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# Hemoglobin catabolism and the killing of intraerythrocytic *Plasmodium falciparum* by chloroquine

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*Abstract.* To evaluate how chloroquine kills malaria parasites, hemoglobin catabolism was studied at the various stages of intraerythrocytic parasite development. We found that hemoglobin catabolism is switched off when *Plasmodium falciparum* parasites mature to the late trophozoite or early schizont stages and is switched on again during the ring stage. When hemoglobin catabolism is switched off, the parasites are resistant to the morphologic effects of chloroquine. Although the ring stage parasites failed to mature in the presence of chloroquine, some of them switched on hemoglobin ingestion and became stuffed with hemoglobin-filled vesicles, indicating a distal block in catabolism. In fact, we demonstrated a high-grade block in hemozoin production during a 22 h incubation of synchronized ring forms; ferriprotoporphyrin IX (FP) incorporation into the $\beta$-hematin of hemozoin decreased from 900 to 50 pmol/$10^6$ parasitized erythrocytes. We propose that the primary effect of chloroquine on hemoglobin catabolism is to block FP polymerization to $\beta$-hematin. Secondarily, toxic FP and FP-chloroquine complexes accumulate and are available to exert their several toxicities, which include inhibition of hemoglobin-degrading proteases and membrane damage. As a consequence, maturation is arrested and eventually the parasites die and lyse.
*Key words.* Antimalarial drugs; heme; hemozoin; malaria; erythrocytes.

A fundamental characteristic of *Plasmodium falciparum* parasites is their ability to grow and multiply in the hemoglobin-rich environment of mature erythrocytes. To survive, they obtain nutrients from their host cells, including especially amino acids, which they obtain in part by catabolizing hemoglobin[1]. Three processes are involved in hemoglobin catabolism: 1) ingestion of host cytosol into food vacuoles[2], 2) subsequent digestion of hemoglobin[3] and presumably other substances with the release of ferriprotoporphyrin IX (oxidized heme, FP), amino acids, and probably other metabolites, and 3) finally, polymerization of FP to $\beta$-hematin[4,5] with the production of hemozoin.

Recently, we reported that exposure to chloroquine reduces hemozoin production by chloroquine-susceptible *P. falciparum*[6]. To understand more completely how chloroquine kills malaria parasites, we turned next to studies of hemoglobin catabolism at the various stages of parasite development. The results of these studies, which are presented here, provide new information about normal hemoglobin catabolism by *P. falciparum* and about stage-specific effects of chloroquine.

## *Materials and methods*

The malaria parasite used throughout these studies was the chloroquine-susceptible HB-3 strain of *P. falciparum*, the initial inoculum of which was supplied by D. E. Goldberg of Washington University, St. Louis, Missouri, USA. The parasites were grown in vitro in erythrocytic cultures using erythrocytes and serum from O-positive human donors as described by Jensen and Trager[7] with slight modifications[6]. Every experiment was conducted with erythrocytes containing parasites which had been synchronized at the ring stage by sorbital lysis[8] at the beginning of the experiment and washed to remove hemozoin debris as previously described[6]. In addition, the parasites were maintained in a synchronous state by sorbitol lysis once or twice each week.

For measurement of hemozoin production or chloroquine accumulation after 22 h or more of incubation, the parasites first were grown in 24-well flat bottom tissue culture plates. Each well contained 0.5 ml of the cell suspension. At 22 h, the cell cultures were pooled in sterile plastic tubes and centrifuged at $530 \times g$ for 10 min, after which the supernatant fluid was discarded and the pellets were resuspended in fresh medium with or without chloroquine. The final suspensions contained 2 to 8 $\times 10^8$ erythrocytes/ml. Two ml aliquots of these suspensions were then transferred to sterile high-speed Oak Ridge centrifuge tubes with screw caps and were either used immediately for hemozoin measurement[6] and studies of chloroquine accumulation or cultured further at 37 °C until the required parasite age was reached. For studies at 0 and 12 h, 2 ml aliquots of parasitized erythrocytes were cultured in Oak Ridge tubes without first incubating in the 24-well tissue culture plates.

To measure chloroquine accumulation, ring-labelled [$^{14}$C]-chloroquine (specific activity of 2.36 mCi per mmole, purchased from New England Nuclear Corporation, Boston, Massachusetts, USA) was added to a suspension of parasitized erythrocytes, after which the suspension was incubated at 37 °C for 60 min. In every case, these suspensions were prepared in fresh medium immediately prior to addition of [$^{14}$C]-chloroquine to ensure that the pH was adequately maintained. At the end of incubation, the suspensions were centrifuged at 27,000 × *g* at 4 °C for 30 min and the supernatant fluid was separated from the pellet. Both supernatant fluid and pellet were assayed radiochemically for chloroquine[9] using Opti-Fluor-O scintillation cocktail (Packard Instrument Co., Inc., Meriden, Connecticut, USA). For electron microscopy, parasitized erythrocytes were fixed for 16 h at 4 °C with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 3% sucrose and 2 mM calcium chloride. They were then washed 2 × 5 min in cacodylate buffer containing 5% sucrose, postfixed for 6 h with 1% osmium tetroxide in cacodylate buffer containing 2% sucrose, and washed 2 × 5 min with distilled water, all at 4 °C. At each step, the erythrocytes were resuspended, mixed on an aliquot mixer and collected by brief centrifugation prior to changing solutions; after this point they were processed as pellets. The pellets were stained en bloc with 2.5% aqueous uranyl acetate for 16 h at 4 °C, and then washed 2 × 10 min with distilled water at room temperature. Next, they were dehydrated 2 × 5 min each with 35%, 50%, 70%, 95% and 100% ethanol and 2 × 10 min with propylene oxide, infiltrated for 3 h with a 50:50 mixture of Polybed (Polysciences, Inc., Warrington, Pennsylvania, USA) and propylene oxide and then with 100% Polybed for 3 h. Finally, the samples were embedded in fresh Polybed and polymerized for 24 h at 70 °C. Sections were cut from trimmed tissue blocks with a diamond knife on a LKB Ultracut E ultramicrotome, collected on copper grids and post-stained with uranyl acetate and lead citrate. They were viewed and photographed with a JEOL 100 CX electron microscope operated at 60 kV.

*Results*

Despite frequent synchronization, an overlap in distribution of trophozoite and schizont stages occurred in these cultures (fig. 1). Apparently, there were always a few hours difference in the ages of individual ring stage parasites after synchronization. Nevertheless, the total amount of FP in hemozoin at 0 h was low: 70 ± 7 pmol/$10^6$ parasitized erythrocytes (mean ± SE of 22 experiments). Younger ring forms contained little or no hemozoin (fig. 5A).

Starting from a low baseline, hemozoin production proceeded slowly during the ring stage before accelerating rapidly as the rings matured to trophozoites between 12 and 22 h (fig. 2). After 22 h, when schizonts became prevalent, hemozoin production decreased. The rate of hemozoin production was minimal by the 28th h. At the trophozoite stage (22 h), the maximal rate of incorporation of FP into hemozoin was in excess of 50 pmol/h/$10^6$ parasitized erythrocytes.

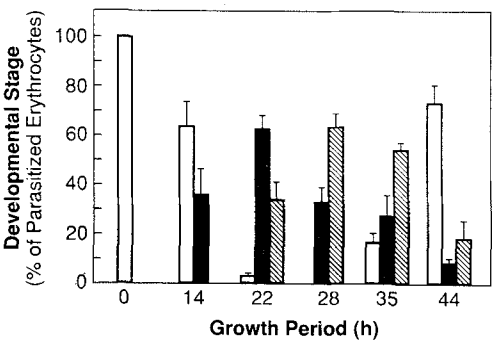


Figure 1. Growth of *P. falciparum* in cultures. Parasites synchronized at the ring stage (0 h) were permitted to grow under normal culture conditions. The means ± SE for 6 experiments are given for ring forms (open bars), trophozoites (solid bars) and schizonts (striped bars). The parasitemias ranged from 1 to 9 percent.

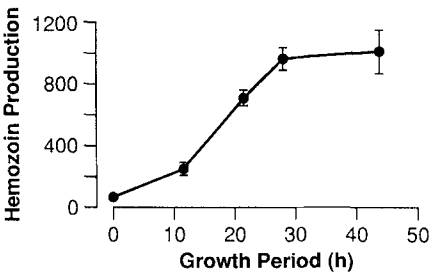


Figure 2. Hemozoin production by *P. falciparum*. Parasites synchronized at the ring stage (0 h) were permitted to grow under normal culture conditions. The pmols of FP of hemozoin in $10^6$ parasitized erythrocytes were measured at various time intervals. The mean ± SE for 6 experiments is shown at each time interval, except at 44 h for which the results of 3 experiments are shown.

Figure 3 demonstrates that chloroquine accumulation correlates positively with hemozoin production. The ring stage parasites achieved a distribution ratio of approximately 300 (ratio of chloroquine in $10^{13}$ parasitized erythrocytes to chloroquine in one liter of medium), which is large relative to the value for uninfected control erythrocytes of 4.6 ± 0.2 (mean ± SE of 10 experiments), but small relative to the value for trophozoites which was over 1800 at 22 h. As the parasites matured past the trophozoite stage, when hemozoin production was minimal, the distribution ratio fell precipitously back to the initial value for ring stage parasites.

Exposure to 25 ng/ml of chloroquine, beginning at the ring stage, virtually eliminated hemozoin production by parasitized erythrocytes (fig. 4). When chloroquine was removed from the incubation medium at 22 h, hemozoin production showed some recovery during the next

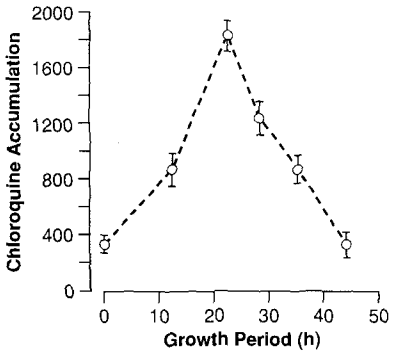


Figure 3. Chloroquine accumulation by *P. falciparum*. Parasites synchronized at the ring stage (0 h) were permitted to grow under normal culture conditions. At selected time intervals, aliquots of the culture were removed and incubated for 60 min at 37 °C in growth medium initially containing 106 nM [$^{14}$C]-chloroquine for measurement of chloroquine accumulation, which is expressed as the ratio of the nanomoles of chloroquine in $10^{13}$ erythrocytes to the concentration (nmole/liter) of chloroquine in the medium; $10^{13}$ erythrocytes represent a volume of approximately one liter. The chloroquine concentration in the medium decreased during incubation, reaching a low value of 25 nM for incubations of erythrocytes infected predominantly with trophozoites. The mean ± SE for 5 to 7 experiments is shown at each growth interval.

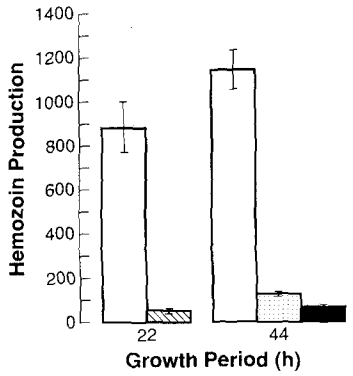


Figure 4. Effect of chloroquine on hemozoin production. The parasites were synchronized at the ring stage (0 h). They were then allowed to grow in normal growth medium with or without the addition of 25 ng/ml of chloroquine. A 22 h chloroquine was removed from the medium of some of the cultures. Incubation without chloroquine, open bars; incubation with chloroquine for 22 h only, striped bar; incubation with chloroquine for the first 22 h and without chloroquine for the next 22 h, stippled bar; incubation with chloroquine for 44 h, solid bar. The mean ± SE for 5 experiments for each group is shown.

22 h, but microscopic examination of the parasites revealed that they did not mature during this period. Some of them survived, however, and began to mature and multiply after another 3 d in culture. If chloroquine was not removed at 22 h, hemozoin production did not recover (fig. 4) and many parasites died and lysed during the next 22 h causing a decrease in parasitemia ranging from 50% to 90% in 7 experiments. These observations are clear evidence that the block of FP polymerization by chloroquine precedes parasite death.

The ultrastructure of malaria parasites cultured with and without chloroquine is shown in figures 5 and 6. In the absence of chloroquine, parasites cultured for 24 h matured from the ring stage (fig. 5A) to healthy trophozoites containing 3 or fewer hemoglobin-filled vesicles per section profile (fig. 5B). With exposure to 25 ng/ml of chloroquine for 24 h, some of the parasites appeared not to have matured beyond the ring stage before they died (fig. 6A). Others grew and accumulated numerous undigested hemoglobin-filled vesicles (fig. 6B). Large

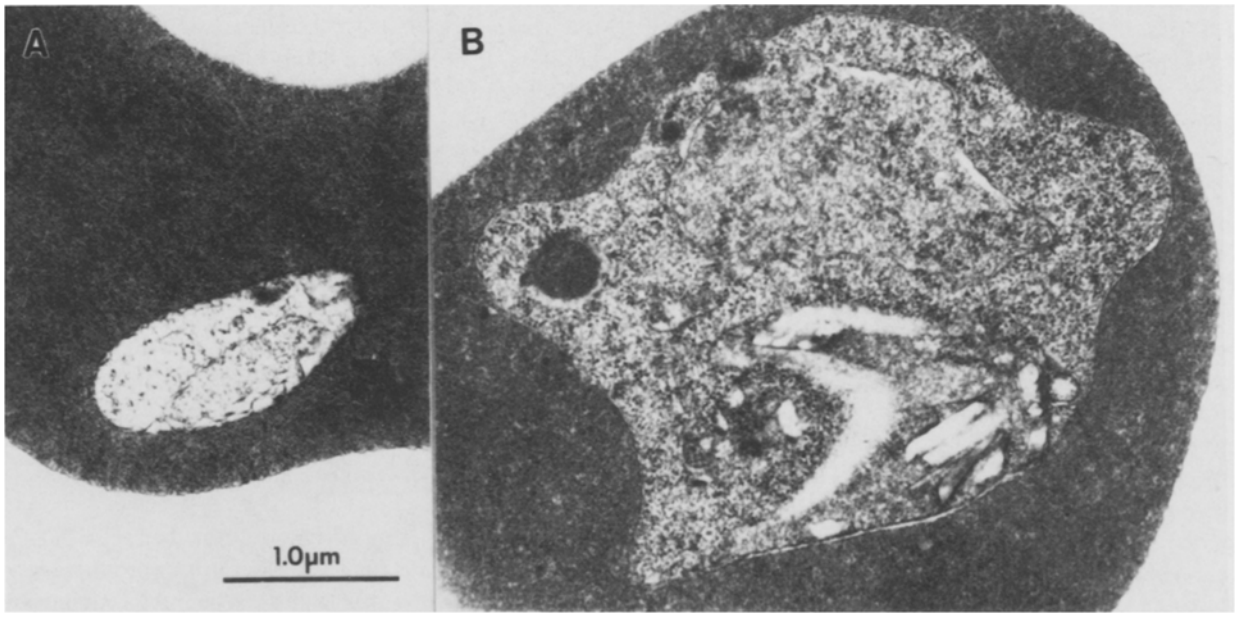


Figure 5. Ultrastructure of *P. falciparum* not exposed to chloroquine. *A* A young parasite (ring stage) at the time of synchronization with sorbitol. *B* At 24 h after synchronization most of the parasites are at the trophozoite stage of development.

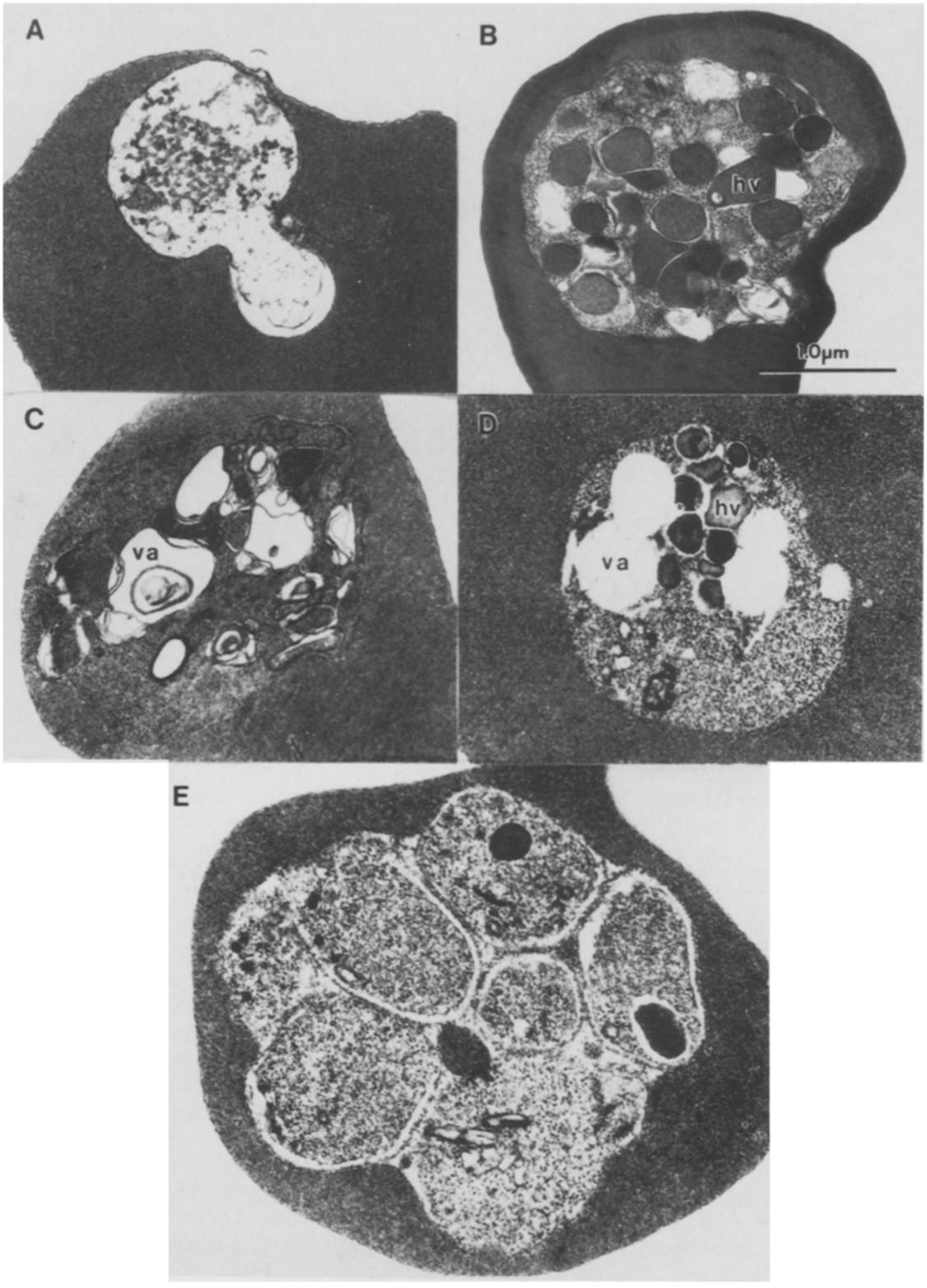


Figure 6. Ultrastructure of chloroquine-susceptible *P. falciparum* exposed to chloroquine. The parasites shown in *A* and *B* were cultured in medium containing 25 ng chloroquine/ml from 0–24 h after synchronization. The parasite in *A* apparently died at the ring stage, but the one in *B* grew and accumulated hemoglobin-filled vesicles (hv). Large vacuoles (va) appeared in parasites exposed to chloroquine for an additional 20 h (*C*). Parasites shown in *D* and *E* were exposed to 100 ng/ml chloroquine during the growth period between 22–30 h after synchronization. The trophozoites (*D*) contained many hemoglobin-filled vesicles (hv) and vacuoles (va). Mature schizonts (*E*) contained neither hemoglobin-filled vesicles nor vacuoles. The merozoites in the schizonts appeared intact.

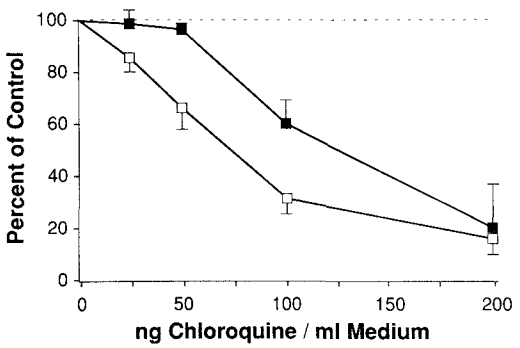


Figure 7. Effect of chloroquine added at the trophozoite stage on parasitemia and hemozoin production by *P. falciparum*. Parasites synchronized at the ring stage (0 h) were permitted to grow for 22 h under normal culture conditions. Chloroquine was then added and the amount of hemozoin produced between 22 and 44 h was measured at 44 h and expressed as a percent of the mean of control values. The broken line represents the control value, i.e., hemozoin produced between 22 and 44 h by parasites grown throughout the experiment in chloroquine-free medium. The means ± SE of 5 experiments are shown at each time interval, except for 200 ng/ml of chloroquine for which the results of 4 experiments are shown. Parasitemia, open squares; hemozoin production, solid squares.

vacuoles appeared in parasites that were exposed longer to chloroquine (fig. 6C).

Exposure to 25 ng/ml of chloroquine had little or no effect on trophozoites and schizonts (fig. 7). Higher concentrations were required to inhibit hemozoin production and parasitemia. The ultrastructure of trophozoites (fig. 6D) but not that of schizonts (fig. 6E) was noticeably affected by 100 ng/ml of chloroquine. Increasing resistance to chloroquine as the parasite matures also has been observed in survival studies[10] and in studies of ($^3$H)-hypoxanthine and ($^3$H)-isoleucine uptake by erythrocytes infected with *P. falciparum*[11].

*Discussion*

Since we measured both hemozoin production and chloroquine accumulation, inferences may be drawn about FP polymerization and hemoglobin digestion. Such inferences are justifiable because FP released from hemoglobin is neither excreted from the erythrocyte nor degraded[12] and must accumulate either in a polymerized form in hemozoin, or in a nonpolymerized form which binds chloroquine[13]. For example, nonpolymerized FP and, hence, chloroquine binding would increase if a decrease in hemozoin production precedes a decrease in hemoglobin digestion. On the other hand, chloroquine binding would decrease if a decrease in hemoglobin degradation precedes a decrease in hemozoin production. We found the latter situation in control cultures of *P. falciparum* at the schizont stage (figs 2 and 3). We conclude, therefore, that the rate of hemozoin production normally is limited by the availability of FP which, of course, is a function of the rate of hemoglobin digestion.

As assessed by hemozoin production, there are major changes in hemoglobin catabolism as *P. falciparum* parasites mature (figs 1 and 2). At the early ring stage, there is little or no hemoglobin catabolism. Then, there is an abrupt switch to a high rate of catabolism. Just as abruptly, hemoglobin catabolism switches off late in the trophozoite stage or early in the schizont stage. The signals responsible for switching hemoglobin catabolism on and off are not yet identified, but their existence undoubtedly is crucial for the survival of malaria parasites.

Chloroquine disrupts hemoglobin catabolism, as has been previously reported[6], and we now report for the first time that this effect is stage-specific. Thus, high concentrations of chloroquine were required to affect hemozoin production as the parasites matured (fig. 7); and, as would be expected when hemoglobin catabolism is switched off in the schizont stage, high concentrations of chloroquine (100 ng/ml) failed to cause hemoglobin filled vesicles to accumulate (fig. 6E). By contrast, when hemoglobin catabolism was switched on in the ring stage, hemoglobin was ingested in the presence of chloroquine, but it was not properly digested, and hemoglobin-filled vesicles accumulated, (fig. 6B) as has been previously reported[14–16]. Low concentrations of chloroquine (25 ng/ml) caused this abnormality in ring forms. These observations indicate that chloroquine at the ring stage disrupts hemoglobin metabolism distal to the process of hemoglobin ingestion.

The present results serve as the basis for filling in certain details on our earlier diagram[17] to explain how chloroquine kills malaria parasites. Also considered in revising the diagram was the evidence that chloroquine causes nonpolymerized FP to accumulate in chloroquine-susceptible *P. falciparum*[13,18], that chloroquine and the FP-chloroquine complex inhibit the hemoglobin-degrading proteases of *P. falciparum* in vitro[19], and that exposure to chloroquine inhibits amino acid production by *P. falciparum*[20]. As the diagram (fig. 8) indicates, the following explanation for the antimalarial action of chloroquine is reasonable. The primary effect of chloroquine on hemoglobin catabolism is to block FP polymerization. FP then accumulates and forms FP-chloroquine complexes. Next, FP and its chloroquine complexes accentuate a feedback loop which, under normal circumstances, would only be required to make fine adjustments in hemoglobin degradation to keep FP concentrations below those known to be toxic[19,22]. With sufficient accumulation of FP and FP-chloroquine complexes, this feedback loop slows hemoglobin degradation enough to cause hemoglobin-filled vesicles to accumulate. As hemoglobin degradation slows, the parasites are deprived of amino acids, which arrests their growth and development and may cause them to die. Despite accentuation of the feedback loop, sufficient hemoglobin degradation may continue to prevent death

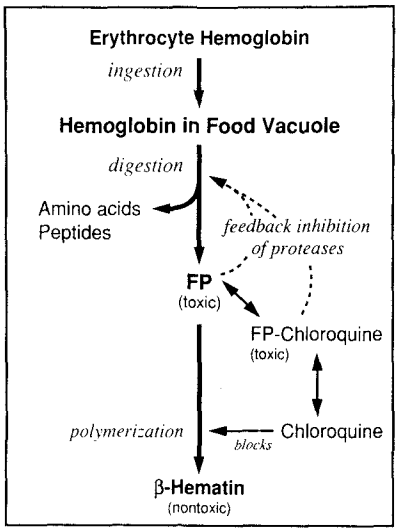


Figure 8. Diagram depicting hemoglobin catabolism and the effect of chloroquine in *P. falciparum*. In addition to accentuation of the feedback loop, the toxic effects of FP and the FP-chloroquine complex include membrane damage resulting in increased permeability and osmotic lysis of cells[21,22].

by starvation. If so, FP and FP-chloroquine complexes would eventually reach the concentrations required to increase membrane permeability[21,22] or to have other toxic effects which would kill the parasites.

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1 Sherman, I. W., Microbiol. Rev. *43* (1979) 453.
2 Rudzinska, M. A., and Trager, W., J. Protozool. *4* (1957) 190.
3 Goldberg, D. E., Slater, A. F. G., Cerami, A., and Henderson, G. B., Proc. natl Acad. Sci. USA *87* (1990) 2931.
4 Slater, A. F. G., and Cerami, A., Nature, Lond. *355* (1992) 167.
5 Chou, A. C., and Fitch, C. D., Life Sci. *51* (1992) 2073.
6 Orjih, A. U., and Fitch, C. D., Biochim. biophys. Acta *1157* (1993) 270.
7 Jensen, J. B., and Trager, W., J. Parasit. *63* (1977) 883.
8 Lambros, C., and Vanderberg, J. P., J. Parasit. *65* (1979) 418.
9 Fitch, C. D., Proc. natl Acad. Sci. USA *64* (1969) 1181.
10 Zhang, Y., Asante, K. S. O., and Jung, A., J. Parasit. *72* (1986) 830.
11 Ter Kuile, F., White, N. J., Holloway, P., Pasvol, G., and Krishna, S., Expl Parasit. *76* (1993) 85.
12 Roth, E. F. Jr., Brotman, D. S., Vanderberg, J. P., and Schulman, S., Am. J. trop. Med. Hyg. *35* (1986) 906.
13 Chou, A. C., Chevli, R., and Fitch, C. D., Biochemistry *19* (1980) 1543.
14 Macomber, P. B., and Sprinz, H., Nature, Lond. *214* (1967) 937.
15 Yayon, A., Timberg, R., Friedman, S., and Ginsburg, H., J. Protozool. *31* (1984) 367.
16 Zhang, Y., Expl Parasit. *64* (1987) 322.
17 McChesney, E. W., and Fitch, C. D., in: Handbook of Experimental Pharmacology, vol. 68: Antimalarial Drugs II. Current Antimalarials and New Developments. p. 3. Eds W. Peters and W. H. G. Richards. Springer-Verlag, Berlin 1984.
18 Fitch, C. D., Science *169* (1970) 289.
19 Vander Jagt, D. L., Hunsaker, L. A., and Campos, N. M., Biochem. Pharmac. *36* (1987) 3285.
20 Zarchin, S., Krugliak, M., and Ginsburg, H., Biochem. Pharmac. *35* (1986) 2435.
21 Chou, A. C., and Fitch, C. D., J. clin. Invest. *68* (1981) 672.
22 Orjih, A. U., Banyal, H. S., Chevli, R., and Fitch, C. D., Science *214* (1981) 667.