinine-resistance were accelerated.

The extrapolation from this rodent model to the human malarias may be erroneous; however, the results with the rodent malaria system should give an indication of what to expect. With the development of *in vitro* culture systems (Trager & Jensen, 1976, 1977) the results obtained with murine malarias could either be confirmed or proved to be unique to rodents. Drug tests using *in vitro* culture systems of *P. falciparum* have already started using drug sensitive parasites (Richards & Maples, 1979). Comparative studies between strains of *P. falciparum* from different areas are in progress in this laboratory. Artificially selected chloroquine-resistant lines of *P. falciparum* have also been established *in vitro* (Nguyen-Dinh & Trager, 1978). Thus, it is now possible to study the mechanisms of drug resistance directly on human malaria parasites.

The application of molecular biological techniques to malarial systems is still in its infancy. Apart from its application in systematics where species of rodent malaria have been demonstrated to differ in their buoyant density and hybridization properties (Chance *et al.*, 1978), little has been done in studying the molecular biology of Plasmodia. This approach should provide insights into the basic genetic organisation of the parasites as well as information on gene expression. Investigations at the level of the 'gene' could provide conclusive evidence for the basis of drug resistance. In addition, genes which code for antigens could be found and cloned which would be

of value for vaccine production. Such aims are probably ambitious but nevertheless this approach is still a valid one.

It would be naive to believe that the malaria situation will not persist or even get worse as chloroquine-resistant strains of P. falciparum continue to develop and spread throughout the endemic regions of the world. The development of malaria vaccines against various stages of the parasite is a long term project, although studies indicate that this is theoretically feasible (Cohen, 1979; Murphy, 1979; Murphy & Lefford, 1979). Should such vaccines be developed, it would take many years before their application is widespread. At present, where even the production and marketing of a new drug, such as mefloquine, is viewed as uneconomic, the chance of an expensive vaccine reaching the countries affected by the disease in the immediate future is small. If such a vaccine were developed, its manufacture and administration would require international support and cooperation.

Chemotherapy, therefore, is likely to remain as the practical treatment against malaria for the foreseeable future. Research on the nature of drug interaction, the fate of drugs in hosts and parasites, on drug action and mechanisms and the genetics of drug resistance are therefore essential. Such studies are especially important with existing drugs such as chloroquine and potentially promising drugs such as mefloquine. It is hoped that this investigation has helped to elucidate some of the questions about resistance to these two drugs and hence contribute to the solving of the malaria problem.

REFERENCES

AIKAWA, M. (1972). High resolution autoradiography of malarial parasites treated with ^{3}H-chloroquine. Am.J.Path. 67, 277-280.

ALLISON, A.C. and YOUNG, M.R. (1964). Uptake of dyes and drugs by living cells in culture. Life Sci. 3, 1407-1414.

ALLISON, J.L., O'BRIEN, R.L. and HAHN, F.E. (1965). DNA reaction with chloroquine. Science 149, 1111-1113.

ANSDELL, V.E., BOOSEY, C.H., GEDDES, A.M. and MORGAN, H.V. (1974). Malaria in Birmingham, 1968-73. Brit.Med.J. ii, 206-208.

BAFORT, J. (1968). Primary exo-erythrocytic forms of Plasmodium vinckei. Nature, Lond. 217, 1264-1265.

BAHR, G.F. and MIKEL, U. (1972). The arrangement of DNA in the nucleus of rodent malarial parasites. Proc.Helm.Soc.Wash. 39, special issue, 361-372.

BALCERZAK, S.P., ARNOLD, J.P. and MARTIN, D.C. (1972). Anatomy of Red Cell Damage by Plasmodium falciparum in Man. Blood 40, 98-104.

BEALE, G.H. and KNOWLES, J. (1978). Extranuclear Genetics. Edward Arnold, Lond.

BEALE, G.H., CARTER, R. and WALLIKER, D. (1978). Ch.5. Genetics. In "Rodent Malaria". (R. Killick-Kendrick and W. Peters, eds). Acad. Press, Lond., N.Y. and San Francisco, pp. 213-245.

BEAUSOLEIL, E.G. (1968). Studies on alleged chloroquine resistance of malaria parasites in Axim and Obuasi. Bull.Wld Hlth Org. 38, 488-491.

BENAZET, F. (1965). Resistance de Plasmodium bergei à la chloroquine et aux amino-4 quinoleines. Ann.Soc.belge Med.trop. 45, 445-458.

BIGGERS, J.D., McLAREN, A. and MITCHIE, D. (1958). Variance Control in the Animal House. Nature, Lond. 182, 77-80.

BISHOP, A. (1958). Drug resistance in Protozoa. Biol.Rev.Camb.Phil. Soc. 34, 445-500.

BISHOP, A. and McCONNACHIE, E.W. (1952). Failure to produce resistance to chloroquine in Plasmodium gallinaceum in chicks. Parasitology 42,52-5

BLODGETT, L.W. and YIELDING, K.L. (1968). Comparison of chloroquine binding to DNA, and polyadenylic and polyguanylic acids. Biochim. Biophys.Acta 169, 451-456.

BODAMMER, J.E. and BAHR, G.F. (1973). The initiation of a "metabolic window" in the surface of host erythrocytes by Plasmodium berghei. NYU-2. Laboratory Investigations 28, 6, 708-718.

BOX, E.D., BOX, Q.T. and YOUNG, M.D. (1963). Chloroquine-resistant Plasmodium falciparum from Porto Velho, Brazil. Am.J.Trop.Med. Hyg. 12, 300-304.

BRUCE-CHWATT, L.J. (1970). Imported Malaria - a growing world problem. Trans.R.Soc.Trop.Med.Hyg. 64, 2, 201-209.

BRUCE-CHWATT, L.J. (1973). In "Advances in Parasitology", Vol. 11 (Ben Dawes, ed.), pp. 75-114. Acad.Press, Lond. and N.Y.

BRUCE-CHWATT, L.J. (1974). Resurgence of malaria and its control. 9th Int.Cong.trop.Med.Malar., Athens. J.Trop.Med. and Hyg. 77, 4, 62-66.

CANFIELD, C.J., HALL, A.P., MacDONALD, B.S., NEUMAN, D.A. and SHAW, J.A. (1973). Treatment of falciparum malaria from Vietnam with a phenanthrene methanol (WR 33063) and a quinoline methanol (WR 30090). Antimicrob.Agents chemothr., 3, 224-227.

CANNING, E.U. and ANWAR, M. (1968). Studies on meiotic division in coccidial and malarial parasites. J.Protozool. 15, 290-298.

CANNING, E.U. and MORGAN, K. (1975). DNA synthesis, reduction and elimination during life cycles of the eimeriine coccidian, Eimeria tenella and the haemogregarin, Hepatozoon domerquei. Exp.Parasitol. 38, 217-227.

CARTER, R. (1970). Enzyme variation in Plasmodium berghei. Trans. R.Soc.Trop.Med.Hyg. 64, 401-406.

CARTER, R. (1972). Effect of PABA on chloroquine resistance in Plasmodium berghei yoelii. Nature, Lond. 238, 98-99.

CARTER, R. (1973). Enzyme variation in Plasmodium berghei and Plasmodium vinckei. Parasitology 66, 297-307.

CARTER, R. (1978). Studies on enzyme variation in the murine malaria parasites Plasmodium berghei, P. yoelii, P. vinckei and P. chabaudi by starch gel electrophoresis. Parasitology 76, 241-267.

CARTER, R. and DIGGS, C.L. (1977). Plasmodia of Rodents. Parasitic Protozoa III, 359-465.

CARTER, R. and WALLIKER, D. (1975). New observations on the malaria parasites of rodents of the Central African Republic - Plasmodium vinckei petteri subs. nov. and Plasmodium chabaudi Landau, 1965. Ann.Trop.Med.Parasitol. 69, 187-196.

CHANCE, M.L., MOMEN, H., WARHURST, D.C. and PETERS, W. (1978). The chemotherapy of rodent malaria, XXIX. DNA relationships within the subgenus Plasmodium (vinckeia). Ann.Trop.Med.Parasitol. 72, 13-22.

CLYDE, D.F., MILLER, R.M., DUPONT, H.L. and HORNICK, R.B. (1970). Treatment of falciparum malaria caused by strain resistant to quinine. J.Am.Med.Assoc. 213, 2041-2045.

CLYDE, D.F., McCARTHY, V.C., SHUTE, G.T. and SANGALANG, K.P. (1971). Characterization of a drug resistant strain of Plasmodium falciparum from the Philippines. J.Trop.Med.Hyg. 74, 101-105.

CLYDE, D.F., McCARTHY, V.C., DU PONT, H.L. and HORNICK, R.B. (1973a). Characterization of a drug resistant strain of Plasmodium falciparum from Burma. J.Trop.Med.Hyg. 76, 56-60.

CLYDE, D.F., McCARTHY, V.C., DU PONT, H.L. and HORNICK, R.B. (1973b). Characterization of drug resistant strain of Plasmodium falciparum from Sabah. J.Trop.Med.Hyg. 76, 226-230.

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. and HORNICK, R.B. (1976a). Chloroquine resistant falciparum malaria from Irian Jaya (Indonesia New Guinea). J.Trop.Med.Hyg. 79, 38-41.

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. and HORNICK, R.B. (1976b). Suppressive activity of mefloquine sporozoite-induced human malaria. Antimicrob. Agents and Chemother. 9, 384-386.

COHEN, S. (1979). Immunity to malaria. Proc.R.Soc.Lond. B, 203, 325-345.

COHEN, S.N. and YIELDING, K.L. (1965). Spectrophotometric studies of the interaction of chloroquine with DNA. J.Biol.Chem. 240, 3123-3131.

COLWELL, E.J., PHINTUYOTHIN, P., SADUDEE, N., BENJAPONG, W. and NEOYPATIMANONDH, S. (1972). Evaluation of an in vitro technique for detecting chloroquine resistant falciparum malaria in Thailand. Am.J.Trop.Med.Hyg. 21, 6-12.

CONTACOS, P.G., LUNN, J.S. and COATNEY, G.R. (1963). Drug-resistant falciparum malaria from Cambodia and Malaya. Trans.R.Soc.Trop. Med.Hyg. 57, 417-424.

COULSTON, F. and MANWELL, R.D. (1941). Single parasite infections and exoerythrocytic schizogony in Plasmodium circumflexum. Am.J. Hyg. 34, 187-196.

DAVIDSON, M.W., GRIGGS, B.G.Jr., BOYKIN, D.W. and WILSON, W.D. (1975). Mefloquine, a clinically useful quinoline methanol antimalarial which does not bind with DNA. Nature, Lond. 254, 632-634.

DE DUVE, C., BARSY, T., POOLE, B., TROUET, A., TULKENS, P. and VAN HOOF, F. (1974). Lysosomotropic agents. Biochem.Pharmacol. 23, 2495-2531.

DEMEREC, M. (1957). Genetic aspects of drug-resistance. In "Drug resistance in micro-organisms", CIBA Found.Symp.Lond., 47-63.

DEMIDOWA, L.W. (1934). Ueber die Geringste zur Erzengung der experimentellen Malaria notige Plasmodium praecox Zahl. Giornale di Batteriol.Immunology B, 872-877. (As cited by Beale et al., 1978).

DENNIS, D.T., DOBERSTYN, E.B. and SISSAY, A. (1974). Chloroquine tolerance of Ethiopian strains of Plasmodium falciparum. Trans. R.Soc.Trop.Med.Hyg. 68, 3, 241-245.

DIGGENS, S.M. (1970). 1. Single-step production of pyrimethamine-resistant Plasmodium berghei. 2. Cloning erythrocytic stages of P. berghei. Trans.R.Soc.Trop.Med.Hyg. 64, 9-10.

DIGGENS, S.M., GUTTERIDGE, W.E. and TRIGG, P.I. (1970). Altered dihydrofolate reductase associated with a pyrimethamine resistant Plasmodium berghei produced in a single step. Nature, Lond. 228, 579-580.

DOBERSTYN, E.B., PHINTUYOTHIN, P., NOEYPATIMANONDH, S. and TEERAKIARTKAMJORN, C. (1979). Single-dose therapy of falciparum malaria with mefloquine or pyrimethamine-sulfadoxine. Bull.Wld.Hlth Org. 57, 2, 265.

DOWNS, W.G. (1947). Infections of chicks with single parasites of Plasmodium gallinaceum Brumpt. Am. J.Hyg. 46, 41-44.

EARLE, D.P.Jr. and BERLINER, R.W. (1948). Chloroquine. Proc. 4th Int.Congr.Trop.Med.Malar., Wash. 1, 725-733.

EBISAWA, I. and FUKUYAMA, T. (1975). Chloroquine resistance of Plasmodium falciparum in West Irian and East Kalmantan. Ann. Trop.Med.Parasitol. 69, 3, 275-282.

EHRLICH, P. (1910). Die Grundlagen der experimentallen. Chemotherapie 2. Angerv. Chem. 23, 2-8 (as cited by Peters, 1970).

EYLES, D.E., HOO, C.C., WARREN, M.W. and SANDOSHAM, A.A. (1963). Plasmodium falciparum resistant to chloroquine in Cambodia. Am.J. Trop.Med.Hyg. 12, 840-843.

FALCONER, D.S. (1960). Introduction to Quantitative Genetics. Oliver and Boyd, Edin., pp. 1-360.

FEDORKO, M.E., HIRSCH, J.G. and COHN, Z.A. (1968). Autophagic vacuoles produced in vitro. II. Studies on the mechanism of formation of autophagic vacuoles produced by chloroquine. J.Cell Biol. 38, 392-402.

FIELD, J.W. and EDESON, T.F.B. (1949). Paludrine resistant falciparum malaria. Trans.R.Soc.Trop.Med.Hyg. 43, 233-236.

FITCH, C.D. (1969). Chloroquine resistance in malaria: a deficiency of chloroquine binding. Proc.U.S.Nat.Acad.Sci. 64, 1181-1187.

FITCH, C.D. (1970). Plasmodium falciparum in owl monkeys. Drug resistance and chloroquine binding capacity. Science, N.Y. 169, 289-290.

FITCH, C.D. (1972). Chloroquine resistance in malaria. Drug binding and cross resistance patterns. Proc.Helm.Soc.Wash., 39, special issue, 265-271.

FITCH, C.D., CHEVLI, R. and GONZALEZ, Y. (1974a). Chloroquine accumulation by erythrocytes: a latent capacity. Life Sci. 14, 2441-2446.

FITCH, C.D., CHEVLI, R. and GONZALEZ, Y. (1974b). Chloroquine-resistant Plasmodium falciparum: effects of substrate on chloroquine and amodiaquine accumulation. Antimicrob.Agents and Chemother. 6, 757-762.

FITCH, C.D., YUNIS, N.G., CHEVLI, R. and GONZALEZ, Y. (1974c). High affinity accumulation of chloroquine by mouse erythrocytes with Plasmodium berghei. J.Clin.Invest. 54, 24-33.

FITCH, C.D., CHEVLI, R. and GONZALEZ, Y. (1975a). Chloroquine resistance in malaria. Variations of substrate stimulated chloroquine accumulation. J.Pharmacol.Exp.Ther. 195, 389-396.

FITCH, C.D., GONZALEZ, Y. and CHEVLI, R. (1975b). Amodiaquine accumulation by mouse erythrocytes infected with Plasmodium berghei. J.Pharm.Exp.Ther. 195, 397-403.

FITCH, C.D., NG, R.C.K. and CHEVLI, R. (1978). Erythrocyte surface: novel determinant of drug susceptibility in rodent malaria. Antimicrob.Agents Chemother. 14, 185-193.

FITCH, C.D., CHAN, R.L. and CHEVLI, R. (1979). Chloroquine resistance in malaria: Accessibility of drug receptors to mefloquine. Antimicrob.Agents Chemother. 15, 2, 258-262.

FOGH, S., JEPSEN, S. and EFFERSOE, P. (1979). Chloroquine resistant Plasmodium falciparum malaria in Kenya. Trans.R.Soc.Trop.Med.Hyg. 73, 2, 228-229.

GARNHAM, P.C.C. (1966). Malaria Parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford.

GLEW, R.H., COLLINS, W.E. and MILLER, L.H. (1978). Selection of quinine resistance in Plasmodium falciparum in Aotus monkeys. Am. J.Trop.Med.Hyg. 27, 1, 9-13.

GODOY, G.A., VOLCAN, G., MARENCO-REALES, O., GUEVARA, R. and TEIXEIRA, A. (1975). Demonstracion de resistencia al disfosfato de cloroquina por cepas de Plasmodium falciparum infectando naturalmente al hombre en un area de estado Bolivar, Venezuela. Revta.Inst.Med.Trop.S.Paulo 17, 1, 38-48 (Cited by Rosario, 1976b).

GREENBERG, J. (1956). Mixed lethal strains of Plasmodium gallinaceum. Drug sensitive transferable (SP) X drug resistant non transferable (B1). Expl.Parasitol. 5, 359-370.

GREENBERG, J. and BOND, H.W. (1954). Resistance of pyrimethamine-resistant strain of Plasmodium gallinaceum to certain 2, 4 diaminopyrimidines and related compounds. J.Parasitol. 40, 472-475.

GREENBERG, J. and TREMBLEY, H.L. (1954a). Infections produced by mixed strains of Plasmodium gallinaceum in chicks. J.Parasitol. 40, 336-340.

GREENBERG, J. and TREMBLEY, H.L. (1954b). The apparent transfer of pyrimethamine-resistance from the B1 strain of Plasmodium gallinaceum to the M strain. J.Parasitol. 40, 667-672.

GUTTERIDGE, W.E. and TRIGG, P.I. (1972). Some studies on the DNA of Plasmodium knowlesi. In "Comparative Biochemistry of Parasites", (H. Van den Bossche, ed.). Acad.Press, N.Y. and Lond., pp. 199-218.

GUTTERIDGE, W.E., TRIGG, P.I. and BAYLEY, P.M. (1972). Effects of chloroquine on Plasmodium knowlesi in vitro. Parasitol. 64, 37-45.

HAHN, F.E., O'BRIEN, R.L., CIAK, J., ALLISON, J.L. and OLENICK, J.G. (1966). Studies on modes of action of chloroquine, quinacrine and quinine and on chloroquine resistance. Milit.Med. 131 (Supplement), 1071-1089.

HALL, A.P. (1976). The treatment of malaria. Brit.Med.J. 1, 323-328.

HALL, A.P., SEGAL, H.E., PEARLMAN, E.J., PHINTUYOTHIN, P. and KOSAKAL, S. (1975a). Amodiaquine resistant falciparum malaria in Thailand. Am.J.Trop.Med.Hyg. 24, 575-580.

HALL, A.P., DOBERSTYN, E.B., METTAPRAKONG, V. and SONKOM, P. (1975b). Falciparum malaria cured by quinine followed by sulfadoxine-pyrimethamine. Brit.Med.J. 2, 15-17.

HALL, A.P., SEGAL, H.E., PEARLMAN, E.J. and PHINTUYOTHIN, P. (1975c). Comparison of a 9-phenanthrene methanol (WR 30090) and quinine for falciparum malaria in Thailand. Trans.R.Soc.Trop.Med.Hyg. 69, 342-349.

HALL, A.P., DOBERSTYN, E.B., KARNCHANACHETANEE, C., SAMRANSAMRUAJKIT, S., LAIXUTHAI, B., PEARLMAN, E.J., LAMPE, R.M., MILLER, C.F. and PHINTUYOTHIN, P. (1977). Sequential treatment with quinine and mefloquine or quinine and pyrimethamine-sulfadoxine for falciparum malaria. Brit.Med.J. 1, 1626-1628.

HARINASUTA, T., SUNTHARASAMAI, P. and VIRAVAN, C. (1965). Chloroquine-resistant falciparum malaria in Thailand. Lancet 2, 657-660.

HAWKING, F. (1953). Milk diet, Para-aminobenzoic acid and malaria. (Plasmodium berghei). Brit.Med.J. 1, 1201-1202.

HAWKING, F. (1966). Chloroquine resistance in Plasmodium berghei. Am.J.Trop.Med.Hyg. 15, 287-293.

HAWKING, F. and GAMMAGE, K. (1962). Chloroquine resistance produced in Plasmodium berghei. Trans.R.Soc.Trop.Med.Hyg. 56, 263.

HAYES, W. (1967). The genetics of bacteria and their viruses. Blackwell Scientific Publication, Oxford and Edin.

HINSHELWOOD, C.N. (1946). The chemical kinetics of the bacterial cell. Clarendon Press, Oxford.

HIPOO, B. and MacCALLUM, J.B. (1967). Possible chloroquine resistant malaria in Zambia. Med.J.Zambia 1, 31-33.

HOMEWOOD, C.A., WARHURST, D.C. and BAGGALEY, V.C. (1971). Incorporation of radioactive precursors into Plasmodium berghei in vitro. Trans.R.Soc.Trop.Med.Hyg. 65, 1, 10.

HOMEWOOD, C.A., JEWSBURY, J.M. and CHANCE, M.L. (1972a). The pigment formed during haemoglobin digestion by malarial and schistosomal parasites. Comp.Biochem.Physiol. 43B, 517-523.

HOMEWOOD, C.A., WARHURST, D.C., PETERS, W. and BAGGALEY, V.C. (1972b). Electron transport in intraerythrocytic Plasmodium berghei. Proc. Helm.Soc.Wash., 39 (special issue), 382-386.

HOMEWOOD, C.A., WARHURST, D.C., PETERS, W. and BAGGALEY, V.C. (1972c). Lysosomes, pH and the antimalarial action of chloroquine. Nature Lond. 235, 50-52.

HOWELLS, R.E. (1970). Mitochondrial changes during the life cycle of Plasmodium berghei. Ann.Trop.Med.Parasitol. 64, 2, 181-187.

HOWELLS, R.E. and MAXWELL, L. (1973a). Further studies on the mitochondrial changes during the life cycle of Plasmodium berghei: electrophoretic studies on isocitrate dehydrogenase. Ann.Trop. Med.Parasitol. 67, 3, 279-283.

HOWELLS, R.E. and MAXWELL, L. (1973b). Citric acid cycle activity and chloroquine resistance in rodent malaria parasites: the role of the reticulocyte. Ann.Trop.Med.Parasitol. 67, 3, 285-300.

INOKI, S. and MATSUSHIRO, A. (1960). Transformation of drug resistance in Trypanosoma. Biken's J. 3, 101-106 (as cited by Peters, 1970).

JACOBS, R.L. (1965). Selection of strains of Plasmodium berghei resistant to quinine, chloroquine and pyrimethamine. J.Parasitol. 51, 481-482.

JEFFERY, G.M. and GIBSON, F.D. (1966). Studies on chloroquine resistance of Plasmodium falciparum in Upper Volta and Liberia, West Africa. Bull.Wld Hlth.Org. 35, 441-449.

JENSEN, J.B. and TRAGER, W. (1978). Plasmodium falciparum in culture: Establishment of additional strains. Am.J.Trop.Med.Hyg. 27, 4, 743-746.

JINKS, J.L. (1964). Extrachromosomal inheritance. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

KAHN, A.A. and MAGUIRE, M.J. (1978). Relative chloroquine resistant Plasmodium falciparum in Zambia. Brit.Med.J. 1, 1669-1670.

KILLICK-KENDRICK, R. and WARREN, M. (1968). Primary exo-erythrocytic schizonts of a mammalian Plasmodia as a source of gametocytes. Nature, Lond., 220, 191-192.

KOLLERT, W. (1963). Experimentelle studien zun resistenz von Malaria-Parasiten gegen chloroquin. Medizin und Chemie. (Verlag Chemie G.M.b.H. Weinbeim/Bergstrasse, ed.) (as cited by Peters, 1970).

KONOPKA, E.A., GOBLE, F.C. and DONOVAN, J.S. (1966). Sex of host as a factor in protozoal chemotherapy. Abstracts 3rd Int.Pharm. Meet.Sao Paulo, 212-213.

KRAMER, P.A. and MATUSIK, J.E. (1971). Location of chloroquine-binding sites in Plasmodium berghei. Biochem.Pharmacol. 20, 1619-1626.

LANDAU, I. (1965). Description de Plasmodium chabaudi n.sp. parasite de rongeurs Africains. C.r. Acad.Sci.Paris, 260, 3758-3761.

LANDAU, I. and KILLICK-KENDRICK, R. (1966). Rodent Plasmodia of the Republique Centralafricaine: the sporogony and tissue stages of Plasmodium chabaudi and Plasmodium berghei yoelii. Trans.R. Soc.Trop.Med.Hyg. 60, 633-649.

LANDAU, I. and BOULARD, Y. (1978). Ch.2. Life cycles and morphology. In "Rodent Malaria" (R. Killick-Kendrick and W. Peters, eds). Acad.Press, Lond, N.Y. and San Francisco.

LANDAU, I., MICHEL, J.C., ADAM, J.P. and BOULARD, Y. (1970). The life cycle of Plasmodium vinckei lentum sub sp. nov. in the laboratory: comments on the nomenclature of the murine malaria parasites. Ann.Trop.Med.Parasitol. 64, 315-323.

LASCH, E.E. and N'GUYEN, T.L. (1965). Observations on an apparent chloroquine resistant strain of Plasmodium falciparum in West Africa. Brit.Med.J. 2, 1219-1222.

LUMSDEN, W.H.R., ROBERTSON, D.H.H. and McNEILLAGE, G.J.C. (1966). Isolation, cultivation, low temperature preservation and infectivity titration of Trichomonas vaginalis. Brit.J.Vener.Dis. 42, 145-154.

MABERTI, S. (1960). Desarallode resistencia a la pyrimethamina. Presentacion de 14 casos estudiados en Trajillo, Venezuela. Archas.Venez.Med.Trop.Parasitol. 3, 239-259 (as cited by Peters, 1970).

McGREGOR, I.A. and SMITH, D.A. (1952). Daraprim in treatment of malaria. A study of its effects in falciparum and quartan infections in West Africa. Brit.Med.J. 1, 730-734.

McLAREN, A. and MITCHIE, D. (1956). Variability of response in experimental animals. J. Genetics 54, 440-455.

MacLEOD, R.A.F. (1977). "The genetics of drug-resistance in malaria parasites". Ph.D. Thesis, University of Edinburgh.

MacLEOD, R.A.F. and BROWN, A. (1976). Development of gametocytes of Plasmodium chabaudi in splenectomised rats. Trans.R.Soc. Trop.Med.Hyg. 70, 4, 286.

McNAMARA, J.V., RIECKMANN, K.H., FRISCHER, H., STOCKERT, T.A., CARSON, P.E. and POWELL, R.D. (1967). Acquired decrease in sensitivity to quinine observed during studies with a strain of chloroquine-resistant Plasmodium falciparum. Ann.Trop.Med.Parasitol. 61, 386-395.

MACOMBER, P.B. and SPRINZ, H. (1967). Morphological effect of chloroquine on Plasmodium berghei in mice. Nature, Lond. 214, 937-939.

MACOMBER, P.B., O'BRIEN, R.L. and HAHN, F.E. (1966). Chloroquine: physiological basis of drug resistance in Plasmodium berghei. Science, N.Y. 152, 1374-1375.

MOMEN, H. (1979). Biochemistry of intraerythrocytic parasites II. Ann.Trop.Med.Parasitol. 73, 2, 117-121.

MONTGOMERY, R. and EYLES, D.E. (1963). Chloroquine resistant falciparum malaria in Malaya. Trans.R.Soc.Trop.Med.Hyg. 57, 409-416.

MOORE, D.V. and LANIER, J.E. (1961). Observations on two Plasmodium falciparum infections with an abnormal response to chloroquine. Am.J.Trop.Med.Hyg. 10, 5-9.

MORGAN, S. (1974). "The genetics of malaria parasites: studies on pyrimethamine resistance". Ph.D. Thesis, University of Edinburgh.

MORRIS, C.R., ANDREW, L.V., WICHARD, L.P. and HOLBROOK, D.J.Jr. (1970). The binding of antimalarial amino-quinolines to nucleic acids and polynucleotides. Mol.Pharm. 6, 3, 240-250.

MOST, H., LONDON, I.M., KANE, C.A., LAVIETES, P.H., SCHROEDER, E.F. and HAYMAN, J.M. (1946). Chloroquine for treatment of acute attacks of vivax malaria. J.Am.Med.Assoc. 131, 963-967.

MURPHY, J.R. (1979). Host defenses in murine malaria: analysis of the mechanism of immunity to Plasmodium berghei generated in response to immunization with formalin-killed blood-stage parasites. Infection and Immunol. 24, 3, 707-712.

MURPHY, J.R. and LEFFORD, M.J. (1979). Host defenses in murine malaria: successful vaccination of mice against Plasmodium berghei using formalized blood parasites. Am.J.Trop.Med.Hyg. 28, 4-11.

NGUYEN-DINH, P. and TRAGER, W. (1978). Chloroquine resistance produced in vitro in an African strain of human malaria. Science 200, 1397-1398.

OLATUNDE, A. (1977). Chloroquine resistant Plasmodium falciparum and malaria in Africa. Trans.R.Soc.Trop.Med.Hyg. 71, 1, 80-81.

OTT, K.J. (1969). The Plasmodium chabaudi-mouse system in chemotherapy studies. Exp.Parasitol. 24, 194-204.

OXBROW, A.I. (1973). Strain-specific immunity to Plasmodium berghei: a new genetic marker. Parasitology 67, 17-27.

PETERS, W. (1964). Pigment formation and nuclear division in chloroquine-resistant malaria parasites (P.b. vincke & Lips 1948). Nature, Lond. 203, 1290-1291.

PETERS, W. (1965). Drug resistance in Plasmodium berghei, vincke and Lips (1948). I. Chloroquine resistance. Exp.Parasitol. 17, 80-89.

PETERS, W. (1967). I. Malaria Rational Methods in the Search for Antimalarial Drugs. Trans.R.Soc.Trop.Med.Hyg. 61, 3, 400-410.

PETERS, W. (1968a). The chemotherapy of rodent malaria. I. Host-parasite relationships, Part 1: The virulence of infection in relation to drug resistance and time elapsed since isolation of the "wild" strain. Ann.Trop.Med.Parasitol. 62, 238-245

PETERS, W. (1968b). The chemotherapy of rodent malaria. II. Host-parasite relationships, Part 2: The relationship between chloroquine sensitivity and the age of the host cell. Ann.Trop. Med.Parasitol. 62, 246-251.

PETERS, W. (1968c). The chemotherapy of rodent malaria. V. Dynamics of drug resistance, Part 1: Methods for studying the acquisition and loss of resistance to chloroquine by Plasmodium berghei. Ann. Trop.Med.Parasitol. 62, 277-287.

PETERS, W. (1970). Chemotherapy and drug resistance in malaria. Acad.Press, Lond., pp. 1-876.

PETERS, W. (1974). Recent advances in antimalarial chemotherapy and drug resistance. Adv.Parasitol. 12, 69-114.

PETERS, W. and FLETCHER, K.A. (1966). Phagotrophy and pigment formation in a chloroquine-resistant strain of Plasmodium berghei. Proc.1st Int.Congr.Parasitol., Rome 1, 253-254.

PETERS, W., FLETCHER, K.A. and STÄUBLI, W. (1965). Phagotrophy and pigment formation in a chloroquine-resistant strain of Plasmodium berghei, Vincke and Lips, 1948. Ann.Trop.Med.Parasitol. 59, 126-134.

PETERS, W., ROBINSON, B.L., RAMKARAN, A.E. and PORTUS, J.H. (1969). A virulent chloroquine resistant strain of Plasmodium berghei berghei transmitted through Anopheles stephensi. Trans.R.Soc. Trop.Med.Hyg. 63, 1, 8.

PETERS, W., HOWELLS, R.E., PORTUS, J., ROBINSON, B.L., THOMAS, S. and WARHURST, D.C. (1977a). The chemotherapy of rodent malaria, XXVII. Studies on mefloquine (WR 142, 490). Ann.Trop.Med.Parasitol. 71, 4, 405-418.

PETERS, W., PORTUS, J. and ROBINSON, B.L. (1977b). The chemotherapy of rodent malaria, XXVIII. The development of resistance to mefloquine (WR 142, 490). Ann.Trop.Med.Parasitol. 71, 4, 420-427.

PETERS, W., CHANCE, M.L., LISSNER, R., MOMEN, H. and WARHURST, D.C. (1978). The chemotherapy of rodent malaria, XXX. The enigmas of the 'NS lines' of Plasmodium berghei. Ann.Trop.Med.Parasitol. 72, 1, 23-36.

POLET, H. (1970). Influence of sucrose on chloroquine-3-H^3 content of mammalian cells in vitro: The possible role of lysosomes in chloroquine resistance. J.Pharmacol.Exp.Ther. 173, 71-77.

POLET, H. and BARR, C.F. (1969). Uptake of chloroquine-^3H by Plasmodium knowlesi in vitro. J.Pharmacol.Exp.Ther. 168, 187-192.

POLLOCK, M.R. (1960). Drug resistance and mechanisms for its development. Brit.Med.Bull. 16, 16-22.

PILLAY, N. and BHOOLA, R.L. (1975). Probable chloroquine resistant Plasmodium falciparum malaria from Mozambique. A case report. S.Afr.Med.J. 49, 35, 1443-1444 (as cited by Trop.Dis.Bull., 1976, 73, 219).

POWELL, R.D., BREWER, G.J. de, GOWIN, R.L. and ALVING, A.S. (1964). Studies on a strain of chloroquine-resistant Plasmodium falciparum from Vietnam. Bull.Wld Hlth Org. 31, 379-392.

POWERS, K.G., JACOBS, R.L., GOOD, W.C. and KOONTZ, L.C. (1969). Plasmodium vinckei: production of chloroquine-resistant strain. Exp.Parasitol. 26, 193-202.

RABINOVICH, S.A. (1968). Experimental investigation of drug resistance in malarial parasites. Abstracts 8th Int.Congr.Trop.Med.Malar., Tehran, 1390-1392 (as cited by Peters, 1970).

RAMAKRISHNAM, S.P., SATYAPRAKASH and CHOUDHURY, D.S. (1957). Selection of a strain of Plasmodium berghei highly resistant to chloroquine. Nature, Lond. 179, 975.

RAMOS, O.L., JACALNE, A.V., CRUZ, F. de la and CUASAY, L.C. (1971). Chloroquine and other anti-malaria drugs resistant Plasmodium falciparum from Palawan, Philippines. J.Philipp.Med.Assoc. 47, 297.

RAY, A.P. and SHARMA, G.K. (1956). Acquired resistance to chloroquine diphosphate in Plasmodium gallinaceum in chicks. Nature, Lond., 178, 1291-1292.

RICHARDS, W.H.G. and MAPLES, B.K. (1979). Studies on Plasmodium falciparum in continuous cultivation. I. The effect of chloroquine and pyrimethamine on parasite growth and viability. Am.J.Trop.Med. Parasitol. 73, 2, 99-108.

RICOSSE, J.H., PICQ, J.J., COZ, J. and CHARMOT, G. (1969). Faits nouveaux relatifs à l'épidemiologie et au Controle du Paludisme en Afrique tropicale franchophone. Trans.R.Soc.Trop.Med.Hyg. 63 (4 Suppl.), S36-S41.

RIECKMANN, K.H., McNAMARA, J.V., FRISCHER, H., STOCKERT, T.A., CARSON, P.E. and POWELL, R.D. (1968). Effects of chloroquine, quinine and cycloquanil upon the maturation of asexual erythrocytic forms of two strains of Plasmodium falciparum in vitro. Am.J.Trop.Med. Hyg. 17, 5, 661-671.

RIECKMANN, K.H., TRENHOLME, G.M., WILLIAMS, R.L., CARSON, P.E., FRISCHER, H. and DESJARDINS, R.E. (1974). Prophylactic activity of mefloquine hydrochloride (WR 142, 490) in drug resistant malaria. Bull. Wld Hlth Org. 51, 375-377.

RIECKMANN, K.H., CAMPBELL, G.H., SAX, L.J. and MREMA, J.E. (1978). Drug sensitivity of Plasmodium falciparum. The Lancet, Jan.1978, 22-23.

RODRIGUES, D.C. (1961). Casos de malaria por Plasmodium falciparum resistentes ao tratomento pel a cloroquina. Arch.Fac.Hig.. Sande Pub., Univ. S.Paulo, 26, 231-235 (as cited by Peters, 1970).

ROSARIO, V.E. (1976a). Genetics of chloroquine-resistance in malaria parasites. Nature, Lond. 261, 585-586.

ROSARIO, V.E. (1976b). "Genetic studies of malaria parasites, especially in relation to drug resistance". Ph.D. Thesis, University of Edinburgh.

ROSARIO, V.E., HALL, R., WALLIKER, D. and BEALE, G.H. (1978). Persistance of drug resistant malaria parasites. Lancet, 185-187.

RUTLEDGE, I.C., WARD, R.A. and GOULD, D.J. (1964). Studies on the feeding response of mosquitoes to nutritine solutions in a new membrane feeder. Mosquito News 24, 407-419.

SAGER, R. (1954). Mendelian and non-mendelian inheritance of streptomycin resistance in Chlamydomonas reinhardi. Proc.Natn Acad.SCi. U.S.A. 40, 356-363.

SANDOSHAM, A.A., EYLES, D.E., PULL, J.H. and SENG, L.D. (1966). Chloroquine-resistant falciparum malaria in a semi-immune indigenous population in North Malaya. Med.J.Malaya 21, 115-124 (as cited by Peters, 1970).

SAUTET, T., ALDIGHIERI,J. and ALDIGHIERI, R. (1959). Etudes sur la production experimentale de la resistance a divers produits antimalariques d'une souche de Plasmodium berghei. Bull.Soc.Path. exot. 52, 331-345.

SCHELLENBERG, K.A. and COATNEY, G.R. (1961). The influence of antimalarial drugs on nucleic acid synthesis in P. gallinaceum and P. berghei. Biochem.Pharmacol. 6, 143-152.

SCHMIDT, L.H. (L969). Chemotherapy of the drug resistant malarias. Ann.Rev.Microbiol. 23, 427-454.

SCHMIDT, L.H. (1973). Infection with Plasmodium falciparum and Plasmodium vivax in the owl monkey - model system for basic biological and chemotherapeutic studies. Trans.R.Soc.Trop.Med.Hyg. 67, 446-474.

SCHMIDT, L.H. (1978). Plasmodium falciparum and Plasmodium vivax infections in the owl monkey (Aotus trivigatus). I. The course of untreated infections. Am.J.Trop.Med.Hyg. 27, 4, 671-702. II. Responses to chloroquine, quinine and pyrimethamine. Ibid. 703-717. III. Methods employed in search for new blood Schizontocide drugs. Ibid. 718-737.

SCHMIDT, L.H., GENTHER, C.S., FRADKIN, R. and SQUIRES, W. (1949). Development of resistance to chlorguanide (Paludrine) during treatment of infections with Plasmodium cynomolgi. J.Pharmacol.Exp.The 95, 382-398.

SCHMIDT, L.H., CROSBY, R., RASCO, J. and VAUGHAN, D. (1978). Antimalarial activities of various 4-quinolinemethanols with special attention to WR-142, 490 (mefloquine). Antimicrob. Agents Chemother. 13, 1011-1030.

SCHNITZER, R.J. (1963). Drug resistance of Protozoa. Int.Rev.Trop. Med. 2, 239-262.

SCHNITZER, R.J. (1966). Drug resistance of pathogenic Protozoa. Trans. New York Acad.Sci. Ser. II, 28, 923-934.

SCHNITZER, R.J. and GRUNBERG, E. (1957). "Drug resistance of microorganisms", pp. 1-395, Acad. Press, N.Y. and Lond.

SCHOENFIELD, C., MOST, H. and ENTNER, N. (1974). Chemotherapy of rodent malaria: transfer of resistance vs. mutation. Exp. Parasitol. 36, 265-277.

SCHREVEL, J., ASFAUX-FOUCHET, G. and BAFORT, J.M. (1977). Étude ultrastructurale des mitoses multiples au cours de la sporogonie du Plasmodium b. bergei. J.Ultrastruct.Res. 59, 332-350 (as cited by Sinden, 1978).

SCHWENDLER, H.P. (1965). Chloroquine resistant strains of Plasmodium falciparum in Ghana. Ghana Med.J. 4, 20-22.

SEATON, D.R. (1951). Failure to induce chloroquine resistance in Plasmodium gallinaceum. Ann.Trop.Med.Parasitol. 45, 99-100.

SERGENT, E. and PONCET, A. (1959). Des variations experimentale de la virulence de Plasmodium berghei. Exaltation-attenuation-mithridatisme. Archs.Inst.Pasteur Alger, 37, 227-2 (as cited by Peters, 1970).

SHUTE, G.T., RAY, A.P. and SANGALANG, K. (1972). Preliminary studies on a Philippine strain of Plasmodium falciparum resistant to amodiaquine. J.Trop.Med.Hyg. 75, 125-132.

SIDDIQUI, W.A., SCHNELL, J.V. and GEIMAN, Q.M. (1972). A model in vitro system to test the susceptibility of human malarial parasites to antimalarial drugs. Am.J.Trop.Med.Hyg. 21, 392-399.

SILVA, J.R. da, LOPES, P.E.A., FERREIRA, L.F., MORTEO, R. and NAVIERA, J.B. (1961). Resistencia do Plasmodium falciparum a acao de chloroquine. Hospital, Rio de Janeiro 60, 581-594.

SIMPSON, M. and WILLIAMS, P. (1978). The spread of chloroquine resistant falciparum malaria in Papua New Guinea. Med.J.Aust, 1, 41-42.

SINDEN, R.E. (1978). Ch.3. Cell Biology. In "Rodent Malaria". (R. Killick-Kendrick and W. Peters, eds). Acad.Press, Lond., N.Y. and San Francisco.

SINDEN, R.E. and CANNING, E.U. (1973). Ultrastructure and cytochemistry of nuclear division in Plasmodium. Progress in Protozool. p. 385 (IV Int.Congr.Protozool. Clermont-Ferrand).

SINDEN, R.E. and STRONG, K. (1978). An ultrastructural study of the sporogonic development of P. falciparum in Anopheles gambiae. Trans.R.Soc.Trop.Med.Hyg. 72, 5, 477-491.

SMITHURST, B.A. (1971). Resistant Plasmodium falciparum malaria cured by high quinine dosage. Med.J.Aust. 1, 436-437.

STEVENSON, D.J. (1966). A case of malaria not responding to chloroquine. Trans.R.Soc.Trop.Med.Hyg. 60, 276-277.

STILLE, W. (1979). Chloroquin-resistente malaria tropica nach Kenia-Reise. Deutsche Medizinische Wochenschrift 104, 26, 954-955.

TERZAKIS, J.A. (1969). A protozoan virus. Milit.Med.134, 10, 916-921.

THEAKSTON, R.D.G., ALI, S.N. and MOORE, G.A. (1972). Electron microscope autoradiographic studies on the effect of chloroquine on the uptake of tritiated nucleosides and methionine by Plasmodium berghei. Ann.Trop.Med.Parasitol. 66, 295-302.

THOMPSON, P.E., BAYLES, A., BUSH, D.L. and LILLIGREN, B.L. (1948). On the ability of Plasmodium lophurae to acquire resistance to chloroguanidine, camoquin and chloroquine. J.Inf.Dis. 82, 250-255.

THOMPSON, P.E., OLSZEWSKI, B., BAYLES, A. and WAITZ, J.A. (1965). Effects of representative antimalarial drugs against three drug-resistant lines of Plasmodium berghei in mice. J.Parasitol. 51, 54.

THONG, Y.H., FERRANTE, A., ROWAN-KELLY, B. and O'KEEFE, D.E. (1979). Effect of mefloquine on the immune response in mice. Trans.R. Soc.Trop.Med.Hyg. 73, 4, 388-390.

TIGERT, W.D. (1966). Present and potential malaria problem. Milit. Med. 131, 9 (Suppl.), 853-856.

TRAGER, W. (1971). Further studies on the effects of antipantothenates on malaria parasites Plasmodium coatneyi and Plasmodium falciparum in vitro. J.Protozool. 18, 232-239.

TRAGER, W. and JENSEN, J.B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

TRAGER, W. and JENSEN, J.B. (1977). Cultivation of erythrocytic stages. Bull. Wld Hlth Org. 55 (2-3), 363-365.

TREMBLEY, H.L. and GREENBERG, J. (1954). Further studies on the hybridization of strain of Plasmodium gallinaceum. J.Parasitol. 40, 475-479.

TREMBLEY, H.L., GREENBERG, J. and COATNEY, G.R. (1951). Strain differences in Plasmodium gallinaceum Brumpt. II. Experiences with the sporozoite and single oocyst passage of the Bl strain. J.Nat.Malar.Soc. 10, 68-75.

TRENHOLME, G.M., WILLIAMS, R.L., DES JARDINS, R.E., FRISCHER, H., CARSON, P.E., RIECKMANN, K.H. and CANFIELD, C.J. (1975). Mefloquine (WR 142,490) in the treatment of human malaria. Science, New York, 190, 792-794.

VALERA, C.V. and SHUTE, G.T. (1976). Preliminary studies on the response of Plasmodium falciparum to chloroquine in the Philippines, with the in vitro technique. Bull.Wld Hlth Org. 53, 391-398.

VAN DYKE, K., SZUSTKIEWICZ, C., LANTZ, C.H. and SAXE, L.H. (1969). Studies concerning the mechanism of action of antimalarial drugs. Inhibition of the incorporation of adenosine-8-^{3}H into nucleic acids of Plasmodium berghei. Biochem.Pharmacol. 18, 1417-1425.

VESSEL, E.S., PASSANANTI, G.T., GREENE, F.E. and PAGE, J.G. (1971). Drug metabolism in man. Ann.N.Y. Acad.Sci. 179, 752-773.

WALLIKER, D. (1972). An infection of Plasmodium berghei derived from sporozoites of a single oocyst. Trans.R.Soc.Trop.Med.Hyg. 66, 543.

WALLIKER, D. (1976). Genetic factors in malaria parasites, and their effect on host-parasite relationships. Symp.Brit.Soc.Parasitol. 14, (A.E.R. Taylor and R. Muller, eds). Blackwell Scientific Publications, pp. 25-44.

WALLIKER, D., CARTER, R. and MORGAN, S. (1971). Genetic recombination in malaria parasites. Nature, Lond. 232, 561-562.

WALLIKER, D., CARTER, R. and MORGAN, S. (1973). Genetic recombination in Plasmodium berghei. Parasitology 66, 309-320.

WALLIKER, D., CARTER, R. and SANDERSON, A. (1975). Genetic studies of Plasmodium chabaudi: recombination between enzyme markers. Parasitology 70, 19-24.

WALLIKER, D., SANDERSON, A., YOELI, M. and HARGREAVES, B.J. (1976). A genetic investigation of virulence in a rodent malaria parasite. Parasitology 72, 183-194.

WARHURST, D.C. (1969). Some aspects of the antimalarial action of chloroquine. Trans.R.Soc.Trop.Med.Hyg. 63, 4.

WARHURST, D.C. (1973). Chemotherapeutic agents and malaria research. In "Chemotherapeutic agents in the study of parasites", Vol. II. Symp.Brit.Soc.Parasitol. (A.E.R. Taylor and R. Muller, eds). Blackwell, Oxford, pp. 1-28.

WARHURST, D.C. and HOCKLEY, D.J. (1967). Mode of action of chloroquine on Plasmodium berghei and Plasmodium cynomolgi. Nature, Lond. 214, 935-936.

WARHURST, D.C. and KILLICK-KENDRICK, R. (1967). Spontaneous resistance to chloroquine in a strain of rodent malaria.(Plasmodium berghei yoelii). Nature, Lond. 213, 1048-1049.

WARHURST, D.C. and WILLIAMSON, J. (1968). Electrophoretic fractionation of ribonucleic acid from Plasmodium knowlesi (laboratory demonstration). Trans.R.Soc.Trop.Med.Hyg. 62, 3-4.

WARHURST, D.C. and WILLIAMSON, J. (1970). Ribonucleic acid from Plasmodium knowlesi before and after chloroquine treatment. Chem. Biol.Interact. 2, 89-106.

WARHURST, D.C. and ROBINSON, B.L. (1971). Cytotoxic agents and haemozoin pigment in malaria parasites (Plasmodium berghei). Life Sciences 10, 755-760.

WARHURST, D.C. and BAGGALEY, V.C. (1972). Autophagic vacuole formation in P. berghei. Trans.R.Soc.Trop.Med.Hyg. 66, 5.

WARHURST, D.C. and THOMAS, S.C. (1975a). Pharmacology of the malaria parasites/ -a study of dose-response relationships in chloroquine induced autophagic vacuole formation in Plasmodium berghei. Biochem.Pharmacol. 24, 2047-2056.

WARHURST, D.C. and THOMAS, S.C. (1975b). Localization of mepacrine in Plasmodium berghei and Plasmodium falciparum by fluorescence microscopy. Ann.Trop.Med.Parasitol. 69, 417-420.

WARHURST, D.C., ROBINSON, B.L., HOWELLS, R.E. and PETERS, W. (1971). The effect of cytotoxic agents on autophagic vacuole formation in chloroquine-treated malaria parasites (Plasmodium berghei). Life Sciences 10, 2, 761-771.

WARHURST, D.C., HOMEWOOD, C.A., PETERS, W. and BAGGALEY, V.C. (1972). Pigment changes in Plasmodium berghei as indicators of activity and mode of action of antimalarial drugs. Proc.Helm.Soc.Wash., 39, (Supplement), 271-278.

WARHURST, D.C., HOMEWOOD, C.A. and BAGGALEY, V.C. (1974). The chemotherapy of rodent malaria, XX. Autophagic vacuole formation in Plasmodium berghei in vitro. Ann.Trop.Med.Parasitol. 68, 265-281.

WATANABE, T. (1963). Infective heredity of multiple drug resistance in bacteria. Bacteriol.Rev. 27, 87-115.

WATANABE, T. and FUKASAWA, T. (1966). Episome-mediated transfer of drug-resistant enterobacteriaceae. 1. Transfer of resistant factors by conjugation. J.Bacteriol. 81, 669-678.

WEIDEKAMM, E., WALLACH, D.F.H., LIN, P.S. and HENDRICKS, J. (1973). Erythrocyte membrane alterations due to infection with Plasmodium. Biochim.Biophys.Acta 323, 539-546.

WELLDE, B.T., BRIGGS, N.T. and SADUN, E.H. (1966). Susceptibility to Plasmodium berghei: parasitological, biochemical and haematological studies in laboratory and wild animals. Milit.Med. 131, 859-869.

WERNSDORFER, W.H. (1979). Long-term aims of malaria research. TIBS, March, 1979, 49-51.

WERY, M. (1968). Studies on the sporogony of rodent malaria parasites. Ann.Soc.belge Med.trop. 48, 1-138.

WICHARD, L.P. and HOLBROOK, D.J. (1970). The effect of chloroquine on the enzymatic hydrolysis of nucleic acids. Mol.Pharmacol. 6, 548-556.

WOLCOTT, G.B. (1954). Nuclear structure and division in the malaria parasite, Plasmodium vivax. J.Morphol. 94, 353-366.

WOLCOTT, G.B. (1957). Chromosome studies in the genus Plasmodium. J.Protozool. 4, 48-51.

WOLFE, H.L. and HUDDLESTON, J.A. (1969). Investigation of suspected resistance of Plasmodium falciparum to chloroquine in Zambia. Med.J.Zambia 2, 173-179.

WHO (1965). Resistance of malaria parasites to drugs. Report of a W.H.O. Scientific Group. Tech.Rep.Ser.Wld Hlth Org. 296.

WHO (1967). Chemotherapy of malaria. Tech.Rep.Ser. 375, Geneva.

WHO (1973). Chemotherapy of malaria and resistance to antimalarials. Tech.Rep.Ser. 529, Geneva.

WHO (1976). Document TDR/1M/76.3. WHO, Geneva. (as cited by Wernsdorfer, 1979).

YOELI, M., UPMANIS, R.S. and MOST, H. (1969). Drug resistance transfer among rodent plasmodia. 1. Acquisition of resistance to pyrimethamine by a drug-sensitive strain of Plasmodium berghei in the course of its concomitant development with a pyrimethamine-resistant Plasmodium vinckei.strain. Parasitology 59, 429-447.

YOUNG, M.D. and MOORE, D.V. (1961). Chloroquine resistance in Plasmodium falciparum. Am.J.Trop.Med.Hyg. 10, 317-320.

YOUNG, M.D., CONTACOS, P.G., STITCHER, J.E. and MILLAR, J.W. (1963). Drug resistance in Plasmodium falciparum from Thailand. Am.J. Trop.Med.Hyg. 12, 305-314.

YUDKIN, J. (1953). Origin of acquired drug resistance in bacteria. Nature, Lond., 171, 541-546.

ZUCKERMAN, A. and YOELI, M. (1954). Plasmodium berghei in male and female rats. J.Inf.Dis. 94, 225-236.

APPENDIX

APPENDIX

TABLE 34: Calculation of Standard Error Estimates

$$\text{Standard Error} = \sqrt{\frac{\sigma^2}{n}}$$

$$\text{Where } \sigma^2 = \frac{\Sigma_1^n (x_i - \bar{x})^2}{n - 1}$$

$$\text{With } \bar{x} = \frac{\Sigma_1^n x_i}{n}$$

$\bar{x}$ is the mean

TABLE 35: Drug Delay Test Using 10 mg kg^{-1} Chloroquine

		% Parasitaemia (± S.E.)							
Day of Infection		D_3	D_4	D_5	D_6	D_7	D_8..... D_{13}		D_{15}
Drug Treatment									
AS(SENS)	DG	0.07 (±0.04)	-	-	-	-	-	0.06 (±0.06)	0.08 (±0.06)
	UND	0.06	0.5 (±0.07)	2.8 (±0.5)	11.4 (±3.3)	50(±0)	PP	PP	PP
AS(3CQ)	DG	0.5 (±0.03)	0.3 (±0.07)	-	-	-	-	0.1 (±0.08)	1.3 (±0.2)
	UND	0.4 (±0.035)	1.8 (±0.3)	10.0 (±3.6)	41.0 (±7.4)	PP	PP	PP	PP
AS(15CQ)	DG	0.2 (±0.07)	0.3 (±0.2)	0.05 (±0.03)	-	-	0.05 (±0.01)	3.7 (±1.02)	0.8 (±0.3)
	UND	0.4 (±0.15)	3.1 (±0.4)	19.7 (±4.6)	49.7 (±3.4)	PP	PP	PP	PP
AS(30CQ)	DG	0.6 (±0.07)	1.2 (±0.5)	1.3 (±0.3)	1.2 (±0.5)	0.9 (±0.3)	2.6 (±0.9)	0.9 (±0.7)	0.1 (±0.06)
	UND	0.6 (±0.15)	3.2 (±0.7)	16.9 (±3.5)	43.3 (±2.9)	8.3 (±1.5)	PP	PP	PP

DG: Drugged animals - mean of 3 mice; drug administered on D_3 - D_6

UND: Undrugged animals - mean of 4 mice

PP: Post Peak

Legend as for Figure 9

TABLE 36: Drug Delay Test Using 20 mg kg^{-1} Chloroquine

		% Parasitaemia (± S.E.)							
Day of Infection		D_3	D_4	D_5	D_6	D_7	D_8..... D_{13}		D_{15}
Drug Treatment									
AJ(SENS)	DG	0.3 (±0.1)	-	-	-	-	-	-	0.7 (±0.3)
	UND	0.06 (±0)	0.5 (±0.07)	2.8 (±0.5)	11.4 (±3.3)	50(±0)	PP	PP	PP
AS(3CQ)	DG	0.6 (±0.07)	-	-	-	-	-	0.03 (±0.3)	0.7 (±0.5)
	UND	0.4 (±0.035)	1.8 (±0.3)	10.0 (±3.5)	41.0 (±7.4)	PP	PP	PP	PP
AS(15CQ)	DG	0.2 (±0.12)	0.05 (±0.03)	0.06 (±0.06)	0.07 (±0.06)	-	0.1 (±0.1)	2.5 (±0.7)	0.3 (±0.09)
	UND	0.4 (±0.2)	3.1 (±0.4)	19.7 (±4.6)	49.7 (±3.4)	PP	PP	PP	PP
AS(30CQ)	DG	0.8 (±0.09)	0.9 (±0.2)	0.5 (±0.1)	0.5 (±0.09)	1.0 (±0.1)	3.8 (±1.6)	3.1 (±1.7)	0.7 (±0.5)
	UND	0.5 (±0.2)	3.2 (±0.6)	16.9 (±3.5)	43.3 (±2.9)	83.0 (±2.9)	PP	PP	PP

DG: Drugged animals - mean of 3 mice; drug administered on D_3 - D_6

UND: Undrugged animals - mean of 4 mice

PP: Post Peak

Legend as for Figure 10

TABLE 37: Response of Different Lines of P. chabaudi to Varying Doses of CQ

	% Parasitaemia (± S.E.)									
Drug Dose ($mg\ kg^{-1}$ for 6 days)	UND	3CQ			5CQ			10CQ		
Day of Infection	D_7	D_5	D_7	D_9	D_5	D_7	D_9	D_5	D_7	D_9
Run I										
AJ(SENS)	44.2 (±14.0)	0.05 (±.025)	0.7 (±0.3)	9.9 (±3.6)	-	-	0.008 (±0.008)	-	-	-
AS(3CQ)	38.0 (±16.5)	9.1 (±6.2)	34 (±22.5)	PP	0.07 (±0.05)	0.6 (±0.4)	6.8 (±4.4)	-	-	-
AS(15CQ)	39.0 (±11.3)	2.8 (±1.7)	20.5 (±11.0)	17.5 (±2.7)	0.08 (±0.08)	1.8 (±1.5)	15 (±8.9)	-	0.005 (±0.003)	0.4 (±0.2)
AS(30Q)	54.0 (±2.6)	8.0 (±2.4)	40.5 (±8.4)	PP	0.2 (±0.2)	7.0 (±2.8)	18.1 (±4.2)	0.01 (±0.006)	0.2 (±0.006)	6.5 (±2.6)
Run II										
AJ(SENS)	50.0 (±0)	2.9 (±1.7)	34.0 (±8.0)	PP	0.1 (±0.1)	0.5 (±0.3)	8.3 (±4.5)	-	-	-
AS(3CQ)	45 (±2.5)	2.4 (±1.2)	18.1 (±8.1)	PP	0.4 (±0.06)	2.5 (±0.5)	25.3 (±1.6)	-	-	-
AS(15Q)	52 (±1.0)	3.3 (±1.3)	17.7 (±2.8)	PP	1.1 (±0.3)	7.6 (±2.2)	37.5 (±4.3)	-	0.2 (±0.08)	5.8 (±2.3)
AS(30Q)	32.0 (±13.0)	4.8 (±1.8)	31.8 (±5.5)	PP	1.2 (±0.2)	16.6 (±3.5)	38.8 (±6.8)	0.005 (±0.003)	0.3 (±0.3)	2.8 (±2.0)

Drugged animals - mean of 4 mice

UND: Undrugged animals - mean of 3 mice

TABLE 38: Drug Tests of Stability Studies on the AS(30CQ) Line

	% Parasitaemia (± S.E.)					
	Drugged					Undrugged
Day of Infection	D_9	D_{10}	D_{11}	D_{13}	D_{15}	D_7
Experiment No.						
1) AS(30CQ)		0.04 (±0.01)		1.0 (±0.14)		PP
AS(3CQ) CONTROL		-		-		16.0 (±1.0)
2) AS(30CQ)	0.008 (±0.009)		0.2 (±0.02)	3.4 (±0.9)		50.0 (±9.9)
AJ(Sens) CONTROL	-		-	-		45.0 (±0)
3) Passage No.1 AS(30CQ)	-		0.09 (±0.01)	0.82 (±0.2)		22.0 (±5.0)
AS(3CQ) CONTROL	-		-	-		42.0 (±14.84)
4) Passage No.5 AS(30CQ)	0.05 (±0.035)		0.5 (±0.3)	0.8 (±0.4)		42.5 (±2.5)
AS(3CQ) CONTROL	-		-	-	-	42.0 (±0)
5) Passage No.6 AS(30CQ)	0.02 (±0.01)		0.5 (±0.4)			30.0 (±9.9)
AS(3CQ) CONTROL	-		-			45.0 (±5.0)
6) Passage No.8 AS(30CQ)	0.006 (±0.006)		0.3 (±0.1)			4.2 (±3.9)
AS(3CQ) CONTROL	-		-			50.0 (±0)
7) AS(30CQ)	-		0.03 (±0.01)	0.2 (±0.05)	3.2 (±1.6)	20.0 (±5.0)
AJ(Sens) CONTROL	-		-	-	-	50.0 (±0)
8) AS(30CQ)	0.006 (±0.006)		0.014 (±0.01)	0.6 (±0.3)	2.9 (±1.0)	45.0 (±0)
AJ(Sens) CONTROL	-		-	-	-	40.0 (±0)

Drugged animals - mean of at least 3 mice, treated with 30 mg^{-1} for 6 days

Undrugged animals - mean of 2 mice

Legend as for Figure 11

TABLE 39: Stability of AS(30MF) Line After Mosquito Transmission

	% Parasitaemia (± S.E.)				
Day of Infection	D_9	D_{11}	D_{13}	D_{15}	UND D_9
AS(30MF)(Before MOS)	-	0.09 (±0.03)	0.3 (±0.1)	1.5 (±0.5)	PP
AS(30MF)(After MOS) MI	-	-	-	-	PP
MII	-	-	-	-	PP

MOS - Mosquitoes

Drugged animals - mean of mice treated with 30 mg kg^{-1} mefloquine for 4 days

UND - Undrugged animals

PP - Post Peak

Legend as for Figure 13

TABLE 40: Stability of AS(30MF) Line

	% Parasitaemia(± S.E.)				
Day of Infection	D_9	D_{11}	D_{13}	D_{15}	UND D_9
AS(30MF) Before MOS	0.4 (±0.2)	5.3 (±2.6)	10.1 (±4.0)	PP	PP
AS(30MF) After 1 MOS PASSAGE	-	0.2 (±0.07)	0.5 (±0.2)	1.7 (±0.7)	PP
AS(30MF) After 2 MOS PASSAGES	-	-	-	-	PP
AS(30CQ)	-	-	-	-	PP

MOS - Mosquitoes

Drugged Animals - mean of 3 mice, treated with 15 mg kg^{-1} mefloquine for 4 days

UND - Undrugged animals

PP - Post Peak

Legend as for Figure 13

TABLE 41: Mefloquine-Resistance of AS(30MF) After Mosquito Transmission

STABILATE NO.	Day of Infection	% Parasitaemia (± S.E.) D_{12}	D_{14}	D_{16}	UND D_{12}
	PASSAGE NO.				
df 870	8	0.6(±2)			PP
	9	0.2 (±0.08)			PP
	15	-	-	0.2 (±0.08)	PP
	16	-	-	-	PP
df 900	4	-	0.1 (±0.05)	0.4(±0.1)	PP
	5	-	-	1.8(±0.5)	PP

Drugged Animals, mean of 3 mice, treated with 15 mg kg^{-1} MF for 4 days

UND - Undrugged Animals, mean of 2 mice

PP - Post Peak

AS(15CQ) Line Control used in all tests showed negative infection throughout

Legend as for Figure 13

mice infected with *S. mansoni* significantly more 21-day-old schistosomula established in the portal system and chloroquine had a reduced depressive effect on the *P. yoelii* parasitaemia and recrudescences occurred.

Development of *Trypanosoma congolense* in tissue sites

A. G. LUCKINS, A. R. GRAY AND J. GREENHORN
Centre for Tropical Veterinary Medicine, University of Edinburgh

Trypanosomes were found in connective tissue in local skin reactions developing in cattle, sheep and rabbits at the sites of bites by *Glossina morsitans* infected with *Trypanosoma congolense*. The parasites developed in the collagen of the deep dermis and were particularly numerous in reactions examined up to 12 days after infection. Substantial numbers of trypanosomes were present at the reaction site in calves up to 21 days after infection and were still present 30 days after infection. The invasion of collagen by the parasites appeared to be focal and, from limited observations, there was no evidence that the infection in the connective tissue became generalized.

Villous atrophy in the mouse: a thymus-dependent phenomenon

D. F. MANSON-SMITH, R. G. BRUCE AND DELPHINE M. V. PARROTT*
*Departments of Zoology and *Bacteriology and Immunology, University of Glasgow, Glasgow G12 8QQ*

An analysis of the response of the small intestine to *Trichinella spiralis* infection in thymus-deprived NIH mice has shown that depletion of T-cells has a dramatic effect on the localization of adult worms in the small intestine and that normal expulsion does not occur. Villous atrophy and crypt hyperplasia which accompany the infection in normal mice is absent or reduced, especially in the jejunum, thus providing clear evidence that in this model villous atrophy is a thymus-dependent phenomenon.

An *in vitro* test for chloroquine-sensitivity in *Plasmodium chabaudi*

R. A. PADUA
Institute of Animal Genetics, University of Edinburgh

Rieckmann *et al.* (1978, *Lancet*, **i**, 22-33) have described an *in vitro* micro-test for assessing the chloroquine-sensitivity of strains of *P. falciparum*. A modification of this test has been applied to chloroquine-sensitive and resistant lines of *P. chabaudi*. In the absence of the drug, parasites of both lines matured to schizonts. At low chloroquine doses, the sensitive line showed delayed development, schizonts being formed later than in undrugged controls. In the resistant line, vacuoles were seen in parasites exposed to chloroquine. Infectivity tests in mice showed that the resistant parasites were viable following incubation with chloroquine, while sensitive forms showed reduced viability.

Mefloquine-resistance in *Plasmodium chabaudi*

R. A. PADUA AND D. WALLIKER
Institute of Animal Genetics, University of Edinburgh

The activity of mefloquine against chloroquine-resistant and sensitive lines of *Plasmodium chabaudi* has been examined. Using a standard four-day test, two lines, one moderately resistant to chloroquine and one highly resistant, were found to be less sensitive to mefloquine than a chloroquine-sensitive line. A mefloquine-resistant line of *P. chabaudi* was developed by exposing sensitive forms to gradual increases in drug pressure during blood passage in mice. Following selection, a line resistant to mefloquine at 30 mg/kg (four doses) was produced. The resistance was moderately stable in the absence of the drug.

We thank the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, for supplying us with mefloquine.

The effects of BCG organisms on the course of *Plasmodium chabaudi*, *P. berghei* and *Trypanosoma brucei* infections in mice

L. J. PANTON AND R. S. PHILLIPS
Department of Zoology, University of Glasgow

C57B1 and CBA male mice were injected intravenously (i.v.) with approximately 10^7 BCG organisms (Glaxo). The C57B1 mice along with controls were subsequently challenged two, three, four or eight weeks later with either 10^6 *Plasmodium chabaudi* (AS strain) or 10^6 *Plasmodium berghei* (KSP11 strain) parasitized red cells. The CBA mice were challenged with 10^5 *Trypanosoma brucei* (TREU 792) two, three, four or eight weeks after BCG.

The C57B1 mice showed some protection against *P. chabaudi* two and three but not four and eight weeks after BCG. In mice challenged with *P. berghei* after BCG the early patent parasitaemias were higher in the BCG-treated mice and those mice injected at four and eight weeks died before the relevant controls. In all the CBA mice the BCG depressed the early *T. brucei* parasitaemias and delayed death.

Cryopreservation of *Plasmodium falciparum* in liquid nitrogen

R. S. PHILLIPS* AND R. J. M. WILSON†
**Department of Zoology, University of Glasgow, and †National Institute for Medical Research, London NW7 1AA*

Human red cells containing ring stages of *Plasmodium falciparum* were washed with medium 199 (PHILLIPS *et al.*, 1972, *Parasitology*, **65**, 525), resuspended in Caucasian serum and cryopreserved (total volume 0·5 ml) in either glycerol in phosphate buffered saline (PBS) (final concentration 17% v/v) or dimethyl sulphoxide (DMSO) (final concentration of 10%, 12% and 15% v/v) by plunging Sterilin ampoules into liquid N_2. Infected blood was thawed for 1 min at 37°C and the cryoprotectant removed by five washings with ice-cold Sorbitol in PBS in which the concentration of Sorbitol was gradually lowered from 17·5% to zero. After a final washing in 199 the cells, resuspended in serum,

using replicate groups of test animals which were infected at different intervals post-implantation, eliminating the possible complication of acquired immunity. At a drug concentration of 0·5% pyrimethamine, protection from infection for > 5, < 6 months was achieved.

Preliminary experiments using other antimalarials in combination with silicone rubber have been performed and the results may be summarized as follows.

Primaquine diphosphate, cycloguanil HCl and menoctone implants gave rise to local tissue necrosis similar to that observed when these drugs are given by normal routes of administration.

Chloroquine implants were ineffective in contrast to those containing pyrimethamine isethionate, sulphadiazine and sulphormethoxine.

These results suggest that the administration of antimalarials in sustained-release delivery systems warrants further investigation.

Genetics of Chloroquine Resistance in Rodent Malaria. By R. A. PADUA (*Department of Genetics, University of Edinburgh, Edinburgh, EH9 3JN*)

Using *Plasmodium chabaudi*, a rodent malaria parasite, the genetic basis of chloroquine resistance was examined. A high level of chloroquine resistance (30 mg/kg for 6 days) was established by gradual increases of drug pressure. This resistance was stable after multiple blood passages and mosquito transmission. The inheritance of the chloroquine resistance was examined by crossing the resistant line to the sensitive line. The progeny of the cross were cloned and tested for drug response. The clones were classified as being either sensitive, intermediate (resistant to either 3 mg/kg or 15 mg/kg for 6 days), or highly resistant (30 mg/kg for 6 days). As there are intermediate levels of resistance resulting from the cross, the high level of resistance can be concluded to be the result of more than one mutation. This system may be a model for the different types of chloroquine response observed in the human malaria parasite *P. falciparum*.

SESSION 1B

Helminth ultrastructure

The Tegumental Surfaces of Some Species of the Genus *Paramphistomum fischoeder*, 1901, as Revealed by Scanning Electron Microscopy. By L. EDUARDO (*Commonwealth Institute of Helminthology, The White House, 103 St Peter's Street, St Albans, Herts, England AL1 3EW*)

Eleven species of *Paramphistomum* from ruminants were examined for surface structures. Most revealed the presence of structures termed here surface 'papillae'. It was observed that there exist some differences as to occurrence, distribution and size of these structures among the species examined but these appear to be consistent in the same species of different ages and from different hosts.

On the basis of occurrence, distribution and size of these surface papillae, it is possible to separate the eleven species examined into four groups as follows: Group 1 – absence of surface papillae, e.g. *Paramphistomum cervi*; Group 2 – presence of surface papillae limited only around the anterior end, e.g. *P. gracile, P. clavula, P. ichikawai*; Group 3 – surface papillae densely arranged around the anterior end and ventrally around the genital pore area, but the size of these papillae in the two areas differs, e.g. *P. microbothrioides, P. sukari*; Group 4 – surface papillae densely arranged around the anterior end and around genital pore area which are of the same structure and almost of the same size, e.g. *P. gotoi, P. leydeni, P. scotiae, P. microbothrium, P. epiclitum*.

A key to separate these species on the basis of tegumental and internal pharyngeal surface structures together with histo-morphological features is given.

Cyst Structure and Excystment of *Asymphylodora dollfusi* Metacercariae. By N. A. EVANS (*King's College London*)

Metacercariae of *Asymphylodora dollfusi* excyst and develop into gravid parasites in the pericardial cavity of certain second intermediate hosts (the prosobranch molluscs *Bithynia*

PUBLICATIONS

1. PADUA, R.A. (1978). An *in vitro* test for chloroquine-sensitivity in *Plasmodium chabaudi*. Trans.R.Soc.Trop.Med.Hyg. 72, 6, 643.

2. PADUA, R.A. and WALLIKER, D. (1978). Mefloquine-resistance in *Plasmodium chabaudi*. Trans.R.Soc.Trop.Med.Hyg. 72, 6, 643.

3. PADUA, R.A. (1979). Genetics of Chloroquine-Resistance in Rodent Malaria. Parasitology 79, iii.