

## MODE OF ACTION OF CHLOROQUINE AND RELATED DRUGS

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*Two hypothesis to explain the mode of action of chloroquine as an antimalarial drug were examined in this review. The first hypothesis proposes that chloroquine acts by discharging the proton gradient of the acidic food vacuole of malaria parasites. The second hypothesis proposes that chloroquine delays the sequestration of ferriprotoporphyrin IX (FP) into malaria pigment and allows FP to exert its intrinsic cellular toxicity. Recent evidence indicates that therapeutic concentrations of chloroquine can discharge the proton gradient of food vacuoles, not because chloroquine acts as a weak base, but because it interacts with some specific component of the parasite. The second hypothesis is supported by evidence showing that (a) the affinity and molecular specificity of chloroquine accumulation in erythrocytes infected with parasites corresponds to the affinity and specificity of chloroquine binding to FP, (b) the release of FP from hemoglobin in normal erythrocytes causes these cells to behave as if they were infected with malaria parasites with respect to the affinity and specificity of chloroquine accumulation, and (c) FP and the chloroquine-FP complex are toxic for malaria parasites. It is possible that these two hypothesis eventually will collapse into a single hypothesis if the chloroquine-FP complex is shown to discharge the proton gradient of food vacuoles.*

*We also present new evidence that the FP of malaria pigment exists in an aggregated state as a coordination complex other than hemin or hematin, possibly as a complex with an organic acid such as a short-chain fatty acid. Accelerated sequestration of FP in this aggregated coordination complex could account for chloroquine resistance in malaria parasites since this form of FP is unavailable to bind chloroquine.*

Two hypothesis to explain the mode of action of chloroquine as an antimalarial drug are under active investigation. The first hypothesis proposes that chloroquine discharges the proton gradient of the acidic food vacuole of malaria parasites (Homewood et al., 1972; Krogstad & Schlesinger, 1986). Presumably, loss of the proton gradient would kill the parasite by interfering with the provision of nutrients, such as amino acids from hemoglobin digestion. The second hypothesis proposes that chloroquine delays the sequestration of ferriprotoporphyrin IX (FP) into malaria pigment and allows the FP to exert its intrinsic cellular toxicity (Banyal & Fitch, 1982; Fitch et al., 1984). If FP is released by hemoglobin digestion in the acidic food vacuole, either hypothesis would explain why the food vacuole is the apparent target for the antimalarial action of chloroquine. In fact, the two hypothesis may eventually collapse into one, since the interaction of chloroquine with FP could cause sufficient damage to the food vacuole to discharge the proton gradient.

**Effect of chloroquine on the food vacuole:** From a morphological standpoint, swelling of food vacuoles and pigment clumping due to coalescence of food vacuoles are the earliest changes induced by chloroquine (Macomber, Sprinz & Tousimis, 1967; Warhurst & Hockley, 1967). These changes led Homewood et al. in 1972 to propose that chloroquine, by virtue of being a weak base, discharges the proton gradient of food vacuoles of malaria parasites. Subsequently this hypothesis was thought to be untenable by the same authors (Warhurst et al., 1972) because it would not explain the molecular specificity of pigment clumping by antimalarial drugs. Their new position received support recently when a reexamination of the hypothesis by Yayon, Cabantchik & Ginsburg (1985) disclosed no evidence that therapeutic concentrations of chloroquine affect the proton gradient of food vacuoles.

Still more recently, however, Krogstad, Schlesinger & Gluzman (1985) reported that therapeutic concentrations of chloroquine do discharge the proton gradient of food vacuoles of malaria parasites, but they noted that effective concentrations are too low for chloroquine to be functioning as a weak base. Although these two groups of investigators asked the same question and came up with two different answers, both agree with the earlier conclusion of Warhurst et al.

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(1972) that chloroquine in concentrations effective against malaria does not act as a weak base to discharge the proton gradient of the food vacuole. If the gradient is discharged, it is an event secondary to an interaction of chloroquine with a component of malaria parasites other than protons. It is appropriate, therefore, to consider the possibility that some component of malaria parasites functions as a specific receptor to mediate the chemotherapeutic effect to chloroquine.

**Do malaria parasites possess a specific receptor for chloroquine?** Evidence for a specific chloroquine receptor in malaria parasites began to accumulate in 1966 when Macomber, O'Brien & Hahn reported that parasitized erythrocytes have the capacity to accumulate much more chloroquine than nonparasitized erythrocytes. Moreover, erythrocytes parasitized with chloroquine-susceptible *P. berghei* accumulated much more chloroquine than those parasitized with chloroquine resistant *P. berghei*. Similar observations subsequently were made in studies of *P. falciparum* (Fitch, 1970; Verdier et al., 1985). Detailed characterization of the process of chloroquine accumulation by erythrocytes infected with malaria parasites has shown it to be saturable (Fitch, 1969; Diribe & Warhurst, 1985) to have high affinity (Fitch, 1969; Diribe & Warhurst, 1985) for chloroquine ( $K_{diss}$  approximately  $10^{-8}$  M), and to have molecular specificity for antimalarial drugs in the same chemotherapeutic class as chloroquine (Fitch, 1972; Fitch et al., 1974). Moreover, drugs that compete with chloroquine accumulation by this specific process are the same drugs to which chloroquine-resistant malaria parasites exhibit cross resistance, and the strength of the competition correlates with the degree of cross resistance (Fitch, 1972; Fitch et al., 1974). All of these results support the existence of a specific receptor for chloroquine in chloroquine-susceptible malaria parasites. This receptor apparently either is deficient or inaccessible in chloroquine-resistant malaria parasites.

The existence of a specific chloroquine receptor also is supported by the work of Warhurst (1973) who followed a different line of experimentation. He evaluated the ability of various anti-malarial drugs to inhibit chloroquine-induced pigment clumping. The specificity of this process corresponded closely to the specificity of chloroquine accumulation, the process was saturable and the apparent affinity for chloroquine was approximately the same as that of the process of chloroquine accumulation. From these observations, Warhurst (1973) suggested that the processes of chloroquine accumulation and of pigment clumping may share a common receptor.

The existence of a specific receptor for chloroquine is now commonly accepted, although Yayan, Cabantchik & Ginsburg (1984) recently argued that chloroquine accumulation by malaria parasites can be accounted for entirely by proton gradients across food vacuoles. Unfortunately, these investigators used quantities of chloroquine large enough to obscure the specific, high-affinity process of chloroquine accumulation, they produced no evidence that proton gradients are smaller across food vacuoles of chloroquine-resistant malaria parasites as would be necessary to account for reduced chloroquine accumulation, and they ignored the fact that the process of chloroquine accumulation has significant molecular specificity. Thus, while there is strong evidence in favor of a drug receptor, there is as yet no evidence for involvement of proton gradients in the specific, high-affinity process of chloroquine accumulation by malaria parasites.

**Ferriprotoporphyrin IX (FP) fulfills the criteria for identification as the chloroquine receptor of malaria parasites:** Hemoglobin digestion is an absolute but not necessarily sufficient precondition for chloroquine susceptibility of malaria parasites. The evidence supporting this assertion is presented in detail in a recent review (McChesney & Fitch, 1984). Briefly, (a) chloroquine and other drugs in the same chemotherapeutic class are effective only as blood schizontocidal agents and (b) malaria parasites which either do not produce pigment or cease to produce it when exposed to the drug are invariably chloroquine resistant. Because of this knowledge, and since hemoglobin digestion by malaria parasites releases FP for storage in pigment in food vacuoles, and since FP is well-known to bind nitrogenous bases including chloroquine, it was reasonable for Macomber, Sprinz & Tousimis (1967) to suggest in 1967 that FP may be a specific receptor for chloroquine.

The suggestion of Macomber, Sprinz & Tousimis (1967) now has extensive experimental support. The binding of chloroquine to pure FP is saturable and has the affinity (Chou, Chevli & Fitch, 1980; Moreau, Charet & Prensier, 1986) and molecular specificity (Chou, Chevli & Fitch, 1980; Moreau, Charet & Prensier, 1986) predicted for the chloroquine receptor. When hemoglobin is experimentally denatured intracellularly in normal erythrocytes, FP is released and is available to bind chloroquine with high affinity (Fitch et al., 1983; Janney, Joist & Fitch, 1986). And similarly, when hemoglobin is digested with proteolytic enzymes, FP is released and is available to bind chloroquine with high affinity (Chou, Chevli & Fitch, 1980). Thus, when FP is added to normal erythrocytes, or when normal erythrocytes are treated with proteolytic enzymes or with agents (such as menadione) which denature hemoglobin intracellularly, the chloroquine binding



characteristics of erythrocytes infected with malaria parasites are duplicated (Fitch et al., 1983; Fitch, Chevli & Gonzalez, 1974). We conclude from these results that FP fulfills the criteria for identification as the specific receptor for chloroquine in erythrocytes infected with malaria parasites.

**Does ferriprotoporphyrin IX mediate the chemotherapeutic effect of chloroquine?** To help answer this question, experiments were first designed to determine whether or not FP and the chloroquine-FP complex are toxic to cells. Erythrocytes were chosen as the model system and were readily found to be lysed by FP and its complex with chloroquine (Chou & Fitch, 1980; Dutta & Fitch, 1983). Both interact with the erythrocyte membrane (Fitch et al., 1984; Yayon et al., 1984) apparently in much the same way as other amphipathic agents (Fitch et al., 1984), causing a massive potassium efflux, swelling of the erythrocytes and finally lysis (Chou & Fitch, 1981; Dutta & Fitch, 1983). In more limited studies we found that both FP and its complex with chloroquine similarly are toxic for the malaria parasites, *P. berghei* (Orjih et al., 1981) and *P. falciparum* (Fitch et al., 1982). The toxicity of FP can be reduced by serum albumin (Banyal & Fitch, 1982; Løvstad, 1986) or by erythrocyte or parasite cytosol (Banyal & Fitch, 1982), presumably in part because of FP binding, and this reduction can be experimentally reversed by chloroquine. Such enhancement of toxicity by chloroquine when FP exists in a complex biological milieu has been demonstrated both for extracellular and for intracellular FP (Banyal & Fitch, 1982; Fitch et al., 1983). The mechanism for enhancement of toxicity has not been studied in much detail, but chloroquine is known to favor the partition of intracellular FP into cellular membranes (Fitch et al., 1984). Thus, FP not only fulfills the criteria for identification as the specific receptor for chloroquine in malaria parasites, it also has direct toxicity for malaria parasites and, under complex biological conditions, chloroquine can enhance the intrinsic toxicity of FP. We conclude that FP is both the receptor with high-affinity for chloroquine in malaria parasites and the mediator of the antimalarial action of chloroquine.

Precisely how the interaction of chloroquine with FP kills malaria parasites remains to be established. For example, there may be widespread damage to parasite membranes or there may be only localized damage to the food vacuolar membrane, possibly discharging the proton gradient and thereby impairing hemoglobin digestion and the provision of nutrients for the parasite. FP and the chloroquine-FP complex also have the potential for interrupting hemoglobin degradation by directly inhibiting the low molecular weight protease of malaria parasites (Vander Jagt, Hunsaker & Campos, 1986). In any case, we have proposed that chloroquine acts as an antimalarial drug by delaying the sequestration of FP into malaria pigment and allowing FP to exert its intrinsic cellular toxicity.

**Mechanism of resistance to chloroquine:** The form of FP accessible to chloroquine apparently exists only transiently in malaria parasites during hemoglobin digestion. It rapidly disappears when experimental conditions are used that would be expected to interrupt hemoglobin digestion, i.e. omitting metabolizable substrate (Diribe & Warhurst, 1985; Fitch et al., 1974) or adding ammonia to alkalize the food vacuole (Yayon, Cabantchik & Ginsburg, 1985). If our hypothesis is correct, chloroquine susceptibility (or conversely resistance) would be determined by the availability of the transient form of FP. If it were available, the parasite would be chloroquine-susceptible; if it were deficient, the parasite would be chloroquine-resistant. A deficiency could result (a) from an inherent inability of parasites to produce FP from hemoglobin, as may occur in rodent malarias which fail to produce malaria pigment (Fitch, 1983; McChesney & Fitch, 1984) or (b) from superior ability to bind FP in an inaccessible form or otherwise to sequester it in malaria pigment (Fitch, 1983; McChesney & Fitch, 1984). We favor the latter possibility as the most probable explanation for chloroquine resistance in malaria parasites that continue to digest hemoglobin during exposure to chloroquine, as may be the case for *P. falciparum*.

**Sequestration of FP in malaria pigment:** With one notable exception, there is general agreement that malaria pigment contains large amounts of FP. The exception was published in 1984 by Yayon et al., who based their conclusions on the results of Mössbauer effect spectroscopy of erythrocytes infected either with chloroquine-susceptible or with chloroquine-resistant *P. berghei*. They conclude that most of the iron in malaria pigment exists in a form other than porphyrin iron and that there is no difference in the total amounts of malaria pigment produced by chloroquine-resistant and chloroquine-susceptible malaria parasites. Unfortunately, these investigators studied the *P. berghei* NYU-2 strain in rats, in which only reticulocytes are infected (Ladda & Sprinz, 1969). Consequently, even the chloroquine-susceptible line of this strain produces little or no visible pigment in this host. Indeed *P. berghei* NYU-2 is chloroquine-resistant in the rat (Ladda & Sprinz, 1969). Therefore, caution is in order when trying to evaluate the significance of a lack of a difference between the two strains. Furthermore, it is improbable that the Mössbauer effect spectroscopy was measuring malaria pigment in the first place. Yayon and associates de-

monstrated only that the spectrum of interest to them was different from the spectrum of hemoglobin iron. They provided no comparison with authentic pigment iron.

Our studies of malaria pigment in mouse erythrocytes infected either with the chloroquine-susceptible or with the chloroquine-resistant line of *P. berghei* are in agreement with previous studies of others (Fulton & Rimington, 1953; Balasubramanian, Mohan Rao & Panijpan, 1984) and are at variance with the conclusions of Yayon et al. (1984). In mouse erythrocytes, the chloroquine-susceptible line produces abundant amounts of visible pigment and the chloroquine-resistant line produces little or none (Ladda & Sprinz, 1969). Moreover the iron in malaria pigment can be accounted for by the presence of FP. With a parasitemia of approximately one parasite per two erythrocytes, we found  $1.02 \pm 0.33$  (mean  $\pm$  S.D.)  $\mu$ moles of pigment FP per ml of packed erythrocytes for 10 different preparations of erythrocytes infected with chloroquine-susceptible *P. berghei* NYU-2. The pigment was isolated by density gradient centrifugation and analyzed by polyacrylamide gel electrophoresis to ensure that it was not significantly contaminated with hemoglobin. FP was quantitated by measuring absorbance at 404nm after dissolving the pigment in water with the aid of 2.5% SDS in 0.05N NaOH. These values are greater than previously thought (Fitch et al., 1984), because in the earlier work, the aggregated FP of malaria pigment was not treated with NaOH to convert it to monomeric FP prior to measuring absorbance at 404nm.

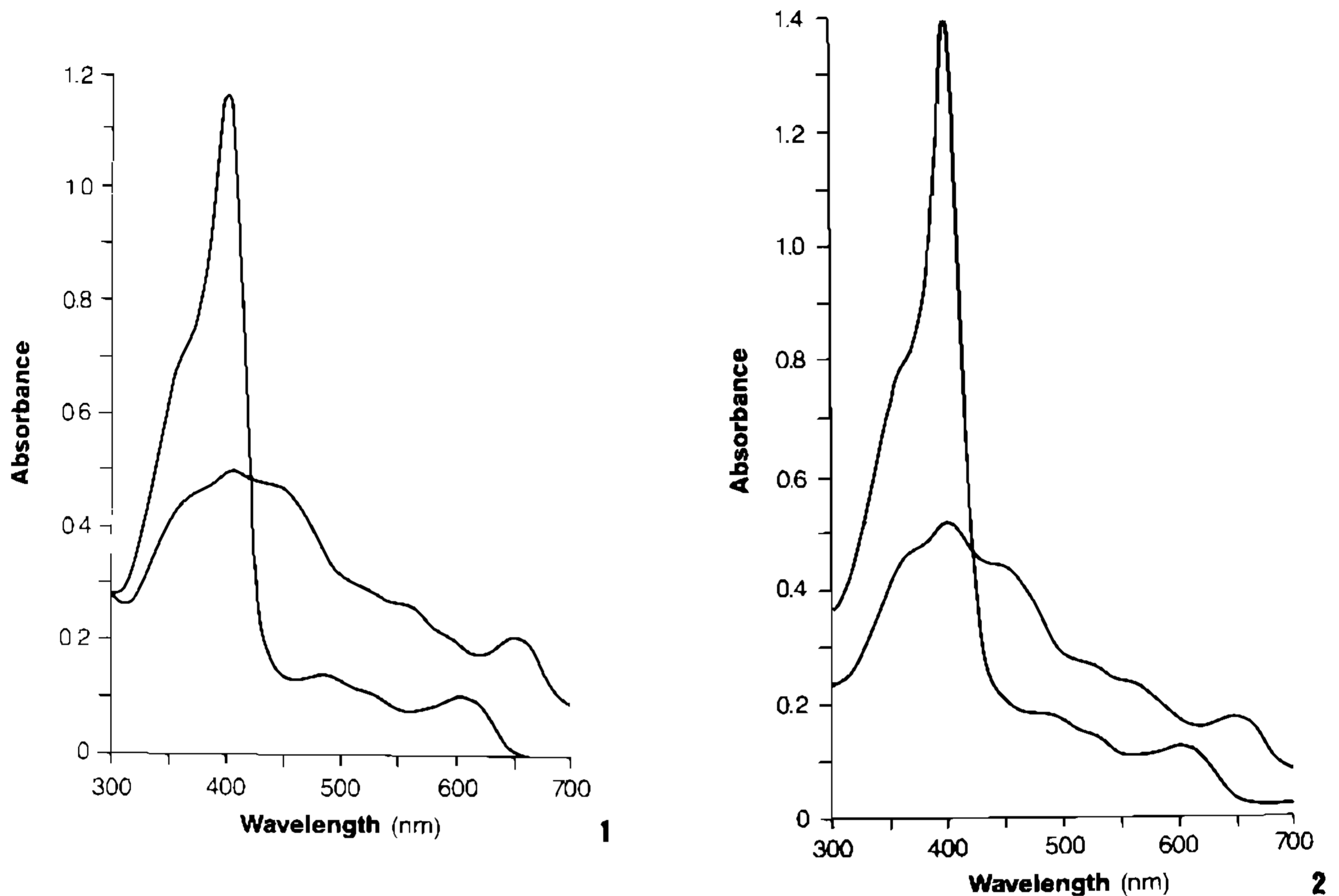
Although it is easy to demonstrate low-affinity interactions of FP with cytosolic preparations from malaria parasites (Banyal & Fitch, 1982), a thorough search by our laboratory for the putative protein with high affinity for FP (Fitch, 1983) has been unsuccessful. In the absence of a high affinity protein to account for the sequestration of FP, we decided to purify the FP of malaria pigment to determine whether it exists as hemin, hematin, or some other coordination complex. Absorption spectra from a partially purified pigment preparation are shown in Fig. 1. In the absence of NaOH the spectrum is similar to the spectrum reported by Belasubramanian, Mohan Rao & Panijpan (1984) for lyophilized preparations of whole parasites of chloroquine-susceptible *P. chabaudi* and is typical of aggregated hemin. After addition of 0.05N NaOH, the spectrum of monomeric FP appears.

One other characteristic of purified pigment preparations is a failure to bind chloroquine. However, after the FP in an aqueous suspension of the preparation was treated with NaOH and then neutralized with HCl, equilibrium dialysis studies demonstrated high-affinity chloroquine binding of the same order of magnitude as for pure hematin. For example, a purified preparation of pigment bound only 0.004 nanomoles of chloroquine per nanomole of FP at a concentration of 2  $\mu$ M chloroquine in the medium. Under identical conditions except for treatment of the pigment with 0.02N NaOH the same preparation of pigment bound 0.32 nanomoles of chloroquine per nanomole of FP. This result is in agreement with a previous report by Jearnpipatkul et al. (1980).

To further purify the FP, we treated the partially purified pigment with pronase to remove contaminating protein and with chloroform-methanol to remove lipid. These treatments have no effect on the absorption spectrum of the preparation (Fig. 1) and they do not cause the preparation to bind chloroquine. Clearly it is possible to purify the FP of malaria pigment extensively without disturbing its aggregation state. Such purified preparations are reproducibly 88% FP by weight.

In other studies we found that the behavior of FP purified from malaria pigment differs from that of hemin and hematin when suspended in 2.5% SDS. Both of the latter compounds develop the spectrum typical of monomeric FP in SDS whereas the FP purified from malaria pigment retains the spectrum typical of aggregated FP (Fig. 1). To obtain additional information about the FP purified from malaria pigment, a sample was submitted to Galbraith Laboratories, Inc. of Knoxville, Tennessee for elemental analysis. The results, shown as the values found compared with the percent calculated for hemin in parentheses, were as follows: carbon 62.9% (62.4), nitrogen 9.0% (8.6), oxygen 16.0% (9.8), and chlorine 0.45% (5.4). From these results we conclude that the FP of malaria pigment does not exist as hemin or hematin. We suspect that it exists as a coordination complex with one or more organic acids, such as shortchain fatty acids or amino acids.

To determine how closely a coordination complex of FP with a shortchain fatty acid would resemble the FP of malaria pigment, we treated human erythrocytes with propionic acid, collected the FP aggregates, digested the aggregates with pronase and extracted them with chloroform-methanol. This preparation behaved identically to the FP of malaria pigment with respect to spectral characteristics when suspended in 2.5% SDS and to chloroquine binding. The absorption spectrum (Fig. 2) retains the features characteristic of aggregated FP, which is in contrast to hemin and hematin and similar to the behavior of FP from malaria pigment. After addition of NaOH the



**Fig. 1: Spectra of FP in malaria pigment.** The preparation was suspended in 2.5% SDS in the presence or absence of 0.05N NaOH. The spectrum with the peak at 645-650nm and the low peak at 400nm was obtained in the absence of NaOH. **Fig. 2: Spectra of FP aggregates prepared by treating human erythrocytes with propionic acid.** The preparation was suspended in 2.5% SDS in the presence or absence of 0.05N NaOH. The spectrum with the peak at 645-650nm and the broad low peak at 400nm was obtained in the absence of NaOH.

typical spectrum of monomeric FP is obtained (Fig. 2). This preparation, like the FP of malaria pigment, also did not bind chloroquine with high-affinity but, as expected, did bind chloroquine with high-affinity after treatment with NaOH. The fact that this preparation of FP duplicates the characteristics of FP purified from malaria pigment supports the possibility that malaria pigment essentially consists of aggregations of one or more coordination complexes of FP with organic acids. This possibility merits experimental verification because accelerated formation of such a complex could confer chloroquine resistance on a parasite by causing a deficiency of the transient form of FP which interacts with chloroquine.

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