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In Vitro Activity of Riboflavin against the Human Malaria Parasite *Plasmodium falciparum*

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The human malaria parasite *Plasmodium falciparum* digests hemoglobin and polymerizes the released free heme into hemozoin. This activity occurs in an acidic organelle called the food vacuole and is essential for survival of the parasite in erythrocytes. Since acidic conditions are known to enhance the auto-oxidation of hemoglobin, we investigated whether hemoglobin ingested by the parasite was oxidized and whether the oxidation process could be a target for chemotherapy against malaria. We released parasites from their host cells and separately analyzed hemoglobin ingested by the parasites from that remaining in the erythrocytes. Isolated parasites contained elevated amounts (38.5% ± 3.5%) of oxidized hemoglobin (methemoglobin) compared to levels (0.8% ± 0.2%) found in normal, uninfected erythrocytes. Further, treatment of infected cells with the reducing agent riboflavin for 24 h decreased the parasite methemoglobin level by 55%. It also inhibited hemozoin production by 50% and decreased the average size of the food vacuole by 47%. Administration of riboflavin for 48 h resulted in a 65% decrease in food vacuole size and inhibited asexual parasite growth in cultures. High doses of riboflavin are used clinically to treat congenital methemoglobinemia without any adverse side effects. This activity, in conjunction with its impressive antimalarial activity, makes riboflavin attractive as a safe and inexpensive drug for treating malaria caused by *P. falciparum*.

Malaria infects 300 to 500 million people and kills about 1 million people, mostly children, each year. In most areas of endemicity, there are significant levels of resistance to many antimalarial agents, and in extreme cases, the disease can be resistant to all known antimalarial agents. Hence, the importance of discovering new drugs that can be used to combat resistant strains cannot be overemphasized.

The malaria parasite ingests 25 to 80% of its host erythrocyte hemoglobin (2, 11, 24, 27) and digests it in an acidic organelle called the food vacuole (fv). Multiple enzymes catalyze this digestion, and the released heme is detoxified into hemozoin in the fv. The degradative enzymes, as well as the process of heme detoxification, have been considered targets of several antimalarial agents. Drugs that act against these targets include quinoline-based compounds (28), protease inhibitors (26), and free-radical generators, such as artemisinin and its derivatives (16, 23). However, redox mechanisms and treatments that regulate the valence state of hemoglobin and their antimalarial potential remain largely unexplored.

In erythrocytes, hemoglobin is converted from the ferrous to the ferric or methemoglobin state at a very slow rate under physiological conditions. This auto-oxidation reaction is accelerated by chloride and other small anions that may function by displacing the superoxide anion from oxyhemoglobin. Lower pH and polyanions also accelerate the process (20, 21). Levels of methemoglobin in *Plasmodium falciparum*-infected erythrocytes have been previously measured. Friedman et al. showed that cultures with high levels of parasitemia contained 3 to 10 times more methemoglobin than those with low levels of parasitemia (10). More recently, Vander Jagt et al. found elevated methemoglobin levels in isolated fv's but not in crude lysates of infected or uninfected erythrocytes (32). In contrast, Hempelmann et al. reported no change in the methemoglobin content of *P. falciparum*-infected versus uninfected erythrocytes (14).

Since the end product of hemoglobin digestion in the fv is hemozoin, a polymer of hematin in the oxidized Fe^{3+} state, at some step during ingestion and/or digestion, Fe^{2+} from hemoglobin has to be oxidized to Fe^{3+}. If methemoglobin is formed, since it has a net positive charge and hemoglobin is a neutral molecule, the charge difference may affect the mechanism of hemoglobin digestion. We therefore reexamined the possibility that ingested hemoglobin may be oxidized to methemoglobin. We used two independent methods to determine the methemoglobin content of isolated parasites and erythrocytes and report that the malaria parasite *P. falciparum* does indeed induce an increase in the oxidation of hemoglobin to methemoglobin. This activity occurs only in parasites and not in erythrocytes and is thus restricted to ingested hemoglobin. We also show that treatment with riboflavin, which can reduce methemoglobin to hemoglobin in vitro (15, 18, 22), results in a decrease in the methemoglobin content of the parasite, inhibits fv development and function, and inhibits asexual parasite growth in erythrocytes. A mechanism for the antimalarial action of riboflavin is proposed.

MATERIALS AND METHODS

Culturing of parasites and drug treatments. RPMI 1640 medium and A^+ human serum were obtained from GIBCO/BRL and Gemini Biological Products (Calabasas, Calif.), respectively. Riboflavin was obtained from Sigma (St. Louis, Mo.). Chloroquine-resistant *P. falciparum* FCB was cultured in vitro by a modification of the methods of Trager and Jensen (31) and Haldar et al. (12). The parasites were synchronized by incubation in 5 volumes of 5% sorbitol for 10 min at 37°C. The effect of riboflavin was determined by incubating cultures of young ring-stage parasites (9 to 15 h) at 5 to 20% parasitemia with 10 to 100 μM riboflavin for 16 to 48 h. Inhibition of parasite growth was measured by examining Giemsa-stained blood smears after 48 h of treatment.

Isolation of parasites and separation of associated proteins. Cells from infected cultures were washed three times with phosphate-buffered saline (PBS). The erythrocytes were mixed with 10 volumes of 0.01% saponin in PBS and

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incubated at 4°C for 10 min. This procedure selectively lysed erythrocyte but not parasite membranes. The lysate was subjected to centrifugation at 2,200 × *g* for 10 min, and the supernatant (enriched in erythrocyte cytosolic content) was removed. The pellet containing isolated parasites was washed three times with cold PBS, solubilized in 5 volumes of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 3 to 5 min, and analyzed by SDS-PAGE (19).

Spectrophotometric assay for determination of methemoglobin in cell lysates. The methemoglobin content of cells was determined by a modification of the method of Evelyn and Malloy (9). Briefly, 10^9 uninfected erythrocytes or 10^8 to 10^9 isolated parasites were lysed in 500 μl of distilled water at room temperature. A total of 400 μl of 0.5 M phosphate buffer (pH 6.1) was added to 600 μl of the cell lysate, and the mixture was centrifuged at 16,000 × *g* for 5 min to sediment debris. A total of 700 μl of the supernatant fraction was used to measure the optical density at 630 nm (the absorbance maximum for methemoglobin), and the reading was recorded as S1. A total of 50 μl of 10% KCN was added, and after 3 to 5 min at room temperature, a second reading (S2) was recorded. KCN converts methemoglobin to cyanomethemoglobin, which does not absorb at 630 nm; hence, the difference between absorbance readings S1 and S2 represents the absorbance due to methemoglobin. To measure total hemoglobin levels, all of the hemoglobin was converted to methemoglobin, the absorbance of the sample at 630 nm was recorded, and then KCN was added to form cyanomethemoglobin. Specifically, 70 μl of the supernatant fraction was diluted 10-fold into 600 μl of 0.1 M phosphate buffer (pH 6.1). Next, 30 μl of freshly prepared 20% $K_3Fe(CN)_6$ (potassium ferricyanide) was added and incubated for 3 to 5 min at room temperature, and an initial reading (T1) was recorded. A total of 50 μl of 10% KCN was subsequently added, and a second reading (T2) was recorded. The percent methemoglobin in the sample was calculated as $[100(S1 - S2)]/[10(T1 - T2)]$.

Oxidation of hemoglobin in vitro. Erythrocytes (5×10^7) were lysed in 500 μl of distilled water at room temperature for 5 min. A total of 5 μl of 20% potassium ferricyanide was added (to convert hemoglobin to methemoglobin) to the lysate at room temperature for 5 min. Erythrocyte ghosts were removed by centrifugation at 16,000 × *g* for 5 min, and the supernatant (containing 1×10^6 to 2×10^6 erythrocyte equivalents) was analyzed by SDS-PAGE and Western blotting.

Peroxidase activity and hemoglobin content after SDS-PAGE. Proteins from saponin-lysed parasites, supernatants of sorbitol-lysed infected erythrocytes, or supernatants of saponin-lysed uninfected erythrocytes were subjected to SDS-PAGE under nonreducing conditions. The separated proteins were transferred to nitrocellulose membranes at 100 V for 1.5 h at 4°C.

Peroxidase activity was detected by chemiluminescence as described by Dorward (6). Briefly, the filters were washed with PBS and incubated for 1 min with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, N.J.). The filters were then covered with plastic wrap and exposed to film for 1 to 30 min. For hemoglobin content determination, the filters were washed with PBS and incubated with 5% nonfat dry milk for 30 min to quench all endogenous peroxidase activity. They were subsequently incubated with antihemoglobin antibody (1:500) for 1 h at room temperature, washed with Tris-buffered saline containing 0.05% Tween 20, and incubated with peroxidase-conjugated secondary antibody (1:1,500) for 1 h at room temperature. The bands were developed with ECL solutions in accordance with the manufacturer's protocol. Polyclonal rabbit anti-human hemoglobin antibody was from Dako (Carpinteria, Calif.).

Quantitative measurement of hemoglobin ingested and hemozoin produced in the presence or absence of riboflavin. Cells were collected by centrifugation, washed three times with PBS, and resuspended in 5 volumes of 5% sorbitol for 20 min at room temperature. This procedure released hemoglobin from infected cells. The cell lysate was subjected to stepwise centrifugation at 600 × *g* for 7 min and 2,200 × *g* for 10 min to separately sediment uninfected erythrocytes and parasites contained within erythrocyte ghosts. The hemoglobin content of the supernatant fraction was determined by measuring the optical density at 540 nm.

Uninfected cells obtained after the first centrifugation step at 600 × *g* were treated with 0.01% saponin in RPMI 1640 medium at room temperature for 10 min. This procedure released intracellular hemoglobin, which was quantitated by measuring the absorbance of the cleared supernatant at 540 nm. The number of moles of hemoglobin in infected and uninfected erythrocytes was calculated based on the number of erythrocytes in the culture, the percent parasitemia, and the millimolar extinction coefficient of 14.61 for hemoglobin (7). The amount of hemoglobin ingested by parasites was obtained by subtracting the amount of hemoglobin released from the erythrocyte cytosol of infected cells by sorbitol treatment from the total amount released from uninfected cells by saponin.

To release hemozoin from parasites, infected cells were first lysed with 0.01% saponin for 10 min at room temperature to release parasites from erythrocyte ghosts. The parasites were washed three times with PBS, resuspended in 2.5% SDS in PBS, and subjected to centrifugation at 20,000 × *g* for 1 h. The supernatant was discarded, and the insoluble pellet was washed in 2.5% SDS in PBS and then dissolved in 20 mM NaOH. The hemozoin content was measured by determining the absorbance at 400 nm and using a standard curve generated with hematin.

Electron microscopy. Cultures of ring-infected erythrocytes were incubated with riboflavin (15 to 100 μM) for 16 to 48 h. Mock- and riboflavin-treated infected erythrocytes were fixed in PBS containing 2% glutaraldehyde at 4°C for 30 min and processed to be embedded in Spurr's resin, and thin sections were examined in a Philips CM-12 microscope. fv diameters were measured, and their statistical variation was assessed by a one-way analysis of variance with the Tukey test for multiple comparisons between means.

RESULTS

Methemoglobin content of isolated parasites and *P. falciparum*-infected and uninfected erythrocytes. Oxidation of hemoglobin to methemoglobin results in marked changes in the chemical and spectral properties of the molecule. For instance, methemoglobin does not bind oxygen, a major physiological function of hemoglobin. The absorbance maximum of methemoglobin is 630 nm, which is red shifted about 100 nm relative to that of hemoglobin. Thus, measurement of the absorbance at 630 nm affords a simple spectrophotometric method of distinguishing between the two species. We therefore exploited this difference to determine the fraction of methemoglobin present in isolated parasites and uninfected erythrocytes. We found that in isolated parasites, the methemoglobin content was 20 to 42% the total hemoglobin content. In contrast, in erythrocytes, this fraction was 0.5 to 1%. These results suggested that the parasite induced an increase in methemoglobin. The fact that increased methemoglobin was detected in isolated parasites reflects oxidation of ingested hemoglobin.

Although the spectrophotometric assay is a standard method for determining the methemoglobin content of blood samples, it is possible that some parasite proteins and products, such as hemozoin, could interfere with the determinations. Thus, we developed a second method in which we exploited the intrinsic peroxidase activity of heme-containing compounds, which is dependent on the oxidation state of heme iron and can be measured by chemiluminescence (6).

We first established the elevated peroxidase activity of methemoglobin produced in vitro. This was done by treating hemoglobin obtained from erythrocyte cytosol with potassium ferricyanide [$K_3Fe(CN)_6$] at room temperature for 5 min. As shown in Fig. 1A, panel I, the peroxidase activity was higher in ferricyanide-treated hemoglobin than in untreated hemoglobin, even though there were comparable amounts of protein (as measured by immunoblotting with antibodies; Fig. 1A, panel II) in the two samples. (The intrinsic peroxidase activity of hemoglobin did not interfere with the immunoblot determination because the endogenous activity is completely quenched after blocking with nonfat dry milk, a standard precaution of the ECL method). This result indicated that chemiluminescence associated with the 14-kDa gel-purified hemoglobin band may be used to determine the relative methemoglobin content of a sample.

In this assay, parasites released from erythrocytes by sorbitol treatment had higher levels of peroxidase activity than the sorbitol supernatant, containing hemoglobin from the host cytoplasm (Fig. 1B, panel I). However, the total amount of hemoglobin in the sorbitol supernatant was much higher than that in isolated parasites (Fig. 1B, panel II). These data suggest that isolated parasites contain a larger fraction of methemoglobin than erythrocyte cytosol, are consistent with those obtained by the standard spectrophotometric assay (see above), and support the hypothesis that *P. falciparum* oxidizes ingested hemoglobin.

As a consequence of hemoglobin degradation, free heme is released and polymerized into hemozoin. To correlate the increase in hemoglobin oxidation to hemozoin formation during the asexual life cycle, peroxidase activities and levels of hemoglobin and hemozoin were measured at the different intracellular stages of the parasite. To do this, cells were collected from synchronized cultures every 6 h, parasites were

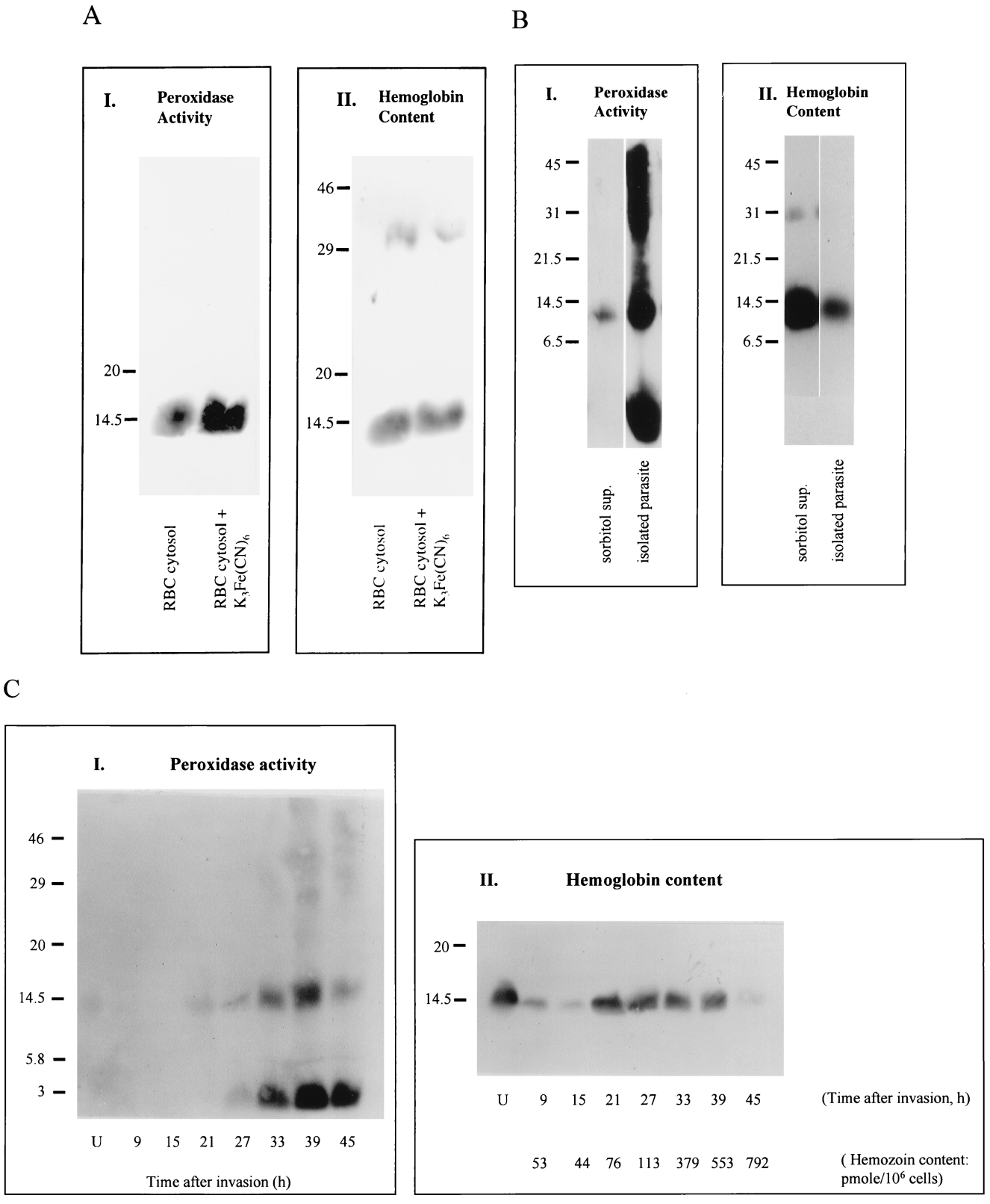


FIG. 1. Peroxidase activity associated with methemoglobin formed in vitro and in vivo. (A) Uninfected erythrocytes (RBC) were lysed hypotonically, the cytosolic fraction was incubated in the absence or presence of potassium ferricyanide (to form methemoglobin), and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and assayed for peroxidase activity or immunoblotted for hemoglobin content. Molecular masses are indicated in kilodaltons. (B) Trophozoite-infected erythrocytes were lysed with sorbitol, and the supernatant fraction (sup.) was separated from isolated parasites in the pellet fraction. Both fractions were assayed for peroxidase activity and hemoglobin content as described for panel A. (C) Uninfected erythrocytes (U) and parasites at the indicated times of growth were isolated by lysing infected erythrocytes with saponin and analyzed for peroxidase activity and hemoglobin content as described for panel A. Hemozoin was also determined, as indicated at the bottom of panel II.

separated from erythrocyte membranes by treatment with 0.01% saponin, and proteins were separated by SDS-PAGE. As shown in Fig. 1C, hemoglobin was detected in association with parasites as early as 9 h after invasion. This result suggests that even early ring-stage parasites may ingest low levels of hemoglobin. However, there was no increased peroxidase activity symptomatic of induced hemoglobin oxidation in ring-stage parasites relative to uninfected cells. In contrast, parasite-associated peroxidase activity increased during the trophozoite and schizont stages (>27 h) of development. This result indicated that a higher level of hemoglobin oxidation occurred at these later stages. This increase in peroxidase activity paralleled the increase in hemozoin production (see lower half of Fig. 1C, panel II), suggesting that the two may be linked.

The levels of hemoglobin detected in the saponin-isolated parasites in our study are much higher than those previously reported by Rosenthal (25). However, the conditions of parasite isolation in that study differ from those in our study. Whereas Rosenthal isolated parasites with 0.1% saponin at 37°C for 15 min, we used a milder isolation procedure of 0.01% saponin at 4°C for 10 min. This mild isolation protocol minimized protein degradation during isolation, possibly explaining why larger amounts of hemoglobin were detected by our method.

Treatment with riboflavin inhibits the formation of methemoglobin and hemozoin, reduces the size of the fv, and blocks parasite proliferation in cultures. Since riboflavin can reduce methemoglobin to hemoglobin in vitro, we investigated its effects on methemoglobin in infected erythrocytes. In these assays, ring-stage parasite (9 to 15 h)-infected cells were cultured with or without riboflavin for 16 to 24 h. Parasites were isolated from erythrocytes by treatment with 0.01% saponin, and the methemoglobin content was measured by both the spectrophotometric and the peroxidase assays. The former indicated that the methemoglobin level in treated cells was 17.5% ± 2.5%, considerably lower than that found in control incubations (38.5% ± 3.5%. The peroxidase activity in treated parasites was also significantly reduced (Fig. 2A, panel I), although the total amounts of hemoglobin were comparable in both samples (Fig. 2A, panel II). These results indicate that riboflavin-treated parasites contain less methemoglobin than untreated parasites. The chemiluminescence and spectrophotometric assays both indicated a 50% reduction in methemoglobin formation. This result further supports the notion that chemiluminescence associated with the 14-kDa band from isolated parasites is due to methemoglobin. There was a concomitant 50% reduction in hemozoin levels, although no significant change was detected in amounts of hemoglobin ingested by parasites (Fig. 2B). These results suggest that riboflavin treatment reduces hemozoin formation and are consistent with the hypothesis that hemoglobin oxidation is important for hemozoin formation in *P. falciparum*.

Hemozoin production is restricted to the fv. To examine whether riboflavin treatment affects the ultrastructure or size of the fv, ring-stage parasite-infected cells were incubated in the absence or presence of riboflavin. The cells were processed for transmission electron microscopy after 24 or 48 h of incubation. Examination of thin sections showed that the average diameter of the fv was significantly reduced in treated cells. The average fv diameter after 24 h of treatment was 1.0 ± 0.08 μm, compared to 1.86 ± 0.1 μm in mock-treated cells (P, <0.001) (Fig. 3A, B, and D). Treatment for 48 h caused a further reduction in the diameter of the fv (0.66 ± 0.06 μm) (Fig. 3C and D), which was statistically significant (P, <0.009) compared to the effect seen after 24 h.

The digestion of hemoglobin and hemozoin production are thought to be essential for parasite growth. Since exposure to

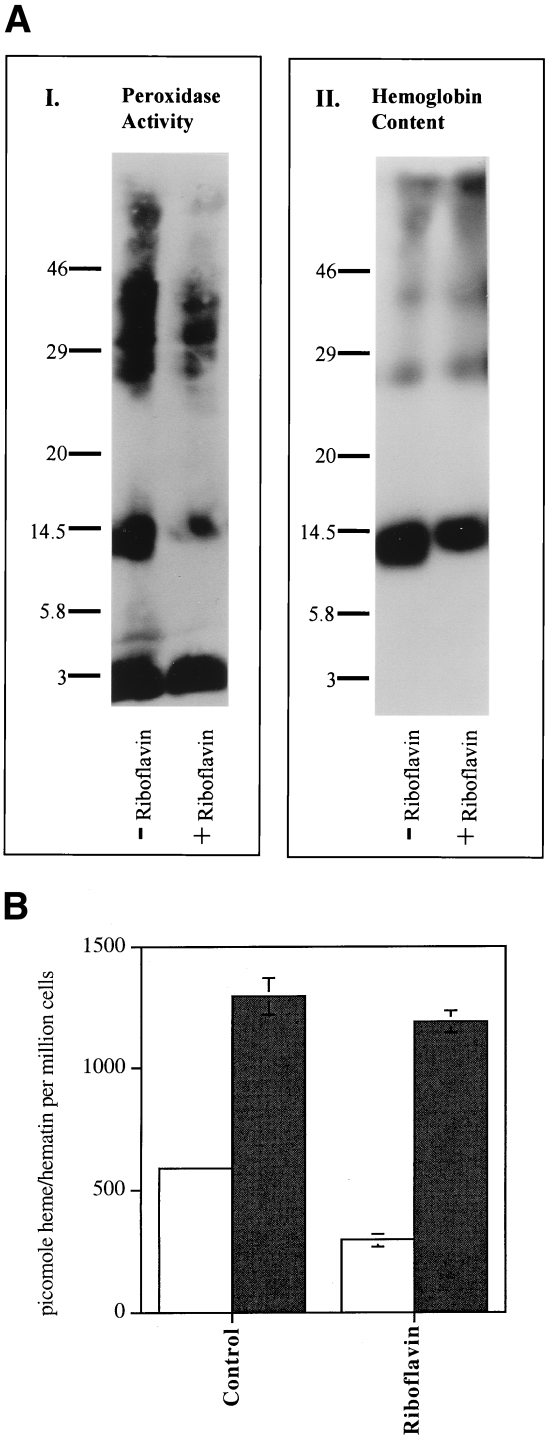


FIG. 2. Effects of riboflavin on the peroxidase activity of methemoglobin, hemozoin formation, and hemoglobin uptake. Cultures of ring-stage parasite-infected erythrocytes were incubated in the absence or presence of 100 μM riboflavin for 24 h, and parasites were isolated by saponin lysis. (A) Peroxidase activity and hemoglobin content were determined as described in the legend to Fig. 1A. (B) Levels of hemozoin produced (open bars) were determined by conversion to hematin. The amount of hemoglobin ingested (by the parasite) (solid bars) was derived by subtracting the amount of hemoglobin released (by sorbitol lysis) from the cytosol of infected erythrocytes from the total hemoglobin content found in uninfected erythrocytes. The bars represent the mean of three independent determinations ± the standard error of the mean.

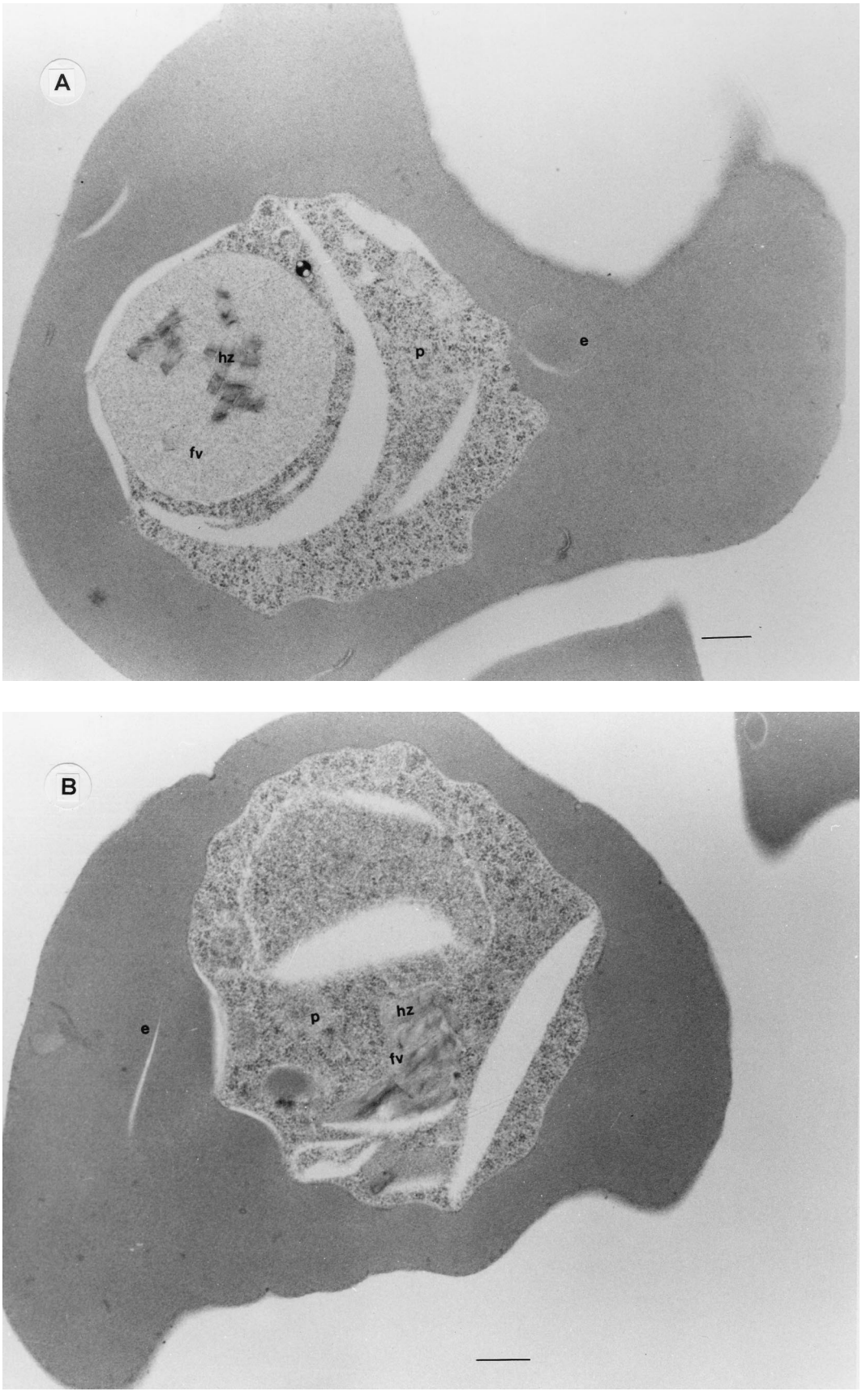
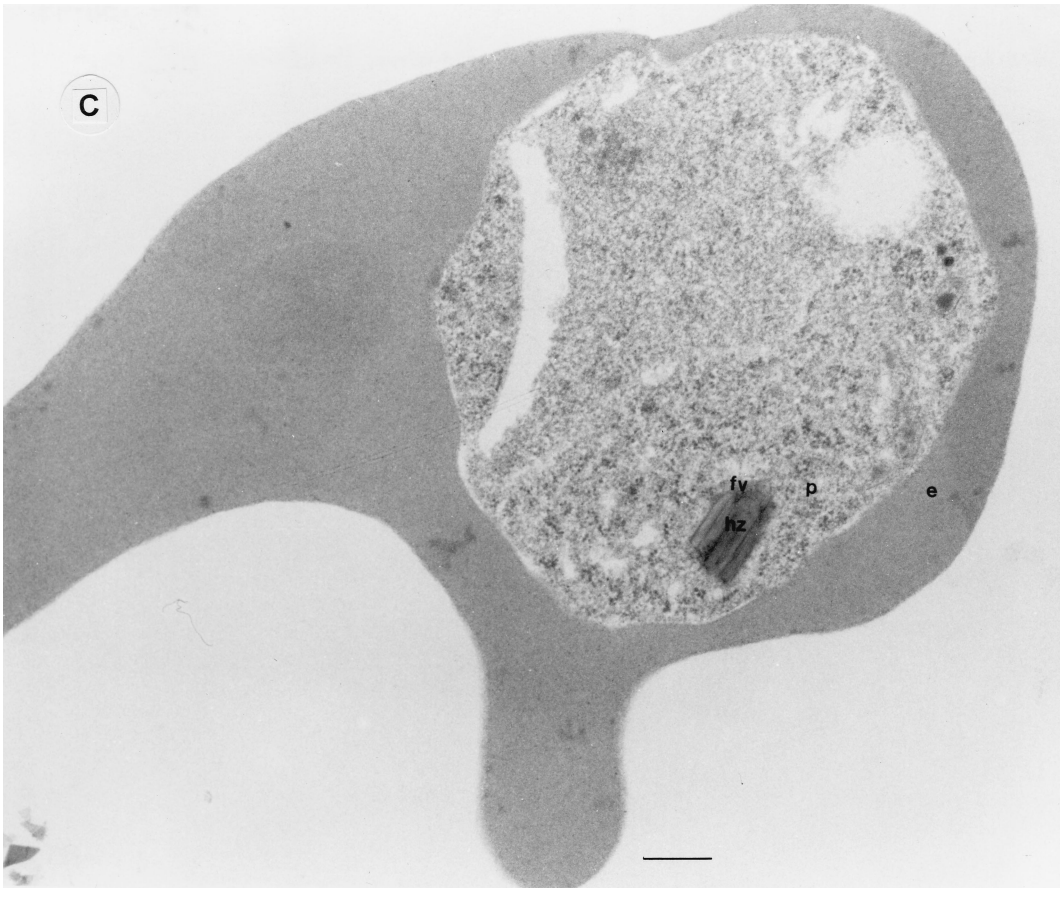


FIG. 3.



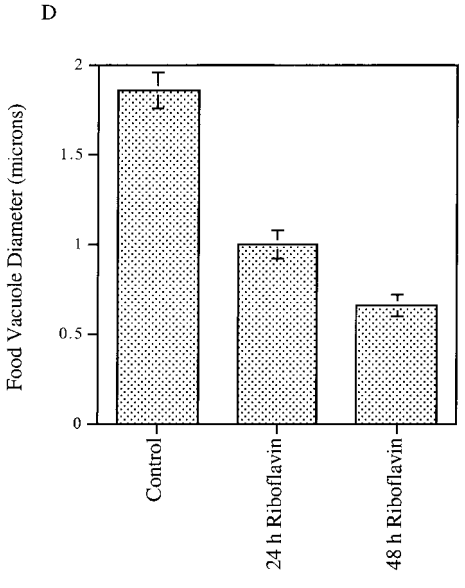


FIG. 3. Effects of riboflavin on the morphology of the fv. Infected erythrocytes were treated with or without riboflavin (100 μM) for the indicated times and prepared for electron microscopy by embedding in Spurr's resin, and thin sections were obtained. (A) Mock-treated infected erythrocyte. (B and C) Infected erythrocytes treated with riboflavin for 24 and 48 h, respectively. hz, hemozoin; p, parasite; e, erythrocyte. Scale bars, 5 μm. (D) fv diameters measured in ultrathin sections from cells that were mock treated or treated with riboflavin for 24 or 48 h. The bars represent the mean ± standard error of the mean. A total of 48 sections were measured.

riboflavin is inhibitory to both, we examined its effects on parasite maturation in the asexual life cycle. In a dose-response study, erythrocytes infected with ring-stage parasites were incubated with 10 to 100 μM riboflavin for 48 h. As shown in Table 1, 15 to 100 μM riboflavin inhibited parasite growth, but parasites in the different treatment groups were not at the same stages of development. At 10 to 15 μM riboflavin, the parasites had largely progressed to form new ring-stage parasites, like untreated parasites. However, at 20 to 25 μM riboflavin, trophozoites from the first cycle that failed to mature were also detected in the culture. Incubation with 50 to 100 μM riboflavin completely inhibited asexual parasite maturation, such that no multinucleated trophozoites or schizonts or new ring-stage parasites were seen in the culture. A significant proportion of parasites in this group was surrounded by ghost erythrocyte membranes, and the cytosol showed an abnormal pink stain in Giemsa-stained smears, consistent with the accumulation of undigested hemoglobin.

To examine whether riboflavin affects another target(s) in addition to fv, trophozoite-stage parasites that contained a fully developed fv were cultured for 24 h with different concentrations of riboflavin. As shown in Table 2, at all concentrations of riboflavin tested, parasitemia was significantly reduced (85 to 93%). This result suggests that there may be another riboflavin-sensitive target(s) besides the fv.

In summary, there may be at least two riboflavin-sensitive sites in the parasite. One is the fv, where the inhibition of methemoglobin and hemozoin formation and a reduction in fv size correlate with a block in asexual parasite maturation to

TABLE 1. Effects of riboflavin on parasite growth after 48 h in culture

Riboflavin concn (μM)	% Parasitemia with:		
	New rings	Trophozoites	Schizonts
0	38.5	0.4	0.1
10	34.6	0.2	0.2
15	21.9	0.6	0.3
20	4.8	1.1	0.4
25	1.3	2.6	0.0
50	0.0	4.4	0.0
100	0.0	4.4	0.0

[a] Ring-stage parasites were cultured with the indicated concentrations of riboflavin for 48 h. Parasitemia was determined by counting the number of parasites in 1,000 erythrocytes on Giemsa-stained blood smears.

schizogony. Additional targets presently unknown remain sensitive to riboflavin after the fv is formed.

DISCUSSION

We provide strong evidence that the malaria parasite converts hemoglobin to methemoglobin and that this process can be inhibited by riboflavin. Although the heme pocket, with its hydrophobic structure, shields the ferrous iron against oxidation, low levels of hemoglobin are continuously oxidized in erythrocytes. Under normal conditions, methemoglobin in erythrocytes is reduced by NADH-cytochrome b_5/cytochrome b_5 reductase and maintained at levels below 1%. However, when methemoglobin content increases due to the presence of oxidant drugs, increased pH, or other exogenous factors, the reducing capacity of the enzyme may be exceeded. Methylene blue or riboflavin is used to treat patients with high levels of methemoglobin. The proposed mechanism of action of these compounds involves their reduction by NADPH-methemoglobin reductase and the subsequent reduction of methemoglobin by reduced methylene blue or riboflavin. The K_m of human NADPH-methemoglobin reductase for riboflavin is 50 μM (33); therefore, 10 to 100 μM riboflavin was used in our experiments.

The malaria parasite ingests a significant proportion of host cell hemoglobin in the fv, whose acidic pH is favorable to the oxidation of hemoglobin to methemoglobin (20). Friedman et al. used spectrophotometric methods to show that a higher level of infection results in a higher methemoglobin content (10). However, Hempelmann et al. compared the methemoglobin levels of uninfected and *P. falciparum*-infected erythrocytes by isoelectric gel electrophoresis (14) and found no difference. Thus, the oxidation state of hemoglobin in *P. falciparum* remains unresolved. Specifically, Hempelmann et al. did not account for monomers and dimers of methemoglobin formed as a result of partial digestion of hemoglobin in the fv, and Friedman et al. did not resolve whether the increase in hemoglobin oxidation occurred in the erythrocyte or in the parasite.

We used two different methods to show that the methemoglobin content in the malaria parasite was indeed elevated and that ingested hemoglobin was oxidized during processing. In the spectrophotometric method, the difference in absorbance at 630 nm with or without KCN was used to establish that the methemoglobin content of isolated *P. falciparum* was higher than that of infected or uninfected erythrocyte cytosol. Hemozoin interferes with hemoglobin measurement at 540 nm (27), raising the issue of whether it affects the spectrophotometric measurement of methemoglobin. However, this is unlikely for two reasons. First, methemoglobin is measured as a net difference in absorbance. Second, the measurement is done at an acidic pH, at which hemozoin is insoluble and thus is not present in the high-speed supernatant that contains hemoglobin.

We have developed a new method of estimating the relative methemoglobin content of unknown samples based on the observation that methemoglobin has higher peroxidase activity than hemoglobin. The major advantages of this method over the spectrophotometric determination are that it uses 10- to 100-fold less material and measures the activity of proteins that are purified by SDS-PAGE, thus minimizing contamination from other proteins and hemozoin. Using the peroxidase assay, we confirmed that the parasite induces an increase in hemoglobin oxidation inside the parasite and not in the infected erythrocyte cytosol. The induction of oxidation is higher in trophozoites and schizonts than in ring-stage parasites. Since hemozoin formation occurs in trophozoites and schizonts, it is possible that the oxidation process is necessary for hemozoin formation. Our results are consistent with those of Vander Jagt et al., who showed by visible-absorption-spectrum measurements that isolated fv's contained a mixture of hemoglobin and methemoglobin, whereas infected or uninfected erythrocyte lysates contained only native hemoglobin (32).

In preliminary studies, we found that 1 nM methylene blue killed ring-stage parasites (data not shown), as previously reported in the literature (1). However, since we detected no appreciable methemoglobin formation during the ring stage, the mechanism of action of methylene blue may not involve the reduction of methemoglobin in the parasite. It is possible that methylene blue and riboflavin have distinct targets in the parasite. Unlike treatment with methylene blue, treatment with riboflavin (up to 500 μM) had no effect on ring-stage parasites. In contrast, treatment of ring-stage parasites with 50 to 100 μM riboflavin for 24 h reduced methemoglobin content and hemozoin formation by more than 50%. Thus, methemoglobin reduction, inhibition of hemozoin formation, fv development, and arrest of asexual parasite development to schizogony are correlated, suggesting that these events may be linked.

The effects of riboflavin reported here apparently differ from those reported in previous studies showing that the ability of infants from Papua New Guinea to suppress *P. falciparum* infections is correlated with riboflavin deficiency (30). *P. berghei*-infected rats fed a riboflavin-deficient diet were also able to suppress the initial infection better than control rats (17). These results led Dutta et al. (8) to suggest that riboflavin deficiency could be used to treat human malaria. However, as pointed out by Thurnham (29), the effect of nutrient deficiency on parasite growth is not limited to riboflavin but, in general, nutrient deprivation is detrimental to both parasite and host. The reason for the antimalarial activity of riboflavin deficiency may be due to a decrease in the activity of reductive enzymes such as glutathione reductase, which require ribofla-

TABLE 2. Effects of riboflavin on trophozoite development

Riboflavin concn (μM)	% Parasitemia with:	
	Rings	Trophozoites
0	13.4	0.0
50	2.0	0.0
100	1.9	0.3
150	2.3	0.5
200	2.2	0.2
300	0.7	0.2

[a] Trophozoite-stage parasites were cultured with the indicated concentrations of riboflavin for 24 h. Parasitemia was determined by counting the number of parasites in 1,000 erythrocytes on Giemsa-stained blood smears.

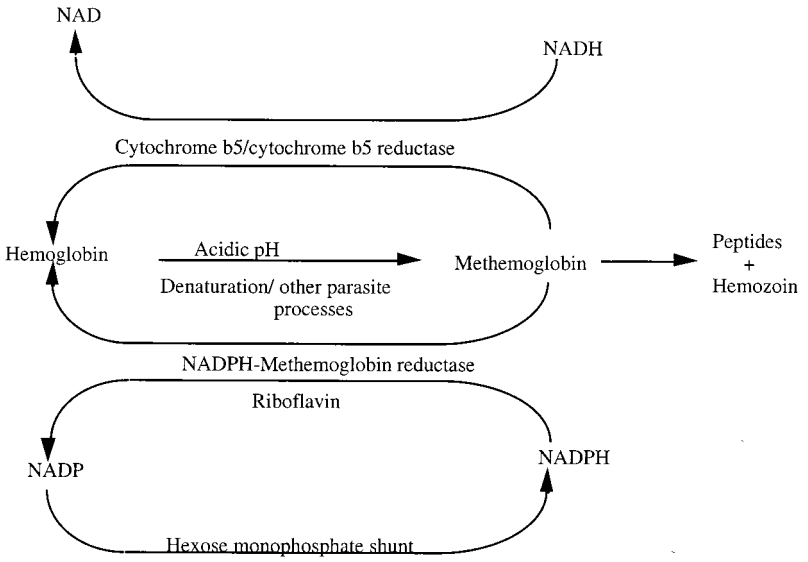


FIG. 4. Proposed model for hemoglobin oxidation and riboflavin action in *P. falciparum*-infected erythrocytes.

vin as a cofactor. This decreased activity lowers glutathione levels, resulting in increased lipid peroxidation (5), which is detrimental to the parasite (oxidative stress hypothesis). Consistent with the oxidative stress hypothesis, several riboflavin and flavin analogues have been shown to have antimalarial activity (3, 4, 13), but their mode of action may not be through the inhibition of the glutathione reducing system (13).

We propose that the continued reduction of methemoglobin by riboflavin creates a futile cycle (Fig. 4) in which hemoglobin is continually oxidized (since the conditions in the fv favor oxidation) and reduced. The model predicts that hemozoin formation will be inhibited, since the cycle reduces the amount of methemoglobin and/or hemoglobin available for further processing. The continued utilization of NADPH means that its availability to enzymes, such as glutathione reductase, that require this cofactor for activity will be severely limited. The sum total of these effects would culminate in the arrest of parasite maturation and differentiation.

In conclusion, riboflavin severely affects the development and function of the fv of *P. falciparum*. The concentrations required for its antimalarial activity are similar to those used to treat human patients with congenital methemoglobinemia over several years with no adverse effects. The presence of two or more targets for riboflavin in *P. falciparum* favors its use as an antimalarial agent because this characteristic decreases the probability of the emergence of resistant strains.

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