

# Heme Biosynthesis by the Malarial Parasite

IMPORT OF  $\delta$ -AMINOLEVULINATE DEHYDRASE FROM THE HOST RED CELL\*

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The mouse and human malarial parasites, *Plasmodium berghei* and *Plasmodium falciparum*, respectively, synthesize heme *de novo* following the standard pathway observed in animals despite the availability of large amounts of heme, derived from red cell hemoglobin, which is stored as hemozoin pigment. The enzymes,  $\delta$ -aminolevulinic dehydratase (ALAD), coproporphyrinogen oxidase, and ferrochelatase are present at strikingly high levels in the *P. berghei* infected mouse red cell *in vivo*. The isolated parasite has low levels of ALAD and the data clearly indicate it to be of red cell origin. The purified enzyme preparations from the uninfected red cell and the parasite are identical in kinetic properties, subunit molecular weight, cross-reaction with antibodies to the human enzyme, and N-terminal amino acid sequence. Immunogold electron microscopy of the infected culture indicates that the enzyme is present inside the parasite and, therefore, is not a contaminant. The parasite derives functional ALAD from the host and the enzyme binds specifically to isolated parasite membrane *in vitro*, suggestive of the involvement of a receptor in its translocation into the parasite. While, ALAD, coproporphyrinogen oxidase, and ferrochelatase from the parasite and the uninfected red cell supernatant have identical subunit molecular weights on SDS-polyacrylamide gel electrophoresis and show immunological cross-reaction with antibodies to the human enzymes, as revealed by Western analysis, the first enzyme of the pathway, namely,  $\delta$ -aminolevulinic synthase (ALAS) in the parasite, unlike that of the red cell host, does not cross-react with antibodies to the human enzyme. However, ALAS enzyme activity in the parasite is higher than that of the infected red cell supernatant. We therefore conclude that the parasite, while making its own ALAS, imports ALAD and perhaps most of the other enzymes of the pathway from the host to synthesize heme *de novo*, and this would enable it to segregate this heme from the heme derived from red cell hemoglobin degradation. ALAS of the parasite and the receptor(s) involved in the translocation of the host enzymes into the parasite would be unique drug targets.

Chloroquine resistance in malarial parasite is assuming serious proportions, and the necessity to discover new drugs to treat malaria is a major challenge. This calls for the identification of newer drug targets.

Studies in this laboratory have revealed that the human malarial parasite, *Plasmodium falciparum*, synthesizes heme *de novo*. The parasite is able to incorporate [2-<sup>14</sup>C]glycine and 4-<sup>14</sup>C-labeled  $\delta$ -aminolevulinic acid (ALA),<sup>1</sup> but not [1-<sup>14</sup>C]glutamate into heme, indicating that the parasite manifests the glycine pathway observed in liver and erythrocytes. ALA synthase and ALA dehydratase activities were also detected in the parasite (1). The synthesis of heme *de novo* by the parasite in the intraerythrocytic stage is rather surprising, since the parasite obtains a surplus of heme from the degradation of the red cell hemoglobin. However, this heme is stored as the inert hemozoin pigment (2, 3). Further, most hemoflagellates, parasitic protozoa in both plants and animals, need preformed heme for growth (4).

*Leishmania tarentolae* was found to contain free porphyrins but required intact iron-protoporphyrin for growth in culture, suggesting that it may lack ferrochelatase, the enzyme required to incorporate iron into protoporphyrin (5). *Trypanosoma cruzi* was shown to require hemin/hemoglobin for growth. It was reported to manifest ALA dehydratase activity, but porphobilinogen deaminase was absent. However, functional ferrochelatase could be detected, leading to the conclusion that the parasite has lost part of its heme pathway due to mutations in some of the enzyme sequences, necessitating preformed hemin requirement (6).

The malarial parasite contains cytochromes (7, 8), and studies in this laboratory have demonstrated a requirement for heme to sustain optimal protein synthesis (9). *De novo* synthesis of heme is vital to the malarial parasite, since treatment of the culture with succinylacetone, a specific inhibitor of ALA dehydratase, leads to death of the parasite. It has, therefore, been concluded that the parasite utilizes the heme synthesized *de novo* for metabolic purposes, whereas it stores the surplus heme, which is toxic, derived from hemoglobin degradation as inert hemozoin pigment (1, 9). In view of these results and the possibility that the heme biosynthetic pathway of the malarial parasite could be a new drug target, studies have been carried out on the possible origin of the enzymes of the pathway. The results indicate the interesting feature that the parasite genome may not code for all the enzymes of the pathway. While

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<sup>1</sup> The abbreviations used are: ALA,  $\delta$ -aminolevulinic acid; ALAS,  $\delta$ -aminolevulinic synthase; ALAD,  $\delta$ -aminolevulinic dehydratase; CPO, coproporphyrinogen oxidase; FC, ferrochelatase; IRS, infected red cell supernatant; URS, uninfected red cell supernatant; SA, succinylacetone; RBC, red blood cell; GST, glutathione-S-transferase; GST-RXR, glutathione S-transferase-retinoic acid X receptor fusion proteins; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBG, porphobilinogen.

the parasite makes ALA synthase, the first enzyme of the pathway, it imports ALA dehydrase and perhaps most of the other enzymes of the pathway from the host red cell to acquire the heme biosynthetic potential.

#### EXPERIMENTAL PROCEDURES

##### Materials

Antibodies raised against purified CPO and FC preparations from the human red blood cells in rabbits (10) were used. Antibodies to human red cell ALAS and full-length mouse erythroid ALAD clone were kind gifts from Dr. Gloria C. Ferreira, University of South Florida and Dr. Terry Bishop, Johns Hopkins University, respectively. [2-<sup>14</sup>C]Glycine and 4-<sup>14</sup>C-labeled  $\delta$ -aminolevulinic acid were obtained from BRIT (Bombay) and NEN Life Science, respectively.

##### Methods

**Parasite Maintenance and Isolation**—Swiss mice were injected with *P. berghei*, and blood was removed at different stages of parasitemia ranging from 30 to 80%. The parasitized red cells as well as the red cells from uninfected mice (control) were spun down and put into overnight culture as described for *P. falciparum* (11). In some experiments, *P. falciparum* maintained on O<sup>+</sup> human red blood cells in culture (about 8% parasitemia) as described earlier (1, 11) was also used. After appropriate treatment of culture, the cells were isolated and lysed with 0.15% (w/v) saponin (12). The parasite pellet as well as the infected red cell supernatant (IRS) were obtained by centrifugation. The red cells from uninfected mice were processed similarly to isolate the uninfected red cell supernatant (URS) and the membrane pellet.

**Heme Synthesis by *P. berghei* in Culture**—Mice were injected with *P. berghei* and blood was removed at different stages of parasitemia ranging from 30 to 80%. The parasitized as well as control red blood cells were spun down and put into overnight culture (8 h), during which time they were incubated with [4-<sup>14</sup>C]ALA (1  $\mu$ Ci) or [2-<sup>14</sup>C]Glycine (12.5  $\mu$ Ci). The red cells were pelleted, and the parasite pellet, IRS, and URS were isolated after lysis. The fractions were extracted with ethyl acetate/acetic acid (3:1). The extract was washed with water and then 1.5 N HCl to remove the precursor used and all the porphyrins. The final ethyl acetate layer was washed twice with water to remove acid and used to measure radioactivity incorporated into heme (13). Separate analysis of the ethyl acetate layer by chromatography has revealed that the radioactivity is entirely accounted for by heme (14). The first water wash and the 1.5 N HCl-extracted fractions were used to analyze for radioactivity in ALA plus porphobilinogen (PBG) and coproporphyrin plus protoporphyrin, respectively (13). ALA and porphobilinogen were separated by fractionation on a Dowex-1 (acetate) column (15).

**Assay of Enzymes**—ALAS, ALAD, and FC activities were assayed in isolated fractions of the parasite, IRS, and URS. The parasite pellet was lysed with 20 mM Tris-HCl (pH 7.5) containing 0.2% Triton X-100 by repeated homogenization, and the membrane fraction was pelleted at 8000  $\times$  g (15 min). This fraction was used to assay ALAS and FC, since the activities were located in this fraction. The assay mixture for ALAS contained the following (16): Tris, pH 7.5 (50 mM), glycine (100 mM), sodium succinate (10 mM), MgCl<sub>2</sub> (5 mM), coenzyme A (500  $\mu$ M), ATP (5 mM), pyridoxal phosphate (200  $\mu$ M), partially purified *Escherichia coli* succinyl-CoA synthase (20  $\mu$ g), and the membrane protein (100  $\mu$ g). The mixture was incubated for 1 h at 37 °C. The product was converted to porphobilinogen by the addition of purified ALAD, and porphobilinogen formed was estimated using modified Ehrlich reagent (17). FC was also assayed in the membrane fraction (18), and the assay mixture contained Tris, pH 7.5 (20 mM), protoporphyrin 1 X (100  $\mu$ M),  $\beta$ -mercaptoethanol (1 mM), <sup>59</sup>FeCl<sub>3</sub> (2.5  $\mu$ Ci), and membrane protein (50  $\mu$ g). The mixture was incubated for 1 h at 37 °C under anaerobic conditions. Radioactive heme formed was then extracted and radioactivity measured. ALAD was assayed in the soluble fractions after passing the same through a DEAE-Sepharose column to remove hemoglobin. The enzyme activity was located in the 0.2 M NaCl eluate, and the assay mixture contained Tris, pH 7.5 (20 mM), ALA (50  $\mu$ M), ZnCl<sub>2</sub> (10  $\mu$ M),  $\beta$ -mercaptoethanol (5 mM), and enzyme fraction (20  $\mu$ g of protein). The assay mixture was incubated at 37 °C for 1 h. The porphobilinogen formed was estimated using modified Ehrlich reagent (17).

**Purification of ALAD from the Parasite (*P. berghei*) and RBCs**—ALAD was purified from the parasite pellet and mouse and human red blood cells by a slight modification of the known procedure (19). The parasite and red cell lysates were spun at 8000  $\times$  g for 15 min, and the soluble fractions were subjected to the following steps: DEAE-Sepharose chromatography, ammonium sulfate fractionation (0–45%), and octyl-

Sepharose and phenyl-Sepharose chromatography. The last two column steps were repeated with the preparation obtained to get the pure protein. The preparation from human red blood cells was used to raise antibodies in rabbits.

**Western Blot Analysis**—The purified ALAD preparations (75 ng) from the parasite and mouse red blood cells were analyzed on SDS-PAGE (10% gels) and transferred to nitrocellulose for immunoblot analysis. Similarly, membrane fractions (8000  $\times$  g pellet, 100  $\mu$ g of protein) from the parasite, IRS, and URS were used for Western analysis of ALAS, CPO, and FC. The crude soluble fraction (75  $\mu$ g of protein) was also used for analysis of ALAD. The blots were screened with antibodies to the human enzyme preparations, diluted in the range 1:200 to 1:1000. The blots were finally analyzed with goat anti-rabbit antibodies conjugated to alkaline phosphatase.

**N-terminal Sequence Analysis**—Purified ALAD from the parasite (*P. berghei*) was subjected to N-terminal sequence analysis using a Shimadzu gas phase protein sequencer (PSQ1).

**Immunogold Electron Microscopy**—Immunogold electron microscopy was carried out (20) with the isolated parasite (*P. berghei*), as well as with the infected and uninfected red cells. These were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and embedded in LR Gold resin. Sections (60–70 nm) were cut and picked on copper grids. In experiments where purified ALAD was incubated with parasite membrane to demonstrate binding, the membrane preparation was first sonicated briefly to get a homogenous preparation. After incubation with ALAD and washing, the membrane pellet was diluted severalfold and used directly for coating copper grids by incubating at room temperature for 1 h. All of the grids were washed with PBS containing 3% bovine serum albumin and incubated for 2 h with ALAD antibodies (1:1000 dilution), washed again, and then incubated with goat anti-rabbit IgG conjugated to gold particles (10 nm in diameter) in PBS containing 1% bovine serum albumin. The grids were stained with 2% osmium tetroxide and examined under the electron microscope.

**Influence of Succinylacetone Treatment of the Host Red Cell on ALAD Activity of the Parasite (*P. falciparum*)**—Succinylacetone is known to be an irreversible inhibitor of ALAD activity (21). Fresh human RBCs (in complete medium) were incubated with succinylacetone (500  $\mu$ M in final concentration) overnight at 4 °C. These as well as RBCs not treated with succinylacetone (control) were washed thoroughly with culture medium and then incubated with PBS containing 2 mM ALA for 2 h at 37 °C to remove any free succinylacetone by converting it nonenzymatically to a pyrrole (22). A 3-day-old culture (1.5 ml) of *P. falciparum* was subcultured in two vials, one receiving succinylacetone-treated RBCs and the other control RBCs. After another 4 days, a second subculturing was done with the respective RBCs. After another 48 h, the cultures were incubated with 1  $\mu$ Ci of [4-<sup>14</sup>C]ALA overnight as described earlier. Radioactivity incorporation into heme was measured in the parasite and red cell supernatant fractions. In another experiment, several vials were pooled after second subculturing, and ALAD activity was assayed in the parasite and red cell supernatant fractions. To ensure that the succinylacetone-treated RBCs used in culturing did not have any carryover of free succinylacetone to inhibit ALAD activity, untreated and succinylacetone-treated RBCs were lysed, and the enzyme activity was assayed in each of the fractions as well as when they were mixed.

**Specific Binding of Host Red Cell ALAD to the Parasite (*P. berghei*) Membrane**—The parasites were isolated from infected mouse red blood cells by saponin lysis as described earlier. The parasites were then lysed in 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% Triton X-100 for 1 h at 4 °C. The lysate was spun down at 12,000  $\times$  g for 30 min, and the pellet was washed with PBS. The membrane pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% Triton X-100 and an aliquot (200  $\mu$ g of protein) was incubated with partially purified ALAD from mouse RBCs at room temperature for 1 h with gentle shaking. A similar incubation was carried out with mouse red cell membrane and trypsin-treated, washed parasite membrane. Another control employed was the incubation of bacterially expressed glutathione S-transferase and glutathione S-transferase-retinoic acid X receptor fusion proteins with the parasite membrane under similar conditions. After the incubations, the membrane fractions were reisolated by centrifugation and washed repeatedly with PBS, and the pellet was then subjected to SDS-PAGE (10% gels) followed by Western analysis with anti-ALAD and anti-fusion protein antibodies. In addition, the membrane fraction was also examined through immunoelectron microscopy as described earlier.

**Determination of the Number of Binding Sites for ALAD on the Parasite Membrane**—Full-length mouse erythroid ALAD-cDNA was cloned and expressed with histidine tag in the T7 polymerase promoter based vector, pRSETB. The transformed *E. coli* cells were grown in 5 ml

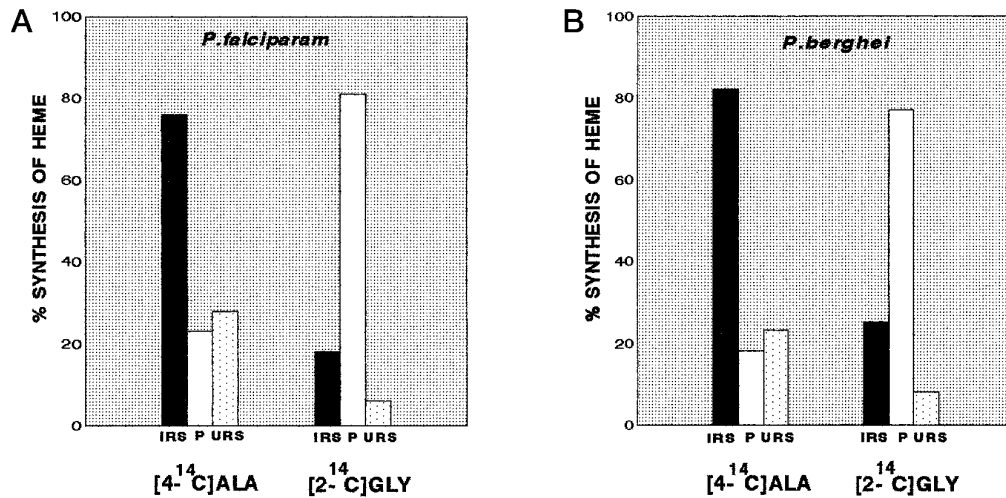


FIG. 1. Heme synthesis by *P. falciparum* and *P. berghei* in culture. *P. berghei*-infected and -uninfected mouse red blood cells were spun down and put into overnight culture in microtiter plates. *P. falciparum* was maintained in culture in O<sup>+</sup> red blood cells. The cultures were incubated with [4-<sup>14</sup>C]ALA (1  $\mu$ Ci) or [2-<sup>14</sup>C]glycine (12.5  $\mu$ Ci) for 8 h in the case of *P. berghei* and 12 h in the case of *P. falciparum*. The red cells were pelleted, and the parasite, IRS, and URS were isolated. The fractions were extracted with ethyl acetate/acetic acid (3:1), and radioactivity incorporated into heme in the ethyl acetate layer was measured after the appropriate washes (13).

of LB medium (inoculated from an overnight culture) and after 2 h of growth (OD 0.6), 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside and 200  $\mu$ Ci of [<sup>35</sup>S]methionine were added. After another 2½ h of incubation, labeled ALAD was isolated from *E. coli* cells and purified on a nickel-nitrotri-acetic acid (Qiagen) column, using a single-step affinity procedure. The parasite membrane (2.4 mg of protein) was solubilized using 2.5% Triton X-100, 0.5% sodium cholate, and 0.5 M NaCl for suspension followed by sonication. The insoluble debris was removed by centrifugation, and the solubilized membrane was loaded onto nitrocellulose membrane filters. These filters were then blocked with 5% skimmed milk powder in TTBS (0.1% Triton X-100, 50 mM Tris-buffered saline) containing 1% bovine serum albumin. The nitrocellulose membrane was then incubated with increasing amounts of labeled ALAD for 1 h in the presence and absence of a 100-fold excess of nonradioactive ALAD. The nitrocellulose membrane filters were washed thrice with TTBS and dried, and radioactivity was measured. The data were used to generate the saturation curve and the Scatchard plot to calculate the dissociation constant and the number of binding sites.

## RESULTS

**De Novo Heme Synthesis in *P. berghei* and *P. falciparum***—In the case of *P. berghei*, blood from infected and uninfected mice was spun down, and the cells were incubated with [2-<sup>14</sup>C]glycine or [4-<sup>14</sup>C]ALA for 8 h in culture. In the case of *P. falciparum*, the parasite maintained in culture was incubated with the radiolabeled precursors overnight. The cells were then pelleted down, washed, and lysed with saponin, and the parasite pellet, IRS, and URS were used for measurement of radioactivity in the heme fraction. The results presented in Fig. 1 (A and B) indicate that similar results are obtained with *P. berghei* and *P. falciparum*. The parasite-infected red cell synthesizes 5–8-fold more heme than the uninfected red cell. Certain interesting features of the incorporation pattern of the precursors into heme are also evident. With [4-<sup>14</sup>C]ALA as precursor, 80% of the radioactive heme is recovered in IRS, whereas with [2-<sup>14</sup>C]glycine as precursor, the bulk of the radioactive heme is recovered in the parasite pellet.

Next, it was of interest to examine the heme biosynthetic potential of the isolated fractions. These experiments were carried out with *P. berghei*, since high yields of the parasite could be obtained from infected mice. The IRS, URS, and the parasite pellet were individually incubated with [4-<sup>14</sup>C]ALA, and the incorporation of radioactivity into heme and intermediates of the pathway was measured. The results presented in Table I indicate certain interesting features. First, maximum

TABLE I  
Synthesis of heme by isolated fractions from infected (*P. berghei*) and uninfected red cells in vitro

Infected or uninfected blood was spun down, pelleted red cells were lysed, and the washed parasite pellet (P), IRS, and URS were incubated with 1  $\mu$ Ci of [4-<sup>14</sup>C]ALA for 8 h. The various intermediates were isolated (13), and radioactivity was measured. The results presented are those obtained in a typical experiment.

Metabolites	Radioactivity incorporated		
	P	IRS	URS
	<i>cpm/4 × 10<sup>8</sup> cells</i>		
ALA (unutilized)	499,950	55,000	62,000
PBG	141,400	37,000	24,000
CPP plus PRP <sup>a</sup>	250,000	177,000	790,000
Heme	193,000	755,000	125,000

<sup>a</sup> Coproporphyrin plus protoporphyrin.

heme synthesis is again seen in IRS that is ~ 4–5-fold more than URS or parasite pellet. The parasite pellet has a substantial amount of [4-<sup>14</sup>C]ALA remaining unutilized compared with URS or IRS. This correlates with the low levels of ALAD detected in *P. berghei* (this study) as well as in *P. falciparum* (1). Nevertheless, the parasite converts the porphyrin intermediates to heme efficiently and is more effective than the URS. This suggests that FC, catalyzing iron incorporation into protoporphyrin, could be present at significant levels in the parasite.

**Assay of Heme Biosynthetic Enzymes in *P. berghei***—The next step was to assay the activities of ALAS, ALAD, and FC in *P. berghei* and mouse IRS and URS fractions. ALAS and FC activities were detected in the 8000 × *g* pellet in each case, whereas ALAD activity was detected in the soluble fractions. The results presented in Table II indicate that ALAS activity in the parasite is 3–4-fold more than in IRS or URS. FC activity in the parasite is as high as that in IRS and is 9–10-fold more than in URS. ALAD activity in the parasite is ~20-fold lower than IRS and 4–5-fold lower than in URS. From the results presented, it is also clear that, whereas the activity of ALAS is the same in infected and uninfected RBC preparations, the activities of ALAD and FC are at least 5-fold greater in IRS than in URS.

**Parasite and Red Cell ALAD Activities Are Due to the Same Protein Species**—ALAD was purified to homogeneity from *P. berghei* (Fig. 2), mouse and human red blood cells. The human

TABLE II  
ALAS, ALAD, and FC activities in isolated fractions from infected (*P. berghei*) and uninfected red cells

The parasite pellet, IRS, and URS fractions were isolated as described earlier. ALAS and FC were assayed in the membrane fraction ( $8000 \times g$  pellet). ALAD was assayed in the soluble fraction after fractionation through DEAE-Sepharose to remove hemoglobin. The assay conditions are given under "Experimental Procedures." The values represent mean  $\pm$  S.D. obtained from three different experiments.

	Enzyme activity		
	ALAS <sup>a</sup>	ALAD <sup>b</sup>	FC <sup>c</sup>
Parasite	0.130 $\pm$ 0.008	0.074 $\pm$ 0.006	1.17 $\pm$ (0.09 $\times$ 10 <sup>7</sup> )
IRS	0.040 $\pm$ 0.012	3.879 $\pm$ 1.023	8.89 $\pm$ (0.81 $\times$ 10 <sup>6</sup> )
URS	0.039 $\pm$ 0.008	0.442 $\pm$ 0.058	1.16 $\pm$ (0.06 $\times$ 10 <sup>6</sup> )

<sup>a</sup> Values for ALAS are in  $\mu$ mol of PBG equivalent/mg of protein.

<sup>b</sup> Values for ALAD are in  $\mu$ mol of PBG/mg of protein.

<sup>c</sup> Values for FC are cpm in heme/mg of protein.

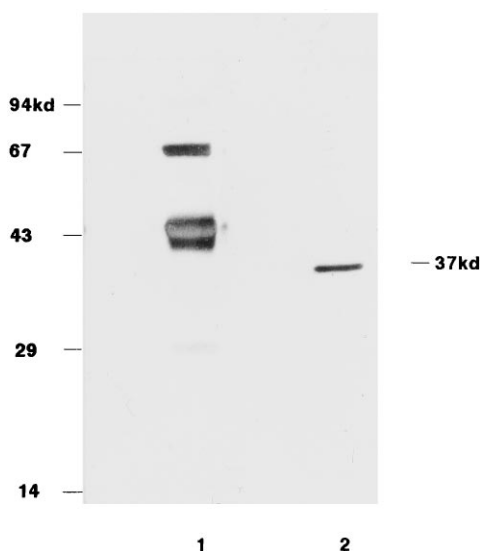


FIG. 2. SDS-PAGE profile of purified ALAD from *P. berghei*. The enzyme was purified from *P. berghei* as described under "Experimental Procedures." The purified preparation was analyzed on SDS-PAGE (10% polyacrylamide gel). Lane 1, molecular weight markers; lane 2, purified ALAD.

enzyme preparation was used to raise antibodies in rabbits. The parasite and mouse red cell (uninfected) enzymes appear identical on the basis of several criteria. Both of the purified preparations show a  $K_m$  of about  $1.5 \times 10^{-4}$  M for ALA. They exhibit a broad pH optimum with a peak at about 7.2. Both preparations show a requirement for zinc. Succinylacetone at a concentration of 50  $\mu$ M brings about 50% inhibition of the activities of both of the enzyme preparations. Both enzyme species have identical subunit molecular mass ( $\sim$ 37 kDa) on SDS-PAGE and cross-react equally well with antibodies to the human enzyme (Fig. 3A). The N-terminal 20-amino acid sequence of both of the preparations is identical (Fig. 3B). In view of the fact that ALAD preparation from humans and mice differ by as many as 3 amino acids and are totally different between mice and yeast in this stretch (23), it has to be concluded that the *P. berghei* and mouse red cell (uninfected) ALAD activities are due to the same protein species.

To further prove that the parasite ALAD is of host red cell origin, attempts were made to demonstrate that when the host red cell ALAD is inhibited irreversibly by succinylacetone, the parasite (*P. falciparum*) cultured with such RBCs also acquires the inhibited enzyme. First, it was found that while the parasite cultured in control RBCs had a detectable ALAD activity, the activity was too low to be detected in parasites derived from succinylacetone-treated RBCs. This was further substantiated



FIG. 3. Western blot analysis and N-terminal sequence of purified ALAD from *P. berghei* and uninfected mouse RBC. A, Western blot was carried out with antibodies to purified ALAD from human RBC. 75 ng of purified protein was analyzed by SDS-PAGE (10% polyacrylamide gel). M, mouse RBC; P, parasite. B, sequence of 20 amino acids at the N terminus of purified ALAD from *P. berghei* and mouse RBC.

TABLE III  
[4-<sup>14</sup>C]ALA incorporation into heme in *P. falciparum* culture maintained on SA-treated RBCs

The experimental details are given under "Experimental Procedures." The parasite was cultured in untreated (control) and SA-treated RBCs. The final culture (1.5 ml) was incubated with 1  $\mu$ Ci of [4-<sup>14</sup>C]ALA overnight, and radioactivity incorporated into the heme fraction isolated from the parasite and red cell supernatant was measured. The results presented are those obtained in a typical experiment.

Fraction	Radioactivity in heme cpm / 1.5 ml culture
Parasite (control RBC)	375
Parasite (SA-treated RBC)	45
RBC supernatant (control)	11,400
RBC supernatant (SA-treated)	1200

by measuring [4-<sup>14</sup>C]ALA incorporation into heme in the parasite and IRS fractions. The results presented in Table III indicate that incorporation of [4-<sup>14</sup>C]ALA into heme in the parasite and IRS fractions derived from succinylacetone-treated RBCs is around 10% of the corresponding fractions derived from control RBCs. ALAD activity assays from control, succinylacetone-treated, and mixed RBC lysates indicate that there is no free succinylacetone left to inhibit the enzyme activity or heme synthesis in the parasite (Table IV). It has also been noted that the parasitemia tends to fall when the parasite is cultured in succinylacetone treated RBCs, and there is a striking inhibition of parasite growth after three or four sub-culturings with such RBCs, indicating that the level of ALAD in the parasite is too low to sustain an optimal rate of *de novo* heme synthesis to support growth.

*Parasite (P. berghei) ALAD Is Not a Contaminant from the Host*—First, the parasite fraction used to assay or purify ALAD was thoroughly washed, till the washings did not carry a detectable enzyme activity. Second, the parasite membrane fraction as isolated after lysis of the intact parasite does not contain detectable ALAD enzyme activity or the protein species detectable by Western analysis under conditions where the activity can be demonstrated in the parasite cytosol fraction. Third, the recoveries of ALAS, ALAD, CPO, and FC proteins in the parasite do not follow the trend seen in the RBC supernatant (Fig. 6). Further, to rule out the presence of ALAD in the parasite fraction as an experimental contaminant, *in situ* immunogold labeling of the enzyme was carried out with the isolated parasite as well as with the infected red cell. In both

cases, immunoelectron microscopy indicates that the grains are seen inside the parasite and not on the periphery, supporting the possibility of import of the host enzyme into the parasite and ruling out experimental contamination (Fig. 4). It is also interesting to note that the infected red cell has many more grains than the uninfected red cell, confirming the enzyme activity model presented in Table II.

**Parasite (*P. berghei*) Membrane Specifically Binds Host Red Cell ALAD**—To examine whether a possible receptor for host red cell ALAD could exist on the parasite membrane, partially purified ALAD from the red cell was incubated with parasite membrane fraction for 1 h at 25 °C, and the membrane fraction was reisolated, washed thoroughly, and then analyzed for ALAD protein by Western analysis after SDS-PAGE. The results presented in Fig. 5A indicate that the parasite membrane can specifically bind to host red cell ALAD. Under identical conditions, the red cell membrane has no sites for binding the protein. Incubation of the parasite membrane with bacterially expressed mammalian glutathione *S*-transferase and glutathione *S*-transferase-retinoic acid X receptor fusion protein does not lead to detectable binding of these proteins. Trypsinized and washed parasite membrane fails to bind ALAD (Fig. 5B, lanes 3–5). The ALAD protein can be recovered in the supernatant fraction, suggesting that the failure to detect membrane binding is not due to degradation of ALAD by leftover trypsin. Finally, the parasite membrane incubated with partially pure ALAD was reisolated, washed, and subjected to immunoelec-

tron microscopy. A specific association of the enzyme with the parasite membrane can be clearly demonstrated, while the red blood cell manifests negligible binding (Fig. 5C).

Attempts were then made to estimate the number of binding sites for ALAD on the parasite membrane. Initial attempts to label the native protein using radiolabeled iodine led to some degradation of the protein and irreproducible binding data. Therefore, the mouse erythroid ALAD was expressed in *E. coli* and biosynthetically labeled with [<sup>35</sup>S]methionine. Since the protein was expressed with a histidine tag, it could be purified on a nickel-nitrotriacetic acid column. Once again, binding experiments with intact parasite membrane gave high background, perhaps due to difficulty in effective washing of the membrane by centrifugation. Therefore, binding of <sup>35</sup>S-labeled ALAD to solubilized parasite membrane loaded on to nitrocellulose membrane filters was carried out. This approach gave reproducible results, and the binding of radioactive ALAD could be chased by the nonradioactive species to an extent of 85–90%. The binding of ALAD to NC filters in the absence of parasite membrane preparation was negligible. The saturation curve and the Scatchard plot are given in Fig. 6 (A and B). The nonspecific binding was deducted to generate the Scatchard plot. The data indicate a fairly high affinity of binding (dissociation constant,  $6.3 \pm 0.4 \times 10^{-9}$ ) with the number of binding sites estimated at  $1550 \pm 26$ /parasite.

**Western Blot Analysis of ALAS, ALAD, CPO, and FC in Parasite, IRS, and URS**—Western analysis of ALAS, CPO, and FC in the membrane fractions of the parasite, IRS, and URS reveals interesting features (Fig. 7). First, antibodies to human erythroid ALAS do not cross-react with the parasite enzyme, although the mouse red cell membrane preparation shows a strong cross-reaction (~72 kDa). However, the membrane preparation used actually shows 3–4-fold more ALAS enzyme specific activity in the parasite than in the mouse red cell. CPO is hardly detectable in the parasite and URS, although strikingly large amounts are seen in IRS (~37 kDa). Separate experiments reveal that when more protein is loaded on gels, the parasite CPO can be shown to move with a mobility identical to that of the host CPO, both cross-reacting with antibodies to the

TABLE IV  
ALAD activity in untreated and SA-treated RBCs used to culture *P. falciparum*

RBCs were treated with SA (500 μM) and then washed with PBS and ALA to remove free SA as described under "Experimental Procedures." ALAD activity was assayed in the red cell lysates. The results represent mean ± S.D. obtained from three different experiments.

Fraction	ALAD activity μmol PBG/mg protein
RBC (untreated control)	0.390 ± 0.048
RBC (SA-treated)	0.003 ± 0.001
Untreated plus treated	0.381 ± 0.052

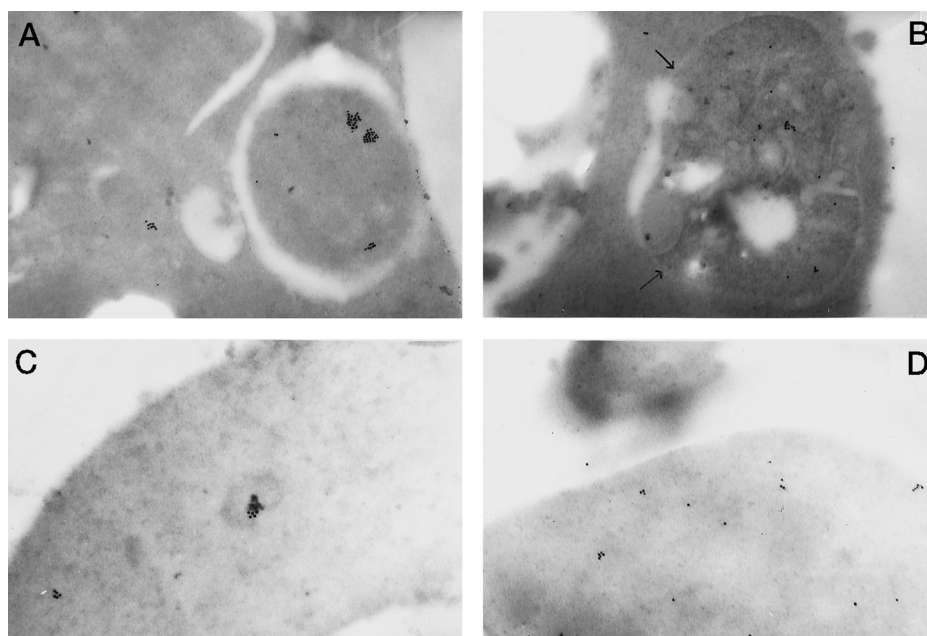
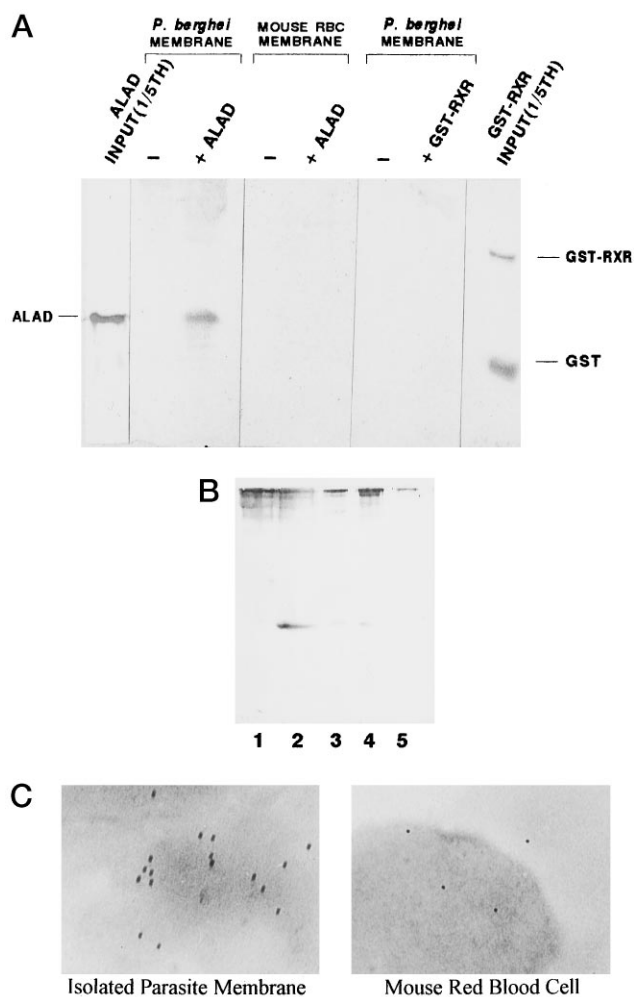


FIG. 4. Immunogold electron microscopy of *P. berghei* trophozoites and infected and uninfected red cells. The isolated parasite as well as the red blood cells were processed as described in the text. The preparations were incubated with ALAD antibodies and then treated with anti-rabbit IgG conjugated to gold particles. A, isolated parasite; B, parasite inside the red cell. Arrow indicates parasite boundary. C, a field of uninfected red cell. D, A field of infected red cell.



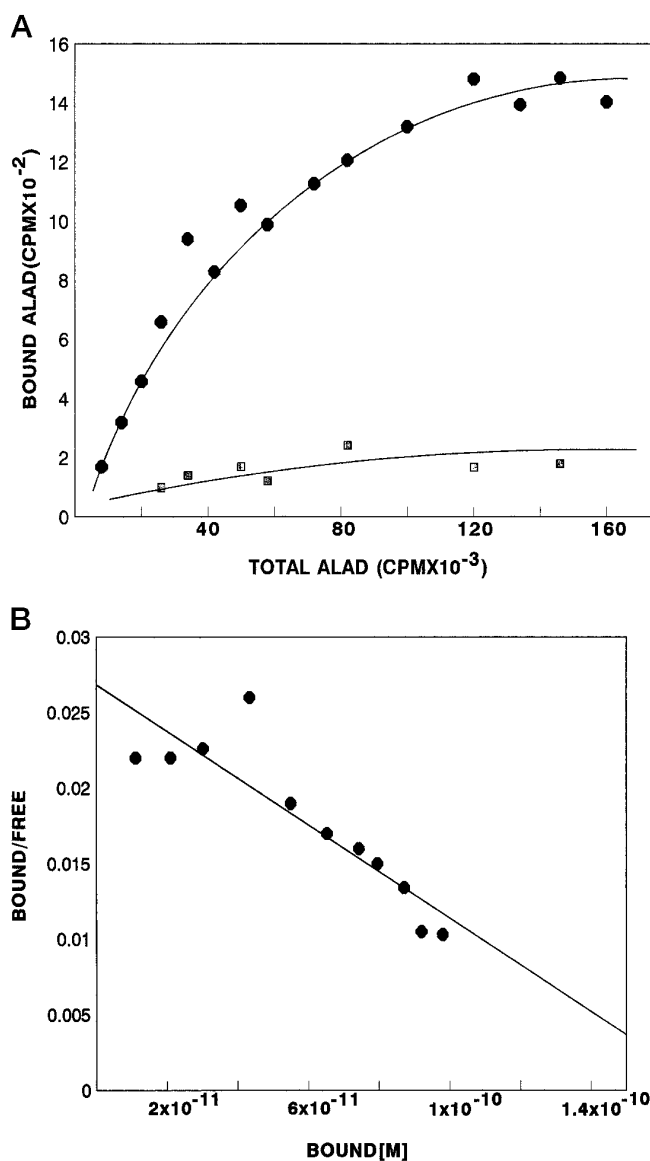
**FIG. 5. Binding of ALAD to parasite membrane.** Binding of partially purified ALAD to parasite membrane, red cell membrane, and trypsin-treated parasite membrane was assessed by Western blot analysis as described under "Experimental Procedures." Binding of bacterially expressed GST and GST-RXR to the parasite membrane was assessed similarly using GST-RXR antibodies. **A**, binding of ALAD, GST, and GST-RXR to parasite and red cell membranes. + and - refer to the data obtained with and without the purified proteins, respectively, added to the incubation mixtures with the membrane preparations. The first and last lanes (left to right) indicate the migration of the purified proteins, ALAD and GST plus GST-RXR, detected using the respective antibodies. **B**, binding of ALAD to trypsin-treated parasite membrane. Lane 1, no protein addition; lane 2, ALAD binding to membrane without trypsin treatment; lanes 3-5, incubation of ALAD with the trypsin-treated membrane preparations. The membrane preparations were pretreated with 50 ng of trypsin at 30 °C for 10, 15, and 30 min, respectively. **C**, immunogold electron micrograph of parasite membrane and RBC incubated with ALAD. The conditions for incubation were as described under "Experimental Procedures."

human enzyme (data not presented). FC protein levels in the parasite and IRS are about the same, with only traces detected in URS. Once again, the protein preparations from all three preparations have identical mobilities in SDS gels (~42 kDa) and cross-react with antibodies to the human enzymes.

The almost equal FC enzyme activity detected in the parasite and IRS (Table II) correlates with the Western blot analysis (Fig. 7). ALAD protein was analyzed in the soluble fraction. The bulk of the protein is seen in the IRS with lower levels in URS and parasite (Fig. 7). This result also correlates well with the enzyme activity pattern shown in Table II.

#### DISCUSSION

The present study on the localization of the enzymes of the heme-biosynthetic pathway in the malarial parasite indicates a



**FIG. 6. Binding of ALAD to parasite (*P. berghei*) membrane.** Mouse erythroid ALAD was expressed in *E. coli* and biosynthetically labeled with [<sup>35</sup>S]methionine. Purified ALAD ( $1.06 \times 10^5$  cpm/ $\mu$ g of protein) was incubated with 2.4 mg of solubilized parasite membrane protein ( $4.2 \times 10^7$  parasites), and binding was measured using the nitrocellulose filter binding assay. **A**, saturation curve with increasing concentrations of ALAD. Binding was carried out in the absence (●) and presence (■) of a 100-fold excess of nonradioactive ALAD. **B**, Scatchard plot derived from data given in **A**. The nonspecific binding was deducted at each concentration of the enzyme used. This plot was used to calculate the dissociation constant and the number of binding sites. The molecular mass for the octomeric protein was taken as 336 kDa.

dual origin for these proteins. The first enzyme of the pathway, namely ALAS, is detectable in the parasite membrane at a level significantly higher than that of the host red cell membrane in terms of activity. However, the parasite enzyme, unlike that of the host red cell enzyme, does not cross-react with antibodies against human erythroid ALAS. This suggests the parasite origin of the ALAS localized in the parasite membrane. This suggestion is supported by a recent report on the successful cloning of the ALAS gene from *P. falciparum* (24). Parasite ALAD, CPO, and FC fall into another category. These are induced in the host (mice) in response to parasite (*P. berghei*) infection, and an amount is then perhaps translocated into the parasite.

Interestingly, there is no induction of ALAS in the IRS, once

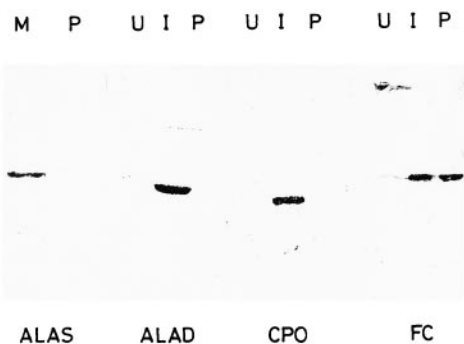


FIG. 7. Western blot analysis of ALAS, ALAD, CPO, and FC in the different fractions. The same enzyme preparations described in Table II were used for this analysis. Membrane preparations (100  $\mu$ g) were used for analysis of ALAS, CPO, and FC. Soluble preparations (75  $\mu$ g) were used for the analysis of ALAD. Equal amounts of protein were loaded on SDS-PAGE (10% polyacrylamide gel) for the analysis of different fractions. The Western analysis was carried out with antibodies to human RBC enzymes. The blot for each enzyme represents a separate electrophoretic run with appropriate molecular weight markers. The bands detected correspond to molecular mass values of 72 kDa (ALAS), 37 kDa (ALAD), 37 kDa (CPO), and 42 kDa (FC). U, uninfected RBC supernatant; P, parasite; I, infected RBC supernatant; M, red cell membrane.

again indicating a difference in its origin in the parasite. Preliminary studies reveal that in *P. falciparum* maintained in culture, there is no significant increase in ALAD in the IRS, unlike the situation seen in mice with *P. berghei* infection *in vivo*. This is to be expected, since the erythrocytes used to culture *P. falciparum* represent a terminally differentiated state and are incapable of RNA or protein synthesis. Therefore, the higher level of heme synthesis in parasite-infected cultures of *P. falciparum* should be due to the contribution of ALAS and perhaps FC from the parasite. The high levels of ALAD, CPO, and FC detected in *P. berghei*-infected mouse erythrocytes should be related to induction of these enzymes during the red cell maturation *in vivo*, perhaps due to stimulus provided by parasite-mediated red cell degeneration.

Detailed studies with ALAD reveal that the parasite (*P. berghei*) enzyme is identical to that of the host red cell but is not a contaminant. The immunogold labeling experiments and the demonstration of specific binding of host red cell ALAD to isolated parasite membrane suggest that this enzyme from the host may be translocated into the parasite through a receptor. These studies indicate that the host ALAD binds to the parasite membrane with high affinity, with the number of binding sites coming to about 1550/parasite. This can only be considered as a minimal estimate, since the binding studies have been carried out with solubilized membrane for reasons mentioned earlier.

A similar mechanism of translocation from the host may also operate, in the cases of CPO and FC, on the basis that these enzymes from the parasite have identical mobilities on SDS-PAGE with those of the host and that the parasite and host enzymes cross-react with antibodies to the human erythroid species, although further studies are needed to establish this point. However, unlike in the case of ALAD and CPO, the parasite has significantly high levels of FC. In mammalian mitochondria, both ALAS and FC, the first and last enzymes of the heme-biosynthetic pathway, are localized in the inner mitochondrial membrane (25). There is a possibility that FC like ALAS may also be coded for by the parasite genome, or it may be of dual origin.

While there is extensive literature on the transport of proteins made by the parasite into the host erythrocyte (20, 26, 27), internalization of host hemoglobin and its degradation in

parasite food vacuoles lead to generation of amino acids for parasite protein synthesis and large amounts of heme, detoxified and stored as inert hemozoin pigment. Superoxide dismutase represents another example, where 20% of the activity in the parasite is accounted for by the parasite gene coding for the protein and the rest is accounted for by the host red cell superoxide dismutase internalized by the parasite. However, the adopted superoxide dismutase has not been proven to participate functionally in the parasite (28, 29). In the case of ALAD, it is clear that the entire functional enzyme of the parasite is derived from the host. Besides, it has not so far been possible to identify the ALAD gene in the parasite genome (studies in this laboratory), although this cannot be taken as an evidence for its absence.

The results obtained with [4-<sup>14</sup>C]ALA incorporation into heme in cultures of *P. falciparum* and *P. berghei* indicate that 80% of the radioactivity is recovered in IRS. This correlates well with the higher levels of ALAD and the subsequent enzymes in IRS than in the parasite. In contrast to this situation, the results obtained with [2-<sup>14</sup>C]glycine incorporation into heme clearly indicate that 80% of the radioactivity is recovered in the parasite fraction. This suggests that this precursor is essentially handled by ALAS in the parasite membrane and that the level of this enzyme is also significantly higher than that of the red cell membrane enzyme. The results also indicate that there is no significant loss of the intermediates from the parasite into the IRS, or perhaps import of the intermediates from IRS into the parasite also does not take place. The parasite has evolved a mechanism to import the required enzymes rather than the biosynthetic intermediates or preformed heme. Heme should be essentially synthesized within the mitochondrion of the parasite, as is the case with mammalian mitochondria, and then distributed to the other parasite compartments. Thus, the parasite has evolved a strategy to segregate and divert the heme derived from red cell hemoglobin into the inert hemozoin pigment while making use of the heme synthesized *de novo* for metabolic purposes.

These studies have also identified new potential drug targets. These would be ALAS species unique to the parasite and the receptor(s) on the parasite membrane to import ALAD and perhaps the other enzymes of the pathway from the host. It would also be of interest to examine in this context whether the parasite employs additional mechanisms for making ALA.

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#### REFERENCES

- Surolia, N., and Padmanaban, G. (1992) *Biochem. Biophys. Res. Commun.* **187**, 744–750
- Scheibel, L. W., and Sherman I. W. (1988) in *Principle and Practice of Malariaology*, (Wernesdorfer, W. H., and McGreger, I., eds) pp. 219–252, Churchill-Livingstone, Edinburgh
- Sullivan, A. D., and Meshnick, S. R. (1996) *Parasitol. Today* **12**, 161–163
- Lwoff, M. (1951) in *Biochemistry and Physiology of Protozoa* (Lwoff, A., ed) pp. 131–182, Academic Press, Inc., New York
- Gaughan, P. L. Z., and Klassner, S. M. (1971) *Comp. Biochem. Physiol.* **39B**, 5–18
- Salzman, T. A., Stella, A. M., Wider De XiFra, E. A., Del, C. Battle, A. M., Docampo, R., and Stoppani, A. O. M. (1982) *Comp. Biochem. Physiol.* **72B**, 663–667
- Fry, M., and Beelsey, J. E. (1991) *Parasitol. Today* **102**, 17–26
- Salganik, R. I. Pankova, T. G., Chekhonadskikh, T. H., and Igonina, T. M. (1987) *Bull. W. H. O.* **65**, 381–386
- Surolia, N., and Padmanaban, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4786–4790
- Taketani, S., Kohno, H., Okuda, M., Furukawa, T., and Tokunaga, R. (1994) *J. Biol. Chem.* **269**, 7527–7531
- Trager, W., and Jensen, J. B. (1976) *Science* **193**, 673–675
- Fitch, C. D., Chevli, R., Banyal, H. S., Phillips, G., Pfaller, M. A., and Krogstad, D. J. (1982) *Antimicrob. Agents Chemother.* **21**, 819–822
- Levin, W., Jacobsen, E., Sernatinger, E., and Kuntzman, R. (1983) *Drug Metab. Dispos.* **1**, 267–274
- Bhat, G. J., and Padmanaban, G. (1988) *Arch. Biochem. Biophys.* **264**, 584–590

15. Gardner, L. C., Smith, S. J., and Cox, T. M. (1991) *J. Biol. Chem.* **266**, 22010–22018
16. Burnham, W. F., Irwing, E. A., and Elliott, W. H. (1970) *Methods Enzymol.* **17A**, 195–204
17. Mauzerall, D., and Granick, S. (1956) *J. Biol. Chem.* **219**, 435–446
18. Dailey, H. A., Jr., and Lascelles, J. (1977) *J. Bacteriol.* **129**, 815–820
19. Anderson, P. M., and Desnik, R. J. (1979) *J. Biol. Chem.* **254**, 6924–2930
20. Shahabuddin, M., Gunther, K., Lingelbach, K., Aikawa, M., Schreiber, M., Ridley, R. G., and Scaife, J. G. (1992) *Exp. Parasitol.* **74**, 11–19
21. Tschudy, D. P., Hess, R. A., and Frykholm, B. C. (1981) *J. Biol. Chem.* **256**, 9915–9923
22. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1382–1390
23. Boese Q. F., Spano, A. J., Li, J., and Timko, M. P. (1991) *J. Biol. Chem.* **266**, 17060–17066
24. Wilson, C. M., Smith, A. B., and Baylon, R. V. (1996) *Mol. Biochem. Parasitol.* **79**, 135–140
25. Jordan, P. M. (1990) in *Biosynthesis of Heme and Chlorophylls* (Dailey, H. A., ed) pp. 55–121, McGraw-Hill Inc., New York
26. Lingelbach, K. R. (1993) *Exp. Parasitol.* **76**, 318–327
27. Elford, B. C., Cowman, G. M., and Ferguson, D. J. P. (1995) *Biochem. J.* **308**, 361–374
28. Fairfield, A. S., Meshnick, S. R., and Eaton, J. W. (1993) *Science* **221**, 764–766
29. Becuwe, P., Gratepanche, S., Fourmaux, M.-N., Beeumen, J. V., Samyn, B., Mercereau-Puijalon, O., Touzel, J. P., Slomianny, C., Camus, D., and Dive, D. (1996) *Mol. Biochem. Parasitol.* **76**, 124–134