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On the molecular mechanism of chloroquine's antimalarial action

(*Plasmodium*/hemozoin/heme/histidine-rich protein)

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ABSTRACT **Chloroquine is thought to exert its antimalarial effect by preventing the polymerization of toxic heme released during proteolysis of hemoglobin in the *Plasmodium* digestive vacuole. The mechanism of this blockade has not been established. We incubated cultured parasites with subinhibitory doses of [^{3}H]chloroquine and [^{3}H]quinidine. These [^{3}H]quinoline compounds became associated with hemozoin as assessed by electron microscope autoradiography and subcellular fractionation. *In vitro*, binding of [^{3}H]quinoline inhibitors to the hemozoin chain depended on the addition of heme substrate. These data counter previous conclusions regarding the lack of quinoline association with hemozoin, explain the exaggerated accumulation of quinolines in the *Plasmodium* digestive vacuole, and suggest that a quinoline–heme complex incorporates into the growing polymer to terminate chain extension, blocking further sequestration of toxic heme.**

The quinolines have been used as specific antimalarial therapy for more than 300 years, yet their mechanisms of action have been difficult to establish (1). In the acidic digestive vacuole, proteolysis of hemoglobin (2) releases toxic heme that is polymerized only in plasmodial species into an inert crystal called malarial pigment or hemozoin (3, 4). Heme polymerization is an elongation reaction with evidence for nucleation by histidine-rich proteins (HRPs) (5). Lipids may also play a role (6). Preformed hemozoin chains can also be extended in the absence of proteins (7, 8). Multiple lines of evidence indicate that quinolines such as chloroquine, mefloquine, quinine, and quinidine act by inhibiting polymerization of the heme that is released during hemoglobin degradation. (*i*) *In vitro* these quinolines block the polymerization of micromolar heme into hemozoin under approximated physiological conditions mediated by crude trophozoite lysates (9), seed crystals of hemozoin (7), or isolated *Plasmodium falciparum*-derived HRPs (5). Chemical polymerization of heme under nonphysiological conditions is similarly inhibited (8). (*ii*) The quinoline inhibition is stage-specific for parasites actively degrading hemoglobin and generating hemozoin (1). (*iii*) Early morphologic effects of drug treatment are digestive vacuolar swelling (10) and pigment clumping (11). (*iv*) Chloroquine and its congeners hyperconcentrate to near millimolar concentration in the acidic digestive vacuole of *P. falciparum* from nanomolar concentration in the plasma (12, 13).

It has been postulated that the chloroquine-induced disruption of heme polymerization results either from binding of drug to heme with sequestration of monomeric substrate or from direct interaction of chloroquine with a polymerization protein. Chloroquine and its congeners bind heme noncovalently (14, 15); ring–ring stacking of the quinoline nucleus with the porphyrin moiety is observed by NMR (16). Photoacoustic spectrophotometry has detected chloroquine–heme complexes *in situ* (17), although they have never been isolated from the parasite. Arguments against substrate–drug interaction lowering available substrate are that quinine, quinidine, and epiquinine interact equally with heme, yet vary 100-fold in their ability to inhibit *in vitro* heme polymerization (1). Likewise chloroquine has a higher heme avidity than quinidine or quinine yet is a weaker inhibitor of *in vitro* heme polymerization (1). Also mefloquine, quinine, and quinidine inhibit *in vitro* polymerization in the presence of 7- to 20-fold excess heme (1). On the other hand, an argument against direct drug interaction with a polymerization protein is that chloroquine blocks the hemozoin-elongation reaction in the absence of protein (7, 8).

In the current study, we have examined the association of subinhibitory doses of [^{3}H]chloroquine and [^{3}H]quinidine with hemozoin in culture and *in vitro*. The data suggest that the quinolines first bind heme and then the drug–heme complex attaches to elongation sites of hemozoin, with accumulation of toxic unpolymerized heme ensuing.

MATERIALS AND METHODS

Parasite Culture. *P. falciparum* clone HB3 (chloroquine sensitive) and strain Indochina 1/CDC (chloroquine resistant) were grown at 1% hematocrit and 10% parasitemia to the early trophozoite stage by the method of Trager and Jensen (18). Synchrony was maintained by sorbitol treatment (19). The early trophozoite cultures in 15-ml plates were incubated for 20–24 h with 0.5 μCi of [^{3}H]chloroquine (1 Ci = 37 GBq) with a specific activity of 27 Ci/mmol (Walter Reed Army Institute of Research, Washington, DC) or with 0.5 μCi of [^{3}H]quinidine with a specific activity of 15 Ci/mmol (American Radiolabeled Chemicals, St. Louis).

Electron Microscopy. Infected erythrocyte cultures (HB3) incubated with [^{3}H]chloroquine were fixed in 1% glutaraldehyde in Hepes saline (20), postfixed in OsO_4, dehydrated through an ethanol series, and embedded in Spurr's resin (Electron Microscopy Sciences, Fort Washington, PA). Sections were cut, placed on carbon-coated Formvar grids, and poststained with uranyl acetate and Reynold's lead. After screening and carbon coating, the grids were coated with a film of EM-1 autoradiography emulsion (Amersham) formed in a wire loop. The grids were dried and left in the dark in a desiccated environment to expose. Grids were developed in D-19 developer (Eastman Kodak) for 2–4 min, fixed in Rapid Fix for 4 min, washed, dried, and examined by electron microscope. Photographic negatives were scored for the number of silver grains over the erythrocyte, parasite, or hemozoin.

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Abbreviation: HRP, histidine-rich protein.

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Each cluster or track in the emulsion was scored as a single event. The example shown in Fig. 1*A* was scored as three.

Sucrose Cushion Hemozoin Purification. The trophozoite cultures incubated with [^{3}H]quinoline were pelleted (1000 × *g*, 15 min), washed once with unlabeled medium, and lysed with 6 ml of hypotonic buffer [5 mM sodium phosphate (pH 7.5)]. The trophozoite lysate was pelleted (2000 × *g*, 15 min) and sonicated in 100 μl of 50 mM Tris·HCl (pH 8.0). The radioactive suspension was placed on top of a 1-ml 1.7 M sucrose cushion in 50 mM Tris·HCl (pH 8.0). Ultracentrifugation (200,000 × *g*, 15 min) pellets hemozoin but not heme in this system. The insoluble pellet was washed once by resuspending in 2 ml of 50 mM Tris·HCl (pH 8.0) with sonication. The 2-ml suspension was ultracentrifuged again and the radioactivity of the pellet was quantitated.

Western Blot Analysis of Sucrose Cushion Fractions. Sucrose cushion fractions were analyzed by SDS/polyacrylamide

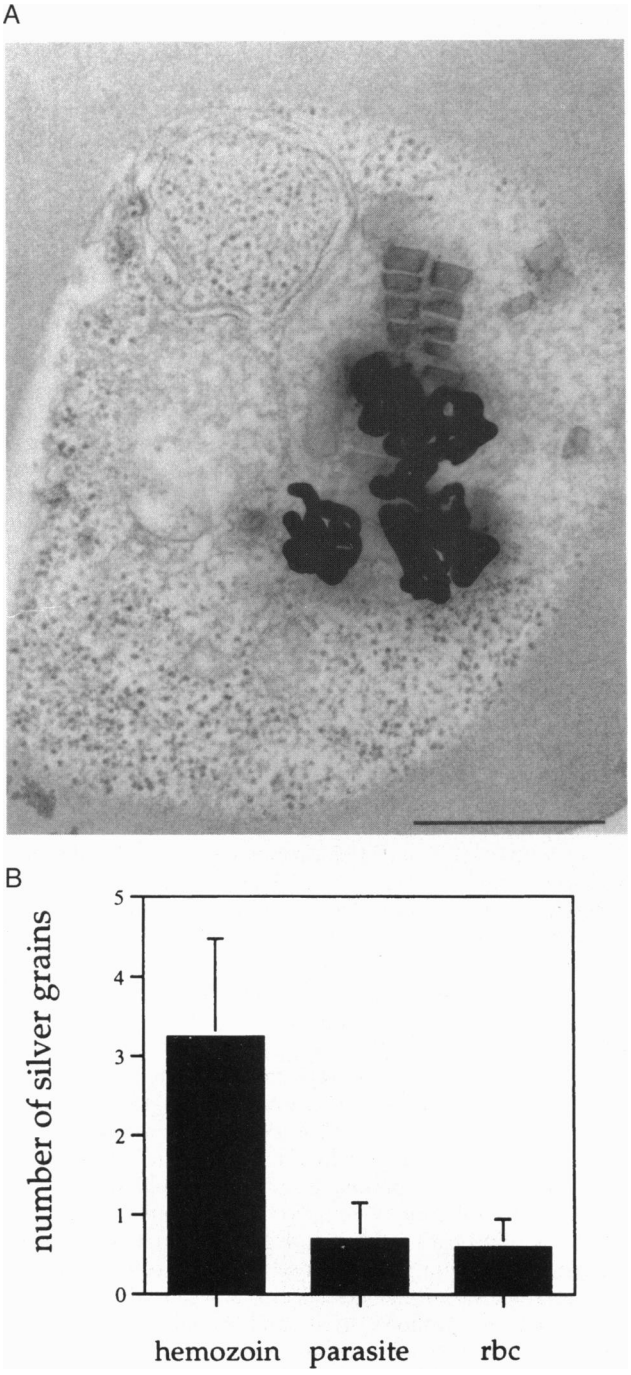

FIG. 1. Chloroquine binding to hemozoin in cultured parasites. (*A*) [^{3}H]Chloroquine is located over the hemozoin pigment crystals situated in the digestive vacuole. (Bar = 0.5 μm.) (*B*) The majority of ^{3}H signal is directly over the crystals. Fifty parasitized erythrocytes were scored for signal distribution. Parasite bar denotes grains located in the parasite but not hemozoin-associated. Data are the mean ± SEM.

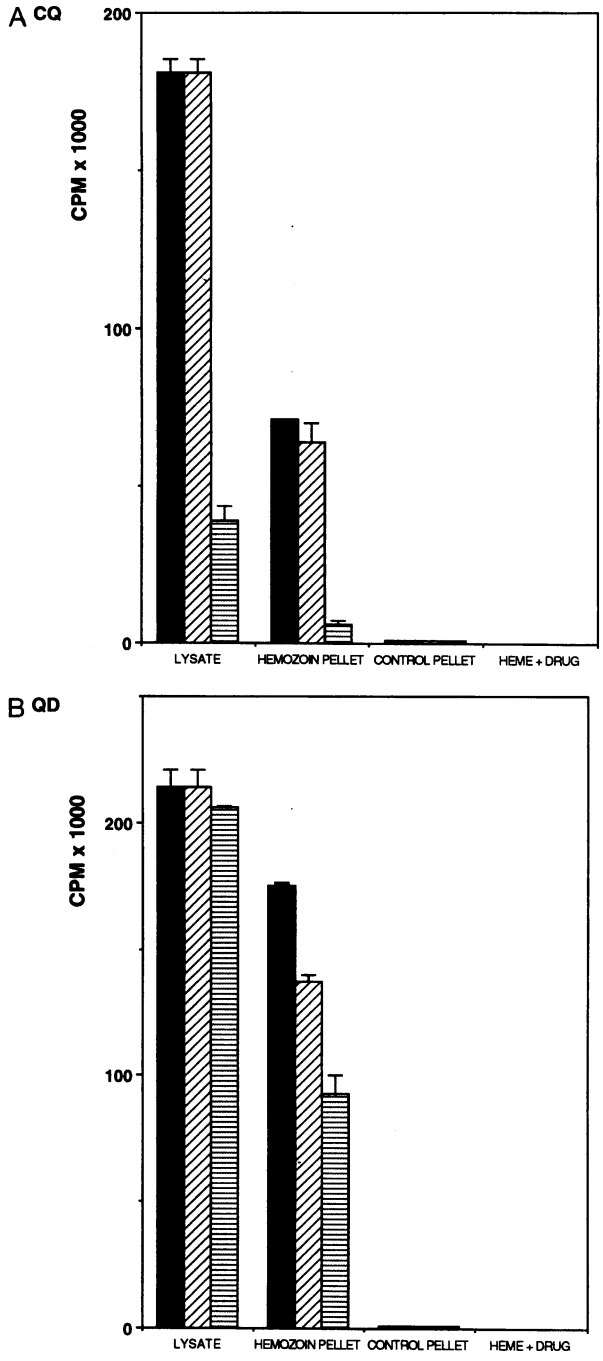

FIG. 2. Fractionation of [^{3}H]quinolines with hemozoin from cultured parasites. Sonicated hypotonic trophozoite lysates were loaded onto a 1-ml 1.7 M sucrose cushion for ultracentrifugation to yield hemozoin pellets. Duplicate HB3 (chloroquine sensitive) lysates were ultracentrifuged without (solid bars) and with (hatched bars) a sucrose cushion. Indochina 1/CDC (chloroquine-resistant) lysates (horizontal striped bars) were ultracentrifuged with the sucrose cushion. Control pellets had 0.5 μCi of drug added to hypotonic lysis solution followed by a 15-min incubation on ice before sedimentation. Another control was 0.5 μCi of drug incubated for 15 min with 50 nmol of heme (heme + drug). (*A*) [^{3}H]Chloroquine. (*B*) [^{3}H]Quinidine. Data are the mean ± SEM (n = 2 experiments).

gel electrophoresis (21) on 10 % gels. Immunoblots (22) with antibodies to erythrocyte band III (Sigma), spectrin (Sigma), erythrocyte catalase (Athens Research & Technology, Athens, GA), HRP I mAb 89 and HRP II mAb 2G12 (23) (Diane Taylor, Georgetown University), plasmespin I (24), and Pfmdr-1 (25) were developed by enhanced chemiluminesence (Amersham).

Hemozoin Synthesis Assays. For hemozoin template assays, 0.05 μCi (50,000 cpm) of [^{3}H]quinoline was incubated with 10 nmol (heme content) of extensively purified hemozoin (3) and 25 nmol of hemin (ferriprotoporphyrin IX chloride) in 500 μl of 100 mM sodium acetate (pH 4.8) overnight at 37°C. Controls omitted hemozoin, heme, or both. The insoluble product was centrifuged for 15 min at 15,000 $\times$ *g*, resuspended by sonication in 100 μl of 50 mM Tris·HCl (pH 8.0), and ultracentrifuged through a 1.7 M sucrose cushion as above.

For HRP-initiated assays, 0.08 μCi of [^{3}H]quinoline was incubated with 1 nmol of HRP II and 40 nmol of heme in 800 μl of 100 mM sodium acetate (pH 4.8) overnight at 37°C (5). Controls omitted HRP, heme, or both. The insoluble product was centrifuged 15 min at 15,000 $\times$ *g*, resuspended by sonication in 1400 μl of 50 mM Tris·HCl (pH 8.0), centrifuged again for 15 min at 15,000 $\times$ *g*, resuspended by sonication in 100 μl of 50 mM Tris·HCl (pH 8.0), and ultracentrifuged as above.

The HRP-produced hemozoin assays used purified HRP-produced hemozoin as template. The product from 24 samples of the HRP-initiated reaction without quinolines (as above) was purified by washing in 2% SDS/0.1 M sodium bicarbonate, pH 9.1, pelleting at 15,000 $\times$ *g* for 30 min, washing again in 2% SDS, pelleting, and washing 2 times with distilled water. This hemozoin was quantitated and 3-nmol (heme content) aliquots were used for hemozoin template incubations as above.

HRP and [^{3}H]Chloroquine Equilibrium Analysis. In 100 mM sodium acetate (pH 4.8), 100 or 1000 nM HRP II was incubated with 10–1000 nM [^{3}H]chloroquine overnight. The solution was concentrated 10-fold by a 15-min centrifugation in Centricon-10 (Amicon), retaining HRP II. The radioactivity in the retentates and filtrates was determined.

Hemozoin Quantitation. Parasite hemozoin or polymerized heme was incubated for 1 h in 2% SDS/20 mM NaOH to solublize polymer into monomeric heme, which has a molar extinction coefficient at 400 nm of 1 $\times$ 10^5.

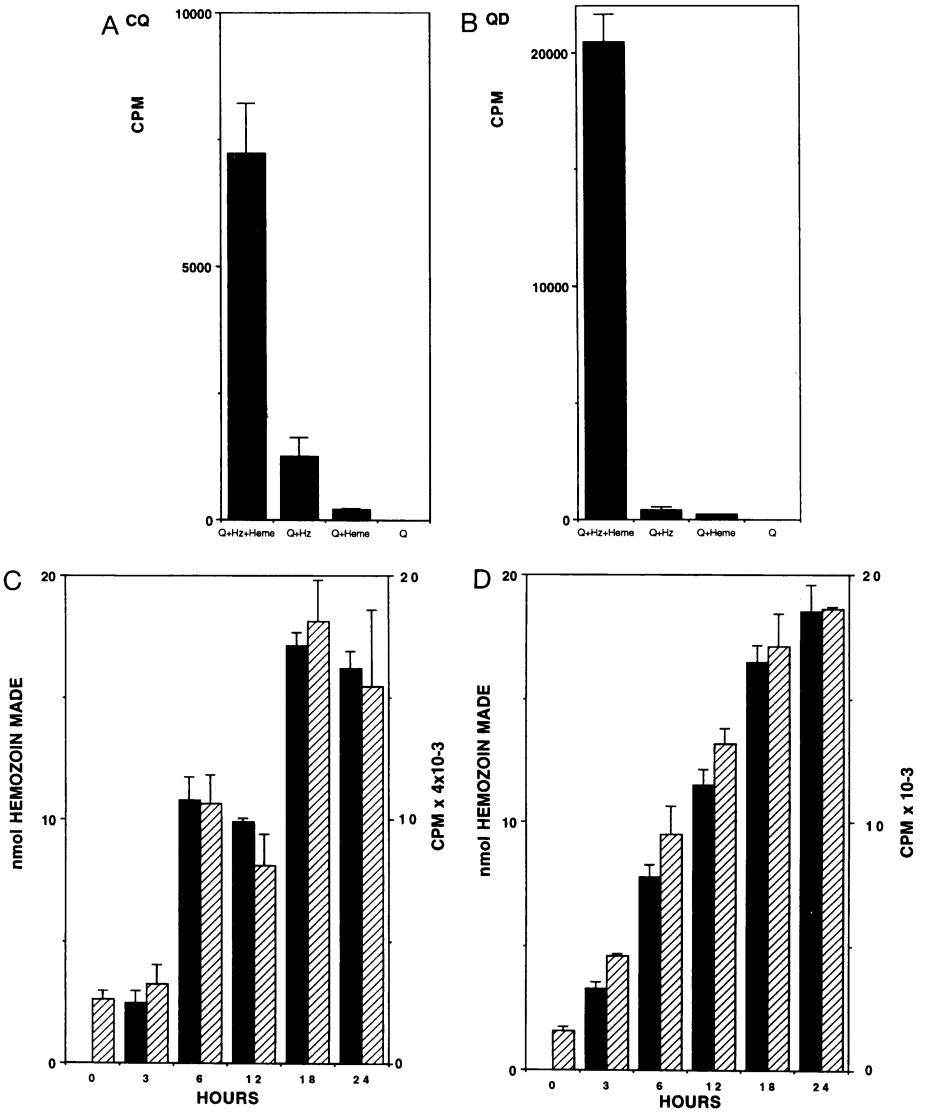

FIG. 3. Binding of [^{3}H]quinoline to hemozoin in the presence of heme *in vitro*. Overnight incubations of drug with purified hemozoin + heme (Q + Hz + heme), hemozoin (Q + Hz), heme (Q + heme), or buffer (Q) alone were ultracentrifuged as in Fig. 2. (*A*) [^{3}H]Chloroquine (CQ). (*B*) [^{3}H]Quinidine (QD). The results are the mean ± SEM (n = 4 experiments). A time course was performed as above for [^{3}H]chloroquine (*C*) and [^{3}H]quinidine (*D*). Bars: solid, nmol of hemozoin synthesized; hatched, cpm of [^{3}H]quinoline (n = 2).

RESULTS

[^{3}H]Quinolines Associate with Hemozoin in Culture. To understand the mechanism of quinoline action, we studied [^{3}H]chloroquine distribution after uptake of subinhibitory doses by cultured intraerythrocytic *P. falciparum*. Electron microscope autoradiography demonstrated that the signal was almost exclusively over hemozoin crystals within the parasite digestive vacuoles (Fig. 1*A*). Statistical analysis of the signal distribution (500 cells) revealed that the infected erythrocytes were 23.4 times more likely to have signal than uninfected erythrocytes. Within infected erythrocytes, signal was strongly associated with hemozoin (Fig. 1*B*).

For further analysis, similar parasite cultures (chloroquine-sensitive clone HB3) incubated with [^{3}H]chloroquine or quinidine were disrupted by sonication in hypotonic buffer. The extracts were purified by centrifugation through a 1.7 M sucrose cushion, with hemozoin pelleting to the bottom. Western blot analysis confirmed separation of hemozoin from >99% of membrane and soluble protein markers (data not shown). The hemozoin pellet contained 35–70% of the parasite-associated [^{3}H]quinoline (Fig. 2). Nearly all the radioactivity sedimentable without sucrose was hemozoin-associated by sucrose cushion analysis. Hemozoin recovery in the sucrose cushion pellet was 75% (12 nmol). Control parasite lysates mixed with [^{3}H]quinoline and incubated for 15 min showed no

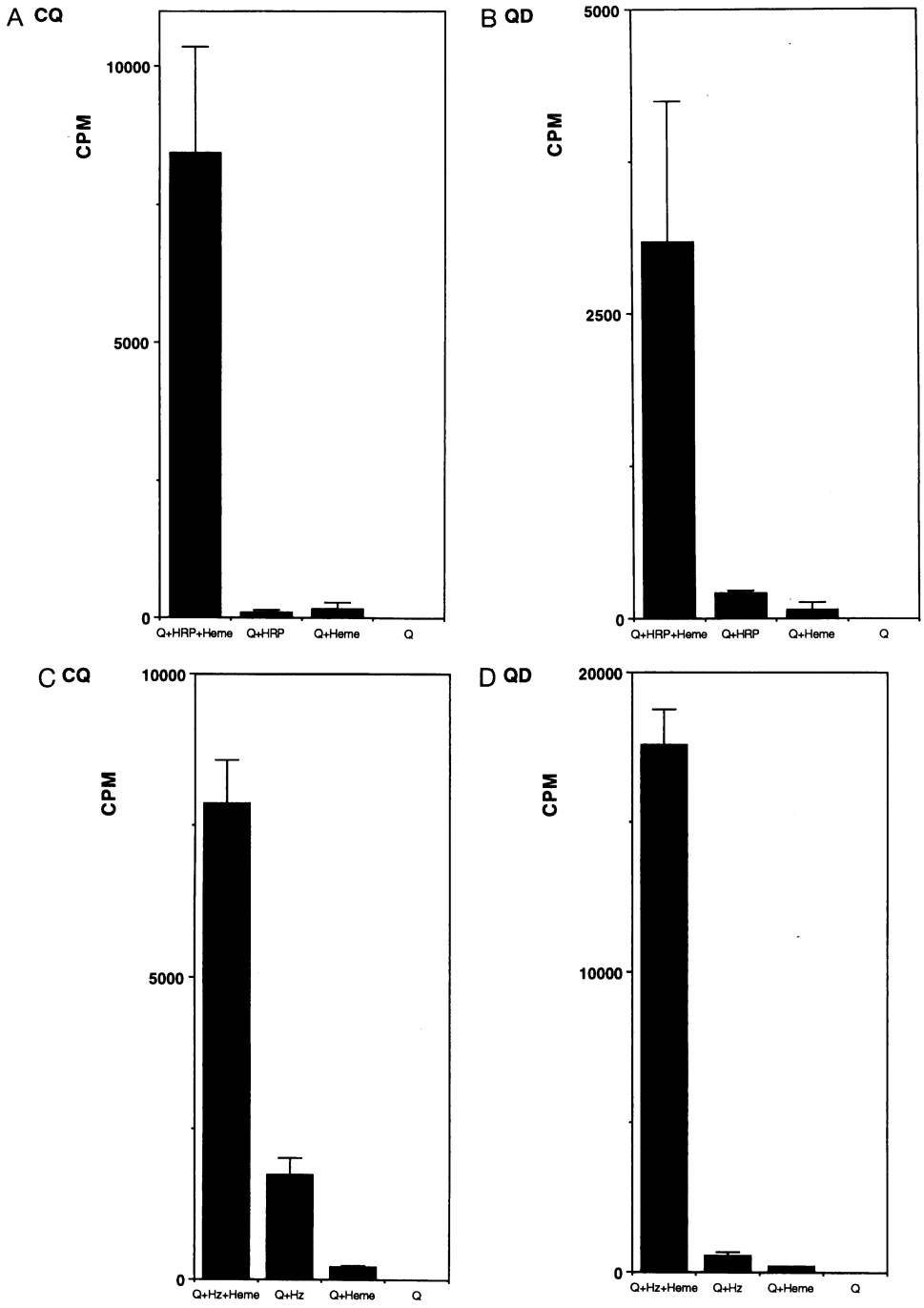

FIG. 4. Binding of [^{3}H]quinoline to HRP-nucleated hemozoin in the presence of heme *in vitro*. Overnight incubations of drug with purified recombinant HRP II + heme (Q + HRP + heme), HRP (Q + HRP), heme (Q + heme), or buffer (Q) alone were ultracentrifuged as in Fig. 2. (*A*) [^{3}H]Chloroquine. (*B*) [^{3}H]Quinidine. Purified HRP II-nucleated hemozoin reincubated with heme in the presence of [^{3}H]chloroquine (*C*) or [^{3}H]quinidine (*D*) is shown. The results are the mean ± SEM (n = 5 experiments.)

[^{3}H]quinoline association with hemozoin. This rules out nonspecific binding during lysis as a possibility. Heme–quinoline complexes also did not pellet in this procedure. Using the chloroquine-resistant Indochina1/CDC strain, which is believed to have reduced drug accumulation due to increased efflux (26), [^{3}H]chloroquine in parasite lysates was reduced and hemozoin-associated radioactivity was minimal (Fig. 2*A*). In contrast, [^{3}H]quinidine accumulation and incorporation into the hemozoin pellet were comparable to the HB3 results (Fig. 2*B*), consistent with Indochina 1/CDC being a quinidine-sensitive strain.

***In Vitro* [^{3}H]Quinolines Interact With Hemozoin.** Hemozoin formation can be nucleated in the test tube with preformed hemozoin (7) or with HRP II and III, which can be found in the parasite digestive vacuole (5). [^{3}H]Chloroquine (4 nM) was incubated with heme (50 μM) and purified malarial hemozoin overnight. The reaction mixture was placed on a 1.7 M sucrose cushion and ultracentrifuged as above. Substantial [^{3}H]chloroquine was found in the hemozoin pellet (Fig. 3*A*). [^{3}H]Chloroquine incubated with hemozoin in the absence of heme did not pellet; nor did [^{3}H]chloroquine incubated with heme without hemozoin as template. [^{3}H]Quinidine behaved similarly in these assays (Fig. 3*B*). A time course demonstrates increasing [^{3}H]quinoline incorporation with increasing hemozoin synthesis (Fig. 3 *C* and *D*). Hemozoin yield was similiar with and without [^{3}H]quinoline.

Initiating the reaction with recombinant HRP II instead of hemozoin also led to incorporation of radioactivity into the polymer pellet using [^{3}H]chloroquine (Fig. 4*A*) or [^{3}H]quinidine (Fig. 4*B*). Coincubation of HRP II with ^{3}H-labeled drug in the absence of heme gave no sedimentable radioactivity. The interaction of HRP II with chloroquine in the absence of heme was further explored under equilibrium conditions and no association could be detected. Labeled chloroquine concentration did not differ significantly between the retentates and filtrates in multiple determinations (data not shown). Interestingly, in the presence of heme, chloroquine incorporation was higher than quinidine incorporation, in contrast to the whole cell results (Fig. 2) and the hemozoin-initiated synthesis (Fig. 3). To explore this, purified HRP II-produced hemozoin was further incubated with fresh heme in the presence of ^{3}H-labeled drug (Fig. 4 *C* and *D*). Quinidine incorporation was greater than chloroquine incorporation (600% greater than quinidine incorporation for HRP II-initiated production), suggesting that drug–heme complexes have differing affinities for the initiating chain than for the extending polymer. Starting with purified HRP II-produced hemozoin, new hemozoin synthesis was similiar to that of Fig. 3 and was minimally (40%) higher than the HRP II-initiated synthesis of Fig. 4 *A* and *B*.

Nonspecific trapping within the *in vitro* growing crystal was ruled out by displacement of bound [^{3}H]quinoline from hemozoin pellets with a 1000-fold excess of unlabeled chloroquine or quinidine incubated for 2 h after the overnight reaction. This result is as expected since up to 30% of the [^{3}H]quinoline is retained by the polymerized hemozoin occupying less than 1% of the reaction volume. Likewise Fitch and Chevli (27) demonstrated that radiolabeled drug in culture could be displaced with unlabeled quinoline incubations.

DISCUSSION

The data suggest that quinolines act by incorporation of drug-heme complex into the growing polymer of hemozoin. Drug does not associate significantly with hemozoin in the absence of polymer elongation. Macomber *et al.* (10) noted that *in vitro* chloroquine did not bind to or dissolve malaria pigment. Later Fitch and Kanjananggulpan (28) showed no binding of chloroquine to hemozoin. Our demonstration of quinoline binding to hemozoin in the presence of heme implicates the drug–heme complex in capping the hemozoin to block polymer extension (Fig. 5). Separating hemoglobin degradation and heme release from heme sequestration initiates the irreversible demise of *Plasmodium*, manifested by the morphologic effects of digestive vacuolar swelling and pigment clumping. This work builds upon the electron microscopic work of Aikawa (29) who localized [^{3}H]chloroquine in the digestive vacuole of *Plasmodium berghei* devoid of pigment due to lethal concentrations of chloroquine.

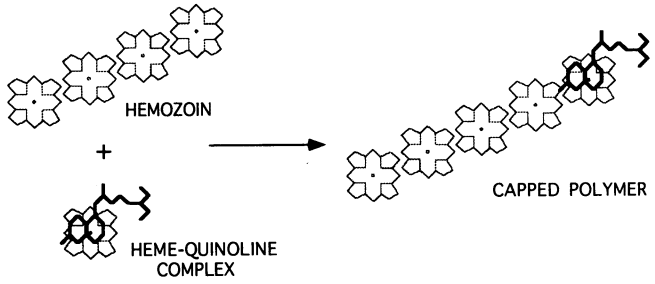

FIG. 5. Model of quinoline inhibition of hemozoin formation. Quinoline binds heme and the drug–heme complex incorporates at the elongation site of the polymer, preventing further additions of heme.

The heme–quinoline complex interaction with hemozoin is clearly noncovalent, as it can be diminished by extensive washing. Yet it is a strong enough interaction to compete for incorporation with free heme in molar excess. Free heme adds on to the growing chain by forming a coordinate iron-carboxylate linkage (3). Kinetic and equilibrium parameters should be interesting to obtain, though this should be a very difficult task since substrate is also part of the inhibitor such that the two cannot be varied independently. Additionally, the low solubility limits of substrate and product make quantitation of the noncovalent interaction technically challenging.

Incorporation of heme–quinoline complex into the growing polymer may well explain the exaggerated accumulation of drug in the *Plasmodium* digestive vacuole. Chloroquine's accumulation to millimolar levels from nanomolar levels in the plasma is out of proportion to that explainable by weak base effects (which account for its more limited accumulation in mammalian lysosomes). The heme in hemoglobin is present in the 80-fl erythrocyte at a concentration of 20 mM. Nearly all the hemoglobin is ingested by the parasite into its 2-μm-diameter (4-fl volume) digestive vacuole. This means that the vacuolar heme concentration is about 0.4 M. Postulating that a quinoline molecule is incorporated into the growing hemozoin chain every 400th heme would yield a 1 mM drug accumulation. Alternatively, if chloroquine is incorporated every 20th heme but the treatment halts polymerization after it is 20% complete, millimolar drug again results.

Future antiheme polymerization drug design studies will need to consider both the drug interaction with heme as well as the heme–drug complex interaction with polymer in the development of new antimalarial agents.

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