Resistance to primaquine in *Plasmodium gallinaceum*, and the problem of resistance to quinoline compounds in malaria parasites*

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**INTRODUCTION**

The occurrence, during the present decade, of resistance to the 4-aminoquinoline compound chloroquine, in infections of *Plasmodium falciparum* in certain regions of South America and South East Asia, has focused attention upon drug resistance as a problem in the therapeutical treatment of malaria, since this drug is widely used in prophylaxis and treatment of the clinical attack. Resistance to other aminoquinolines has been produced experimentally. As early as 1934 Nauck described the development of resistance to the 8-aminoquinoline compound pamaquin (plasmoquine) in *P. knowlesi*, an observation confirmed by Fulton & Yorke (1941), and resistance to this compound was also produced in *P. gallinaceum* (Bishop & McConnachie, 1952b). More recently, resistance to the related 8-aminoquinoline primaquine has been produced experimentally in the Chesson strain of *P. vivax* (Arnold, Alving, Dayman & Hochwald, 1961), and in *P. berghei* (Prakash, Chakrabarti & Choudhury, 1961; Peters, 1965a, 1966).

The following account describes the development of a primaquine-resistant strain of *P. gallinaceum*, its stability, and the effect of other antimalarial drugs upon it; it also describes attempts to produce resistance to chloroquine in this species and discusses the general problem of resistance to aminoquinolines in malaria parasites.

**METHODS**

In order that the stability of primaquine resistance, if it should develop, could be assessed after the strain had been transmitted through mosquitoes, a strain of *P. gallinaceum*, strain G, which had been maintained by alternate passage through young chicks and *Aedes aegypti* and had never previously been treated with a drug, was used in these experiments. Strain A, the strain used in previous experiments on drug resistance, and derived from a single erythrocytic trophozoite (Bishop, 1958), had been maintained by blood inoculation and had ceased to produce significant infections in mosquitoes; it was therefore unsuitable for experiments involving mosquito transmission, but was used in the attempts to produce resistance to chloroquine.

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In testing the sensitivity of the drug-treated strain to drugs, groups of four chicks, 46-65 g in weight, were inoculated intravenously, each with $5 \times 10^7$ parasites. Each group received a different dose of drug, given twice daily for $3\frac{1}{2}$ days, the first dose being given immediately after inoculation; two birds, inoculated similarly but which received no drug treatment, served as controls. The sensitivity of the strain to the drug was assessed by parasite count made on the fourth day following inoculation and expressed as the proportion of parasitized erythrocytes per 500 erythrocytes. The drugs were given by catheter tube into the stomach. Primaquine diphosphate,* mepacrine dihydrochloride, chloroquine diphosphate, quinine dihydrochloride, proguanil hydrochloride and 4,6-diamino-1-$(p$-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride, the active metabolite of proguanil and referred to as dihydrotriazine, were all given in aqueous solution. Since the solubility of pyrimethamine base was slight, a little lactic acid was added to bring about solution. Pamaquin was given as a suspension in water. The dose of drug is expressed in mg per 20 g of body weight.

RESULTS

(1) The development of resistance to primaquine in Plasmodium gallinaceum

The maximum dose of primaquine tolerated by young chicks, when given twice daily over a period of $3\frac{1}{2}$ days, was 0.1 mg; doses above that level caused toxic symptoms and death. When the G strain was tested against primaquine in doses ranging from 0.1 to 0.005 mg, doses down to and including 0.01 mg suppressed the development of the infection so that few or no parasites were observed in the 500 erythrocyte count; if present, they were abnormal in appearance and obviously greatly affected by the drug. Doses of 0.005 mg appeared to be without effect upon the growth rate or morphology of the parasites under the conditions of the tests. The minimum effective dose therefore appeared to be sharply defined. It was, however, later found that parasites were affected by 0.005 mg when exposed to such doses for a longer period than the test.

In attempting to produce resistance to this drug the strain was passaged at 3- or 4-day intervals through birds treated with 0.005 mg doses of the drug twice daily. In the third passage many of the parasites appeared to be affected by the drug; the cytoplasm was vacuolated, the pigment aggregated into relatively large clumps and the chromatin of the nuclei in the schizonts coalesced into large masses which stained deeply. However, by the fifth passage all the parasites appeared normal. The dose was raised to 0.01 mg when the parasites were again similarly affected by the drug and the intensity of infection was low; but by the eighth passage the parasites appeared to be normal and the growth rate had improved. The dose of drug was gradually raised, and with each increase there was a tem-

* Primaquine = 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline.
   Pamaquin = 8-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline.
   Mepacrine = 6-chloro-2-methoxy-9-(4-diethylamino-1-methylbutylamino) acridine.
   Chloroquine = 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline.
   Proguanil = $N_1$,-$p$(chlorophenyl)-$N_2$-isopropyl biguanide.
   Pyrimethamine = 2,4-diamino-5-$p$-chlorophenyl-6-ethylpyrimidine.
Porlary adverse effect upon the parasites, but finally, after a total of 4 months contact with the drug, the strain was maintained in birds treated with the maximum tolerated dose (0.1 mg). Although the parasites were normal in appearance, the growth rate was very low, and inocula as large as 1.0 ml. of heparinized blood were often necessary to keep the strain in a patent state. After 3 months in contact with this dose the growth rate had improved and the strain could be maintained by inocula of 0.1 ml or 0.15 ml.

Table 1. The development of resistance to primaquine in Plasmodium gallinaceum

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<tr>
<th>Expt.</th>
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* The parasite count is expressed as the number of parasitized erythrocytes per 500 erythrocytes.
† The dose is expressed in mg per 20 g of body weight of bird.
D = died before the parasite count was made on the fourth day after inoculation. G = untreated parent strain of P. gallinaceum. PrR = primaquine-resistant strain.

After a total of 7 months treatment with the drug in increasing doses, the sensitivity of the strain was tested against 0.01, 0.05 and 0.1 mg doses of the drug (Table 1, expt. 2). Whereas the infections produced in birds treated with 0.01 mg doses differed little in intensity from those produced in the untreated controls, those produced in birds treated with the higher doses were poor though the parasites were normal in appearance. Similar results were obtained after a further month’s treatment with 0.1 mg doses of the drug (Table 1, Expt. 3). The growth rate in birds treated with higher doses gradually improved over the period of many months during which the strain was passaged through birds treated with this dose, but it never attained that of the untreated controls (Table 1, Expts. 5, 6). Gametocytes were present in the drug-treated as in the untreated birds and pigment was present in the parasites.

(2) The stability of primaquine resistance

The primaquine-resistant strain was passed through Aedes aegypti at frequent intervals during the period that resistance was being developed and studied. The infected mosquitoes were allowed to bite a bird which thereafter was treated with twice-daily doses of drug similar to those to which the strain had previously been exposed, or an untreated bird whose blood, when the infection became patent, provided inocula for birds which received drug treatment. The incubation period
of the sporozoite-induced infections in the drug-treated birds was normal in duration and the parasites were normal in appearance, and no loss of resistance was observed in the infections produced by blood-inoculation from the sporozoite-infected but untreated birds. Since the first mosquito passage into drug-treated birds was made after the strain had been exposed to the drug for only 2 months and at a time when it was resistant to only 0.02 mg doses, it must be concluded that, even in its early stages, resistance was already sufficiently stable to survive cyclical development in the mosquito vector.

In order to discover whether loss of resistance occurred when the primaquine-resistant strain was maintained for a relatively long period in the absence of the drug, a strain was maintained for 41 weeks in a state of acute infection in untreated birds, by inoculation of heavily infected, heparinized blood. Some loss of resistance to the maximum tolerated dose (0.1 mg) was observed over the period, but not to the lower doses (Table 2).

Table 2. The stability of primaquine resistance in P. gallinaceum in the absence of the drug

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* The parasite count is expressed as the number of parasitized erythrocytes per 500 erythrocytes.
† The dose is expressed in mg per 20 g of body weight of bird.
D = died before the parasite count was made on the fourth day after inoculation. G = untreated parent strain of P. gallinaceum. PrR = primaquine-resistant strain.

(3) The sensitivity of the primaquine-resistant strain to other antimalarial drugs

The sensitivity of the primaquine-resistant strain was tested against pamaquin, an 8-aminoquinoline compound which differs from primaquine only in the substitution of ethyl groups in the terminal amine of the side chain. The primaquine resistant strain was cross-resistant to lower effective doses of pamaquin; it was also slightly less sensitive to the lower effective doses of quinine than the normal strain (Table 3). With the lowest doses of mepacrine and chloroquine tested the parasite counts in individual birds were very variable in both strains and no significant difference in sensitivity to mepacrine could be detected; although the primaquine-resistant strain was slightly less sensitive to the minimum effective dose (0·04) of chloroquine than the untreated strain the difference was only marginal.

The sensitivity of the primaquine-resistant strain to proguanil, dihydrotriazine and pyrimethamine was normal (Table 4).
Table 3. The sensitivity of a normal (strain G) and a primaquine-resistant (PrR) strain of *P. gallinaceum* to pamaquin, quinine, chloroquine and mepacrine

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* The parasite count is expressed as the number of parasitized erythrocytes per 500 erythrocytes.
† The dose is expressed in mg per 20 g of body weight of bird.
D = died before parasite count was made on the fourth day following inoculation.
(4) Attempts to produce resistance to chloroquine in Plasmodium gallinaceum

Whereas resistance to chloroquine has been produced relatively easily in *P. berghei*, attempts to produce it in *P. gallinaceum* have either failed (Seaton, 1951; Bishop & McConnachie, 1952a) or the degree of resistance attained has been slight (Ray, Sharma & Misra, 1956). In all three cases large numbers of parasites were passaged through young chicks which were treated with doses of drug sufficient to produce a significant but not complete suppression of parasitaemia. Although one strain was treated with the drug over a period of 54 weeks (Seaton, 1951) and the other for 16 months (Bishop & McConnachie, 1952a) no enhancement of resistance developed, and, in the third strain, the enhancement was only two-fold (Ray et al. 1956).

Table 4. The sensitivity of a normal (strain G) and a primaquine-resistant (PrR) strain of Plasmodium gallinaceum to proguanil, dihydrotriazine and pyrimethamine

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<tr>
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* The parasite count is expressed as the number of parasitized erythrocytes per 500 erythrocytes.
† The dose is expressed in mg per 20 g of body weight of bird.
D = died before the parasite count was made on the fourth day following inoculation.

Since Sautet, Aldighieri & Aldighieri (1959) had reported that resistance to chloroquine developed in *P. berghei* if the strain was treated with large doses of the drug for short periods of time, a further attempt was made to induce resistance to this drug in three substrains of *P. gallinaceum*, each substrain being treated with a different dose of the drug. Three substrains of strain A (p. 755), substrains of which had, in the past, been made resistant to a wide variety of antimalarial drugs, were maintained in young chicks by intravenous inoculation of 1·0 ml of heparinized blood. Three doses of chloroquine were given, the first two on the morning and evening of the day that the infection had attained an intensity in which at least 50% of the erythrocytes were infected, and the third on the following morning. The strains were passaged into normal birds 2 or 3 h after the
third dose had been given, and three doses were given to each recipient, as in the
previous passage, as soon as the infection had reached the required intensity. One
substrain was treated with 0.1 mg doses, the second with 0.5 mg doses and the third
with 1.0 mg doses. These doses all produced a marked effect upon the parasites
within the 26–27 h between the initial dose being given and the passage of the
strain into another bird; the cytoplasm was vacuolated and the nuclei condensed.
In many erythrocytes the only traces of parasites on the second day consisted
of deeply stained granules—presumably the remains of the parasite nuclei.

One substrain was treated with 0.1 mg doses of the drug over a period of 17
months, the second with 0.5 mg doses over a period of 13 months and the third
with 1.0 mg doses over a period of 15½ months, but no change in sensitivity to the
drug could be detected in any of the strains.

Many years ago it was shown that although attempts to produce resistance to
tartar emetic (antimony potassium tartrate) in trypanosomes by treatment with
that compound failed, resistance could be produced readily in an atoxyl-resistant
strain (Mesnil & Brimont, 1908; Yorke, Murgatroyd & Hawking, 1932), and these
observations have been confirmed more recently by Hawking (1966a) using a
tryparsamide-resistant strain of T. brucei. Resistance to chloroquine has been
described in infections in Plasmodium falciparum in individuals in Malaya who have
been undergoing prophylactic treatment with proguanil (Montgomery & Eyles,
1963; Contacos, Lunn & Coatney, 1963). Since resistance to proguanil was known
to occur in Malaya before resistance to chloroquine was encountered, the possi-
bility that resistance to chloroquine might develop more readily in strains already
resistant to proguanil seemed worth exploring. A strain of P. gallinaceum with
maximal resistance (twentyfold) to proguanil was therefore passaged through young
chicks by intravenous inoculation as in the previous experiments, and treated with
0.25 mg doses of chloroquine over a period of 3 months but no change in its
sensitivity to the drug could be detected.

**DISCUSSION**

As compared with resistance induced in *P. gallinaceum* by proguanil, and more
especially by metachloridine and pyrimethamine, resistance to primaquine was
slow in developing; moreover, it was a gradual process characterized by a slow
increase in growth rate in the presence of the drug; there was no evidence of a
sudden enhancement of resistance such as had been observed in some substrains of
*P. gallinaceum* treated with metachloridine, proguanil or pyrimethamine (Bishop,
1958, 1962). In these characteristics it resembled the development of resistance
to the related 8-aminoquinoline compound pamaquin (Bishop & McConnachie,
1952b). A similar gradual increase in growth rate over a prolonged period of time
was observed by Newton (1964) in a quinapyramine-resistant strain of Crithidia
oncopelti which had been made resistant by subculture in a peptone–glucose
medium in the presence of increasing concentrations of the drug. Whereas when
first maintained in a concentration of 300 μg/ml the mean generation time was
24 h as compared with 7-5 h for the untreated parent strain grown in the absence
of the drug, its growth rate increased progressively to a mean generation time of 11.5 h over a period of years during which it was maintained in the same concentration of the drug.

Resistance to primaquine in *P. gallinaceum* appears to be a relatively stable character; thus it was not lost during passage through mosquitoes even though the first of these passages was made after the strain had been treated with the drug for only 2 months and the enhancement of resistance was only twofold; moreover, although resistance to the maximum tolerated dose was diminished over a period of 41 weeks during which the strain was maintained in a state of acute infection in the absence of the drug, its resistance to lower doses was unchanged. In this it resembled the pamaquin-resistant strain of *P. gallinaceum* prepared by Bishop & McConnachie (1950a) more closely than a proguanil-resistant strain. Over a period of 6 months, during which it was maintained by passage through untreated chicks, the pamaquin-resistant strain was found to have lost its resistance to the maximal dose to which it had become resistant but not to lower doses, whereas the proguanil-resistant strain retained its resistance undiminished for more than one year in the absence of the drug. The stability of primaquine-resistance in *P. berghei* seems to be somewhat variable; thus Prakash and his colleagues (Prakash et al. 1961) found that resistance remained stable over a period of 5 months in the absence of the drug, whereas a definite decrease in resistance occurred in Peters’s strain over a period of 30 days (Peters, 1965a, 1966).

The cross-resistance pattern of the primaquine-resistant strain of *P. gallinaceum* to other antimalarial drugs differed in some important respects from that of the primaquine-resistant strain of *P. berghei* prepared by Peters. The two strains closely resembled one another in their sensitivity to mepacrine and chloroquine, no significant change to mepacrine being observed in either strain and the loss in sensitivity to chloroquine in *P. gallinaceum* being only marginal. Greater difference between the strains was observed in their sensitivity to quinine, only a slight decrease in sensitivity being observed in the primaquine-resistant strain of *P. gallinaceum*, whereas the *P. berghei* strain had become relatively resistant to this compound. It was, however, in their sensitivity to pyrimethamine and dihydrotriazine that the strains differed markedly; whereas the sensitivity of the *P. gallinaceum* strain to these compounds was unchanged, the *P. berghei* strain showed a marked enhancement of resistance to pyrimethamine and an almost complete resistance to dihydrotriazine. Unfortunately, the cross-resistance pattern of the primaquine-resistant strain of the Chesson strain of *P. vivax*, produced in human volunteers by Arnold and his colleagues (Arnold et al. 1961), was not studied, nor did Prakash and his co-workers study it in their primaquine-resistant strain of *P. berghei* (Prakash et al. 1961).

Although the resistance to dihydrotriazine exhibited by Peter’s primaquine-resistant strain of *P. berghei* was so pronounced, his dihydrotriazine-resistant strain showed only a slight enhancement of resistance to primaquine (Peters, 1965c).

The sensitivity of Bishop and McConnachie’s pamaquine-resistant strain of *P. gallinaceum* to proguanil, mepacrine and chloroquine was normal but its resistance
Primaquine resistance in *P. gallinaceum* 763
to quinine was enhanced. Its resistance to primaquine, pyrimethamine and
dihydrotriazine was not tested, but in that its sensitivity to proguanil was un-
changed its reaction to this latter compound would appear to have resembled the
primaquine-resistant strain of *P. gallinaceum* rather than Peters’s *P. berghei* strain
which was almost completely insensitive to dihydrotriazine, the active metabolite
of proguanil.

Since his primaquine-resistant strain of *P. berghei* was almost completely in-
sensitive to dihydrotriazine and markedly resistant to pyrimethamine and
diamino-diphenylsulphone (DDS), Peters (1966) suggested that primaquine has
more in common with the antimetabolite antimalarials pyrimethamine, dihydro-
triazine, and DDS than with the 4-aminooquinoline compound chloroquine or the
acridine compound mepacrine. The mechanisms by which primaquine exerts its
inhibitory action upon the malarial parasites are unknown, but the morphological
changes exhibited by parasites of *P. gallinaceum* in birds treated with primaquine
and such compounds as proguanil, sulphadiazine or DDS are quite different. In
birds treated with low but effective doses of primaquine (0.01 mg) the pigment
aggregates into relatively large clumps in both schizonts and gametocytes and the
cytoplasm becomes vacuolated. Later, the nuclei in schizonts appear to coalesce
and form masses which stain deeply with Giemsa’s stain, and the abnormal para-
sites, both asexual and gametocytes, become shrunken and degenerate. The
picture presented is entirely different from that of strains treated with proguanil,
sulphadiazine or DDS where the action of the drug is directed against the dividing
nuclei of the schizonts, and large parasites with a mass of undivided nuclear material
which stains feebly with Giemsa’s stain are frequently seen. In concentrations
which inhibit the development of the asexual parasites these drugs have little or no
apparent effect upon gametocytes; indeed, in strains treated with gradually in-
creasing doses of proguanil, sulphadiazine or 2,4-diamino-6,7-diisopropylpteridine
it has been observed that as the strains became adjusted to an increase in dosage
and the growth rate began to increase, the numbers of gametocytes produced were
much greater than in the normal untreated strain (Bishop, 1954). Although gameto-
cytes were present in the pamaquin-resistant strain of *P. gallinaceum* produced by
Bishop & McConnachie (1952b) and in the primaquine-resistant strain described
above they were never abnormally numerous.

*P. gallinaceum* and *P. berghei* differ markedly in their ability to become resistant
to chloroquine. With the exception of the twofold enhancement of resistance
obtained by Ray and his co-workers after 9 months treatment with the drug
(Ray *et al.* 1956), all attempts to produce resistance to chloroquine in *P. gallin-
aceum* have failed. Resistance to this drug has been obtained more readily in *P.
berghei*, but the period of treatment required for its development has varied. Thus
Ramakrishnan, Prakash & Choudhury (1957) obtained a 200-fold enhancement of
resistance over a period of 7 months treatment. Hawking & Gammage (1962)
obtained maximal resistance after 3-5 months treatment with the drug admini-
stered in the diet, in mice in which the phagocytic function had been suppressed
with ethyl palmitate, but Hawking (1966b) later found that other substrains of his
parent strain failed to become resistant to the drug or developed only a low-grade
resistance after prolonged treatment when the concentration of the drug in the diet was lower. Peters (1965b) obtained maximal resistance after 5 months treatment and Jacobs (1965) after less than 3 months treatment.

The chloroquine-resistant strains of *P. berghei* were unstable when maintained by blood inoculation in the absence of the drug. Thus Jacobs's strain had lost most of its resistance to chloroquine in eight to fifteen passages through untreated mice, depending upon the length of time the strain had previously been treated with the drug, whereas a pyrimethamine-resistant strain prepared by a similar method from the same parent strain proved to be stable whenever tested. Peters (1965b) reported that his chloroquine-resistant strain reverted to normal during ten sequential passages in the absence of the drug, but after further passages through drug-treated mice resistance became stable (Peters, 1966). Hawking (1966b) found that resistance to chloroquine was completely lost after the strain had been maintained for 4 months in the absence of the drug whereas a sulphanilamide-resistant strain retained its resistance to sulphanilamide for 5 months which was the maximal period it was studied.

The stability of drug resistance after mosquito passage has not been studied in *P. berghei*, but in experiments in human volunteers with the chloroquine-resistant strain of *P. falciparum* from Colombia, chloroquine resistance was found to persist after mosquito transmission (Young & Moore, 1961; Powell, Brewer & Alving, 1963). Indeed, if chloroquine resistance were not a stable character in *P. falciparum* there would be no problem in the field. Observations by DeGowin, Eppes, Carson & Powell (1966) upon experimentally induced infections of *P. falciparum* are also of interest in relation to the problem of the stability of chloroquine-resistance in human malaria. A chloroquine-resistant strain from Malaya (the Camp. strain) was passed sequentially through volunteers for more than one year during which no treatment with chloroquine or other 4-aminoquinoline compound, or mepacrine, was given though drugs such as quinine were administered to prevent dangerously high levels of parasitaemia. At the end of this period the response of the strain to 1500 mg of chloroquine over a period of three days was similar to that observed by DeGowin & Powell (1965) with the same strain one or two years previously. Although this chloroquine-resistant strain responded to quinine it has been shown by Jacobs (1965) that a quinine-resistant strain of *P. berghei* was resistant to normally suppressive doses of chloroquine, so in attempting to assess the stability of chloroquine-resistance in *P. falciparum* the possibility that in these experiments contact with quinine may have prevented the loss of resistance to chloroquine cannot be ignored, though the fact that no loss in sensitivity to quinine over the period is recorded—indeed quinine resistance has never been established in human malaria—makes this improbable.

The cross-resistance patterns of chloroquine-resistant strains of *P. berghei* described by Hawking & Gammage (1962), Jacobs (1965), Peters (1965b) and Macomber, O'Brien & Hahn (1966) resemble each other closely; thus all four strains were resistant to mepacrine, Jacobs's, Peter's and Macomber's strains were each resistant to quinine, and the two former strains were each resistant to amodiaquine and primaquine, compounds against which the other strains were not tested. The
Primaquine resistance in *P. gallinaceum*

strains produced by Hawking & Gammage, Jacobs and Peters were tested against pyrimethamine and were sensitive, and Peters’s strain, which was also tested against dihydrotriazine, proved to be sensitive to that compound also.

Some differences in sensitivity to other antimalarial compounds have been observed in strains of *P. falciparum* resistant to chloroquine which have been encountered in different geographical areas. The infections which occurred in Colombia and were described by Moore & Lanier (1961) responded to quinine, an observation which was confirmed by Young & Moore (1961) in experimentally induced infections. In further investigations carried out by Powell *et al.* (1963) in volunteers, the strain was found to be resistant to standard doses of chloroquine, hydroxychloroquine and amodiaquine—all 4-aminquinoline compounds—but radical cure could be effected by pyrimethamine as well as by quinine.

Chloroquine-resistant infections of *P. falciparum* encountered in Commonwealth Forces in Malaya by Montgomery & Eyles (1963) were highly resistant to proguanil, which was not surprising since they had ‘broken through’ intense prophylactic treatment with that drug. Like the Colombian strains they responded favourably to quinine. It was confirmed, in human volunteers, that one of these strains was resistant to chloroquine and proguanil, but proved susceptible to pyrimethamine. Two other chloroquine-resistant strains from Malaya, when studied in volunteers, were resistant to pyrimethamine and one of them responded to mepacrine whereas the other did not, but all three were susceptible to quinine. A strain from Cambodia was resistant to chloroquine, proguanil, mepacrine and pyrimethamine, but this was also susceptible to quinine (Contacos *et al.* 1963). One member of the party of six, from which this chloroquine-resistant strain had been derived, had taken Darachlor (chloroquine + pyrimethamine) prophylactically, though it was questioned whether prophylaxis had been continued sufficiently long to produce resistance, but a pyrimethamine salt project had been employed in this area of Cambodia and discontinued as it was suspected that resistance had developed (Eyles, Hoo, Warren & Sandosham, 1963).

Unless it can be clearly established that proguanil and/or pyrimethamine have not been used in areas from which chloroquine-resistant strains have been derived it is not possible to know whether the broad spectrum resistance to antimalarial compounds observed in some strains of *P. falciparum* is the result of chloroquine treatment *per se*, and is a true cross-resistance in the generally accepted meaning of the term, or whether chloroquine resistance has been superimposed upon a previously established resistance to proguanil and/or pyrimethamine. It must, however, be remembered that the three chloroquine-resistant strains of *P. berghei*, described by Hawking & Gammage (1962), Jacobs (1965) and Peters (1965b) were all sensitive to pyrimethamine, and Peters’s strain, which was tested against dihydrotriazine, was sensitive to that compound also. Only when his chloroquine-resistant strain had been made resistant to dihydrotriazine by treatment with that drug did the strain show an enhanced resistance to pyrimethamine (Peters, 1965d). Although all the chloroquine-resistant strains, whether derived from South America or from South East Asia, have proved susceptible to quinine, DeGowin & Powell (1965) have found some variation in susceptibility to this drug in strains.

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from South East Asia, though fortunately no strain approached absolute resistance.

Like resistance to chloroquine, resistance to quinine and mepacrine appears to be more easily obtained in *P. berghei* than in *P. gallinaceum* or, indeed, in monkey or human malaria. Differences in sensitivity to quinine have long been known to occur in different geographical strains of malaria parasites in man, thus James, Nicol & Shute (1932) found that, in neurosyphilitic patients, it required eight times as much of the drug to control the primary attack with their Roman and Sardinian strains of *P. falciparum* as with their Indian strain of that species, but no well-authenticated accounts of the development of resistance to the drug as a result of its therapeutic use exist, and mepacrine-resistance has only once been recorded—in *P. falciparum* infections in Aitaipe-Wewak in the Second World War (Fairley, 1946).

Attempts by Williamson & Lourie (1947) over a period of 2½ years to produce resistance to quinine and mepacrine in *P. gallinaceum* failed, but the doses used in these experiments were very large and attempts to produce resistance to sulphadiazine also failed though Bishop & McConnachie (1950b) succeeded when their strain was exposed to gradually increasing doses of the drug. Attempts to produce resistance to mepacrine by the same method over a period of 6 months, however, failed (Bishop & Birkett, 1948). Knoppers (1947, 1949) succeeded in producing a twofold enhancement of resistance to quinine in *P. gallinaceum* over a period of 26 weeks, but failed to increase it by further drug treatment, and attempts by Schmidt, Genther, Fradkin & Squires (1949), over a period of 2 years, to produce resistance to quinine or mepacrine in *P. cynomolgi* failed. In view of these failures Jacobs's (1965) success in producing resistance to the maximum tolerated dose of quinine in his strain of *P. berghei*, in seven quinine-treated passages through mice, over a period of 38 days, is surprising and stresses the ease with which drug resistance may be produced in this species of *Plasmodium* as compared with others including those of man. The degree of resistance obtained was of the order of three- to sevenfold. This quinine-resistant strain was also resistant to normally suppressive doses of chloroquine, amodiaquine, primaquine and mepacrine. The sensitivity of the strain to pyrimethamine was normal. Knoppers did not observe any cross-resistance to mepacrine (atabrine) or chloroquine in his quinine-resistant strain of *P. gallinaceum* but the enhancement of resistance to quinine was of a lower order than in *P. berghei*. Jacobs found that quinine resistance was more stable when the strain was maintained by blood inoculation in the absence of the drug than chloroquine resistance, and although Knoppers observed some loss of resistance to quinine after the first mosquito passage there was no further loss after subsequent passages.

Peters (1965a) developed a mepacrine-resistant strain of *P. berghei* by treatment with that drug. This strain was resistant to chloroquine and quinine and slightly resistant to primaquine, but was sensitive to pyrimethamine, dihydrotriazine and DDS and hypersensitive to sulphadiazine (Peters, 1966). Resistance to mepacrine was, however, very labile and was lost after the strain had been maintained in untreated mice for 20 days. It would be of interest to know whether resistance to
Primaquine resistance in *P. gallinaceum*

Mepacrine would become more stable if the strain was treated with the drug for a longer period.

Strains of *P. berghei* resistant to chloroquine, quinine and mepacrine were all characterized by the absence of pigment, which reappeared when resistance was lost in the absence of the drug. Peters, however, did not observe loss of pigment in his primaquine-resistant strain nor was this loss observed in the *P. gallinaceum* strain.

In comparing the development of resistance to antimalarials in different species of *Plasmodium* certain facts emerge. Thus, given suitable methods, resistance to compounds which affect the folic acid metabolic pathway of the parasite, e.g. proguanil, pyrimethamine and the p-aminobenzoic acid-inhibited sulphonamides, can be produced relatively readily, whereas, except in *P. berghei*, it is more difficult or impossible to produce resistance to quinoline compounds or the related acridine compound mepacrine. It must be remembered that although chloroquine-resistance has emerged in *P. falciparum* infections in certain parts of the world, the first cases were not encountered until 1959 (Moore & Lanier, 1961), although the drug was synthesized by German workers before the Second World War and was in use in the U.S. Army from 1945 onwards (Coatney, 1963), and has since been used on an increasing scale in all parts of the world. In comparison with the slow emergence of strains of *P. falciparum* resistant to chloroquine, resistance to proguanil was observed in infections with this species in Malaya in less than 2 years after the introduction of the drug (Edeson & Field, 1950).

A problem of fundamental interest appears to lie in the ability of *P. berghei* to become resistant to drugs to which other species either fail to become resistant or only do so after prolonged exposure to their action. The question arises of what relation the labile resistance to chloroquine, observed in this species, at least in the early stages of the development of resistance to that drug, bears to the development of resistance to chloroquine in *P. falciparum* in the field. Is the labile nature of chloroquine resistance in *P. berghei* a character peculiar to that species, or is it inherent in the mode of action of the compound and its method of producing resistance, and is this the reason why chloroquine resistance has taken so long to develop under natural conditions? The production of resistance in the laboratory, where large numbers of parasites are constantly subjected to drug pressure and passage is effected by syringe, is a very different matter from what must happen in the field where, from its inception, drug resistance must be stable through mosquito transmission if the character is not to be lost. In what features, then, does labile drug resistance, such as occurs in *P. berghei*, differ from the more stable form which is capable of persisting unchanged in the absence of drug pressure and through mosquito transmission? These are fundamental problems in drug resistance in malaria parasites which can only be solved by a knowledge of the biochemical and biophysical changes which occur as drug resistance develops.

A first step in the study of these problems has recently been made. It was shown by Schellenberg & Coatney (1961) that chloroquine inhibited the incorporation of $^{32}$P into both RNA and DNA in *P. gallinaceum* and *P. berghei*. More recently, Ciak & Hahn (1966) found that the drug inhibits DNA and RNA biosynthesis in
**Bacillus megaterium.** They suggested that susceptibility to chloroquine is based upon the capacity of susceptible cells to permit passage and accumulation of critical concentrations of the drug, whereas natural or acquired insusceptibility may be the result of impermeability or an impaired concentration mechanism. This hypothesis was supported by the observation that packed cells of susceptible *B. megaterium*, when exposed to chloroquine, contained ten times as much drug as did equal volumes of insensitive *B. cereus*. Working with chloroquine labelled with $^{14}$C in the quinoline ring, Macomber et al. (1966) have found a marked difference between the concentration of the drug in red blood corpuscles parasitized by a chloroquine-susceptible strain of *P. berghei* and those parasitized by a chloroquine-resistant strain, the concentration of the drug being two to three times higher in the red cells containing chloroquine-sensitive parasites than in those containing chloroquine-resistant parasites. They suggest that resistance to the drug is based on an impairment of the mechanism by which toxic levels of the drug are accumulated. The problem of the nature of the impairment and the mechanism by which it is produced awaits solution.

**SUMMARY**

A tenfold enhancement of resistance to primaquine was obtained by maintaining a strain of *Plasmodium gallinaceum* in a state of acute infection by serial passage of infected blood through young chicks treated with gradually increasing doses of the drug.

No loss in resistance to primaquine was observed when the resistant strain was transmitted through mosquitoes, though there was some loss in resistance to the maximum tolerated dose, but not to lower doses, when the strain was maintained in a state of acute infection through untreated chicks for 41 weeks.

The primaquine-resistant strain was cross-resistant to lower effective doses of pamaquin, and slightly less sensitive to quinine than the parent strain but the loss in sensitivity to chloroquine was only marginal. Sensitivity to proguanil, dihydrotriazine and pyrimethamine was normal.

Attempts were made to produce a chloroquine-resistant strain of *P. gallinaceum* using different doses of the drug but no change in sensitivity was observed though the experiments were continued for more than a year. An attempt to produce a chloroquine-resistant strain by treatment of a proguanil-resistant strain with chloroquine also failed.

The problem of resistance to quinoline compounds in different species of *Plasmodium* is discussed.

**REFERENCES**


