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Antimalarial Activity of Selected Aromatic Chelators. IV. Cation Uptake by *Plasmodium falciparum* in the Presence of Oxines and Siderochromes

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SUMMARY

The growth of *Plasmodium falciparum*, a human malaria parasite, is sensitive to inhibition by chelators of several types. The alkylthiocarbamates and 8-hydroxyquinoline at pharmacologic doses selectively inhibit glycolysis within 6 hr in parasitized erythrocytes. The mechanism attributed to these agents is through the extracellular formation of lipid-soluble 2:1 metal complexes which enter susceptible cells and liberate a lethal 1:1 complex. This study further supports this mechanism since the uptake of ^{59}Fe:oxine complexes within 6 hr occurs at doses corresponding to, or less than, those producing the lethal effects and metabolic changes. Fifty per cent of the uptake occurs in less than 6 hr. The presence of 8-hydroxyquinoline facilitates entry of the radiolabeled cations and uninfected erythrocytes take up less cation, especially in the absence of chelator. The siderochromes, rhodotorulic acid and mycobactin P, when mixed with ^{59}Fe, result in an insignificant uptake, i.e., none of the former and only 12% of the latter penetrate the parasitized cells in 6 hr. Less than 25% of ^{59}Fe:iodochlorhydroxyquin enters infected cells and 8% enters normal erythrocytes, suggesting that very little antimicrobial activity of the iodinated oxines is due to chelation unlike an agent such as KAN-322. In fact, 30% of the oxine complex and possibly more KAN-322 appears to partition in the intracellular parasite itself.

Scheibel and co-workers (1–5) first demonstrated that growth of the human malaria parasite is sensitive to inhibition by low doses of chelators and that this antiparasitic activity depends on chelation. In addition, the alkylthiocarbamates and 8-hydroxyquinoline, at pharmacologic doses, inhibited the glycolysis of infected erythrocytes with little effect on the same processes in normal red cells, but Scheibel and co-workers (1–5) have suggested that the primary effect may be through inhibition of metalloprotein biosynthetic oxidase enzymes. Several of these agents are well tolerated by higher animals and are more plasmodiacidal than quinine *in vitro*, even against chloroquine-resistant plasmodia. Recently, other investigators reported the antiparasitic activity of a number of compounds capable of chelating metal ions (see Ref. 5 for references); unfortunately, the only instance where there is conclusive chemical evidence that this antimicrobial or antiprotozoal activity is directly related to chelation is with the oxine type of chelators (2, 6). Shapiro *et al.* (7) and Raventos-Suarez *et al.* (8) postulated that selected chelators may act by blocking iron or copper utilization by the parasite, thereby depriving the organism of an obligate nutritional requirement. Apparently, the intra-erythrocytic *Plasmodium falciparum* takes up iron from transferrin, and it is this step that explains the sensitivity to desferrioxamine (9, 10). Indeed, it has been suggested that the clinically useful quinoline antimalarials (chloroquine, primaquine, etc.) also block iron uptake in the parasitized cell (11).

Many years ago it was proposed that the antimicrobial action of agents such as the dithiocarbamates and of 8-hydroxyquinoline was by formation of chelate complexes or precipitates with various heavy metal ions indispensable to microbial growth. Growth was thought to be prevented by inhibiting the production of essential enzyme systems containing these elements. Since then, this hypothesized mode of action of these agents has been questioned (12, 13). Evidence against the precipitation theory is offered in the case of copper:8-hydroxyquinoline, which has been reported to be even more active as a fungicide than oxine.

Early work by Albert (14) and later work by Scheibel and colleagues (5, 15) attribute a different mechanism to both the

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ABBREVIATIONS: EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KAN-322, 5-chloro-7-(3-diethylaminopropylaminomethyl)-8-quinolinol dihydrochloride.

thiocarbamate and oxine chelators. Albert *et al.* (16) maintain that toxic complexes are formed with metals of variable valency which are present in the medium. Oxine exerts its lethal effect by combining with these metallic ions without which there is no antibacterial activity (16). The chelates formed from the reaction of oxine with these metals, iron or copper, are themselves as toxic as, or even more strongly antimicrobial than, oxine (17). This was found to be the case in *P. falciparum* cultures by Scheibel and co-workers (3, 15) with the 2:1 chelator:metal complex of quinoline-2-thiol-*N*-oxide:zinc. It presumably penetrates the cell as a lipophilic 2:1 complex with the metal and then reverts to a 1:1 oxine:metal complex, a true toxic agent which initiates oxidative chain reactions inactivating labile enzymes and killing the cell (18, 19). The mode of action of the dialkyl thiocarbamates is closely comparable with that of oxine and pyridine-2-thiol-*N*-oxide (20) except that the latter has a lower stability constant and lower partition coefficient than oxine but is more active biologically, being more able to liberate 1:1 complexes within the cell (18). Scheibel (15) extended this proposed mechanism to suggest that the free chelator (liberated when the 2:1 complex reverts intracellularly to a 1:1 complex) may also exert profound biological effects. In fact, there are significant differences between the results found in the plasmodial system by Scheibel (15) and that by others working on bacteria and fungi. The addition of copper or iron salts does not change the lethal effects of chelators against malaria; the increase in the concentration of chelators does not result in "concentration quenching" of growth curves; an increase in the lipophilicity of the chelating agent does not result, in some instances, in a proportional increase in plasmodiacidal activity (6-hydroxy-*m*-phenanthroline or oxine are less lipid soluble but more potent than 5,6-benzo-oxine, and pyridine-2-thiol-*N*-oxide is less lipophilic but more potent than quinoline-2-thiol-*N*-oxide) (2–4); and EDTA or cobalt added to growing cultures did not antagonize the activity of chelators as had been found in other microbial systems (21). Therefore, it was decided to determine the rate of uptake by *P. falciparum* of 8-hydroxyquinoline and radiolabeled iron-59 (^{59}Fe) under conditions similar to those of our original growth inhibition studies (2). The amount of oxine:metal complex entering the cell per unit time might explain pharmacological changes in the parasitized erythrocyte (i.e., parasite killing, inhibition of glycolysis, and electron microscopic changes). The rationale of this study is to assess whether or not drug activity is due to translocation within the cell of a chelator:labeled cation complex which is formed from metals incidentally present in the growth medium.

Materials and Methods

Chloroquine-resistant *P. falciparum* strain FCB/k$^+$ was grown in a candle jar in Dulbecco's modified Eagle's minimal essential medium/Iscove's modified Dulbecco's medium and schizonts were separated from the immature stages using a plasma gel technique as described in a previous publication (22). No white cells or platelets contaminated the sample. Controls contained uninfected erythrocytes from the same donors as were used to grow the parasites. The hematocrit was adjusted to 4% and tubes contained a volume of 0.25 ml in serum-free medium incubated at room temperature or 37° for varying times. The study was initiated by adding isotope and stopped by the addition of 0.7 ml medium containing 20% sera, similar to a procedure described by Sharefkin and Rich (23). Pellets were washed three times in an Eppendorf model 5412 centrifuge employing 1-min spins at 15,600 × *g*. Both pellets and supernatants were counted on a Beckman Gamma 5500 counter. Large batches of parasites were separated from host erythrocyte material (to delineate uptake by the parasite versus erythrocyte) by saponin lysis using a technique described by Reese *et al.* (24) except that parasites were incubated with 0.04% saponin for 30 min. Free parasites were washed three to four times in a total volume of 4.5 ml and centrifuged as a pellet for counting. Each experiment was repeated two or three times and results were consistent. A representative experiment was selected in each case for presentation.

Ferric chloride (^{59}Fe) was purchased from Amersham Corp. (Arlington Heights, IL). Iron-59 (^{59}Fe) was allowed to complex with chelators in Hepes buffer-bicarbonate solution at the same concentration as contained in growth medium to avoid the precipitation of iron with phosphate in the medium which might render it unavailable for uptake. Protein was assayed by Coomassie blue dye binding, employing bovine serum albumin as the standard (25).

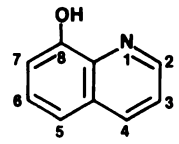
Experiments done in the presence and absence of oxine were conducted in a solution of normal saline with glucose, bicarbonate, and Hepes buffer equal to that contained in standard culture media to avoid the possibility of $^{59}FeCl_3$ precipitating in phosphate containing media.

Vioform (iodochlorhydroxyquin) was obtained as a gift from Ciba Pharmaceutical Co. (Summit, NJ). Diiodohydroxyquin was donated by Glenwood Inc. (Tenafly, NJ). KAN-322 was obtained from Dr. L. M. Werbel, Warner Lambert Co. (Ann Arbor, MI); mycobactin P was from Dr. A. J. White, Imperial Chemical Industries, England; and rhodotorulic acid was from D. R. W. Grady, Cornell Medical Center (New York, NY).

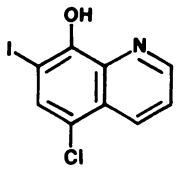
Results

Uptake of ^{59}Fe:oxine complex. There is a rapid uptake of oxine (Fig. 1) at levels similar to those which exert rather profound effects on the growth and metabolism of the parasite (2) when complexed with ^{59}Fe (Table 1). Parasitized erythrocytes take up 17.6% in 1 min and, in 6 hr, approximately 55%; the point at which 50% uptake takes place is 4 hr, 20 min. Normal uninfected erythrocytes take up about half this amount.

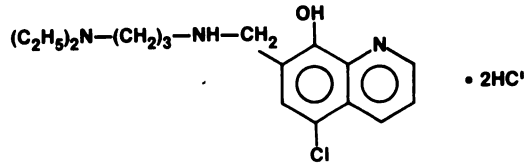
Uptake of ^{59}Fe:rhodotorulic acid complexes and ^{59}Fe:mycobactin P complexes. Aerobic bacteria, yeasts and fungi, are known to meet their biological requirement for iron by excreting siderochromes into the environment. These agents complex with the iron, are in turn reabsorbed by the organism, and then are hydrolysed to liberate iron for the cell (6). One of these siderochromes, desferrioxamine, which is a potent hydroxamic acid chelator of ferric ions, has been shown to inhibit the growth of *P. falciparum*, presumably by iron deprivation (8). Therefore, it might be expected that the uptake of this type of ^{59}Fe:chelator complex would be poor. Scheibel and Stanton (5) have demonstrated two other siderochromes to be inhibitory to the growth of *P. falciparum in vitro*, but at somewhat higher concentrations than is required in the case of the oxine-type chelators in general. One of these chelators is rhodotorulic acid (Fig. 1), a hydroxamic derivative of diketopiperazine found in yeasts. In an attempt to elucidate the probable mechanism of action of this type of agent, 1.7×10^{-7} M ^{59}Fe was allowed to react with 6.0×10^{-6} M rhodotorulic acid in Hepes buffer and $NaHCO_3$ without phosphate. Specific activity was 3.2×10^8 dpm/μmol. This was added to suspensions of schizont-infected erythrocytes as was done in the previous experiment. No ^{59}Fe:rhodotorulic acid complex is taken up within 6 hr of incubation in either parasitized or nonparasitized erythrocytes. These data are compatible with the proposition that the antimalarial effects of this agent in culture are due to iron deprivation. A positive uptake control of ^{59}Fe:iodochlorhydroxyquin



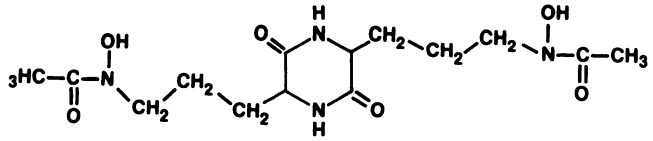
8—Hydroxyquinoline



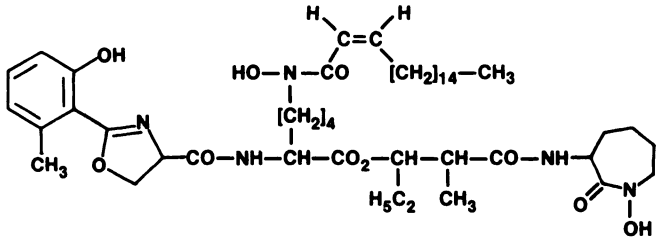
Iodochlorhydroxyquin



5—Chloro—7—(3-Diethylaminopropylaminomethyl)—8—Quinolinol, Dihydrochloride



Rhodotorulic acid



Mycobactin P

Fig. 1. Chelators used in study.

using these cells compared favorably with the experiment in Table 3: uptake in 30 min was 12.1%.

Mycobactin (Fig. 1) is the siderochrome of *Mycobacteria*. It has two hydroxamate groups and another group to aid the three-center binding of ferric iron as strongly as other siderochromes (6). In fact, mycobactin P binds ferric iron even more firmly than does desferrioxamine B, and since this growth factor seemed so specific for mycobacteria, it was postulated that the structure might provide a suitable model for the synthesis of compounds having a specific action against these organisms (for references see Ref. 26). If the mechanism of growth inhibition is by iron deprivation and is similar to that of the other hydroxamic acid siderochromes, rhodotorulic acid and desferrioxamine, then very little radiolabeled complex would be taken up by the cell.

The results of this experiment (Table 2) suggest that little ^{59}Fe:mycobactin P complex is taken up by parasitized cells. Within the first 10 min only approximately 5% is taken up and at 6 hr; this value is only 12.3%. The uptake by normal

TABLE 1

Uptake of ^{59}Fe:oxine complex

Tubes contained 1.2×10^{-7} M ^{59}Fe which was allowed to complex with 1.4×10^{-5} M oxine before being added to incubation medium containing erythrocytes at a 4% hematocrit for a volume of 0.25 ml. The specific activity was 5.3×10^{8} dpm/μmol. There was an average of 1.1 (±0.12) mg of protein in the parasitized erythrocytes (P/time) and 1.4 (±0.20) mg of protein in the normal erythrocytes (H/time).

Pellet designation	Percentage uptake	μmol of Uptake/mg of protein
P/1 min	17.6	4.6×10^{-6}
H/1 min	10.3	2.1×10^{-6}
P/10 min	31.0	8.1×10^{-6}
H/10 min	16.9	3.5×10^{-6}
P/30 min	37.9	1.0×10^{-5}
H/30 min	19.0	3.9×10^{-6}
P/6 hr	55.2	1.5×10^{-5}
H/6 hr	26.2	5.4×10^{-6}

TABLE 2

Uptake of ^{59}Fe:mycobactin P complex

Tubes contained 1.7×10^{-7} M ^{59}Fe which was allowed to complex with 2.5×10^{-7} M mycobactin P before being added to incubation medium containing erythrocytes at 4% hematocrit for a volume of 0.25 ml. The specific activity was 2.8×10^{8} dpm/μmol. There was an average of 2.0 (±0.19) mg of protein in the parasitized erythrocytes and 2.0 (±0.37) mg of protein in the normal erythrocytes.

Pellet designation	Percentage uptake	μmol of Uptake/mg of protein
P/1 min	4.7	1.0×10^{-6}
H/1 min	2.0	4.2×10^{-7}
P/10 min	5.6	1.2×10^{-6}
H/10 min	3.0	6.5×10^{-7}
P/30 min	7.4	1.6×10^{-6}
H/30 min	2.6	5.5×10^{-7}
P/6 hr	12.3	2.6×10^{-6}
H/6 hr	5.4	1.2×10^{-6}

erythrocytes (drawn from the same donor as those used to grow the malaria parasite in this experiment) is about 50% of that taken up by the parasitized erythrocytes at each time frame. Both normal and parasitized cells increase uptake with time. Only 2.5×10^{-7} M mycobactin P (which is rather insoluble) was used in this study compared to higher levels of oxine type chelators (1.4×10^{-5} M) employed in the previous experiments (Table 1).

Uptake of ^{59}Fe:iodochlorhydroxyquin complexes and ^{59}Fe:KAN-322 complexes. The halogenated oxines (iodochlorhydroxyquin or diiodohydroxyquin) are thought to be effective in the treatment of amebiasis by a steady evolution of inorganic iodine which in turn kills the parasite. In support of this hypothesis, several studies have demonstrated that plasma protein-bound iodine is increased after treatment with iodochlorhydroxyquin (27). However, a significant portion of the ingested drug is absorbed and a portion of the antiparasitic activity has been ascribed to chelation. Structurally similar antiprotozoal compounds have been synthesized without iodine which are markedly active *in vitro* and *in vivo* and act totally by chelation (4). One of these is KAN-322 (Fig. 1), and it has been shown by us to be as potent an inhibitor of *P. falciparum in vitro* as is quinine sulfate. Therefore, we compared the rate of uptake of iodochlorhydroxyquin (Fig. 1) or KAN-322 and ^{59}Fe with that of unsubstituted oxine and ^{59}Fe. The drug diiodohydroxyquin was found too insoluble to be included in these studies, and iodochlorhydroxyquin was run at a concentration 10 times lower than that of oxine, due to decreased solubility.

As shown in Table 3, iodochlorhydroxyquin complexed to

TABLE 3

Uptake of ^{59}Fe:iodochlorhydroxyquin complex

Tubes contained 1.7×10^{-7} M ^{59}Fe which was allowed to complex with 1.3×10^{-6} M iodochlorhydroxyquin before being added to incubation medium containing erythrocytes at a 4% hematocrit for a volume of 0.25 ml. The specific activity was 3.1×10^{8} dpm/μmol. There was an average of 1.5 (±0.05) mg of protein in the parasitized erythrocytes and 2.1 (±0.43) mg of protein in the normal erythrocytes.

Pellet designation	Percentage uptake	μmol of Uptake/mg of protein
P/1 min	9.8	2.8×10^{-6}
H/1 min	5.1	1.0×10^{-6}
P/10 min	12.3	3.5×10^{-6}
H/10 min	6.1	1.2×10^{-6}
P/30 min	16.9	4.9×10^{-6}
H/30 min	6.5	1.3×10^{-6}
P/6 hr	22.6	6.5×10^{-6}
H/6 hr	8.4	1.6×10^{-6}

TABLE 4

Uptake of ^{59}Fe:KAN-322 complex

Tubes contained 1.7×10^{-7} M ^{59}Fe which was allowed to complex with 1.4×10^{-5} M KAN-322 before being added to incubation medium containing erythrocytes at a 4% hematocrit for a volume of 0.25 ml. The specific activity was 1.1×10^{8} dpm/μmol. There was an average of 2.1 (±0.06) mg of protein in the parasitized erythrocytes and 2.6 (±0.35) mg of protein in the normal erythrocytes.

Pellet designation	Percentage uptake	μmol of Uptake/mg of protein
P/1 min	22.8	4.7×10^{-6}
H/1 min	17.0	2.8×10^{-6}
P/10 min	23.3	4.8×10^{-6}
H/10 min	16.3	2.7×10^{-6}
P/30 min	27.9	5.7×10^{-6}
H/30 min	18.6	3.1×10^{-6}
P/6 hr	34.9	7.1×10^{-6}
H/6 hr	23.3	3.8×10^{-6}

^{59}Fe is taken up by parasitized red cells ("P/time") but not to the extent of ^{59}Fe:oxine complexes (Table 1). In 1 min, approximately 10% of the ^{59}Fe:iodochlorhydroxyquin complex is taken up and, in 6 hr, approximately 23%. As was seen with ^{59}Fe:oxine, there was less uptake in normal erythrocytes ("H/time") than in parasitized cells.

The non-iodine-containing chelator KAN-322, also developed as an antiamebic drug and found by us to inhibit the *in vitro* growth of *P. falciparum*, is taken up by erythrocytes when complexed with ^{59}Fe to a greater degree than ^{59}Fe:iodochlorhydroxyquin (Table 4). Parasitized erythrocytes take up slightly more than 25% within the first 30 min and normal cells about 19%. This would suggest that, whereas uptake of oxine complexed with ^{59}Fe is somewhat greater over 6 hr, complexes of iodochlorhydroxyquin or KAN-322 with ^{59}Fe are taken up at an appreciable rate, and chelation may explain a portion of their activity. Of interest is the fact that oxine is a more potent inhibitor of the *in vitro* growth of *P. falciparum* than is KAN-322 (2, 4). Whereas the dose of oxine used in our uptake studies (1.4×10^{-5} M) is somewhat higher than that of iodochlorhydroxyquin (1.3×10^{-6} M), the dose of KAN-322 (1.4×10^{-5} M) is the same as that for oxine.

Uptake of ^{59}Fe in the presence and absence of oxine. In an effort to determine what role the oxine chelator plays in the uptake of metal ions, ^{59}Fe was added in the presence and absence of oxine (Table 5). Phosphate-containing media could precipitate $FeCl_3$ which might make it unavailable to be taken up by the erythrocyte. To minimize this possibility, a phosphate-free incubation medium was employed.

TABLE 5

Uptake of ^{59}Fe in the presence and absence of oxine

Tubes contained 1.7×10^{-7} M ^{59}Fe which was allowed to complex with 1.4×10^{-6} M oxine or in the absence of oxine in a total volume of 0.25 ml containing erythrocytes at a 4% hematocrit. Specific activity was 2.7×10^{8} dpm/μmol. There was an average of 2.0 (±0.27) mg of protein in parasitized erythrocytes and 2.0 (±0.35) mg of protein in the normal erythrocytes.

Pellet designation	Percentage uptake	μmol of Uptake/mg protein
P/1 min	6.6	1.8×10^{-6}
P/1 min + oxine	7.1	4.0×10^{-6}
H/1 min	1.5	3.3×10^{-7}
H/1 min + oxine	9.1	2.0×10^{-6}
P/10 min	8.1	1.8×10^{-6}
P/10 min + oxine	20.2	4.4×10^{-6}
H/10 min	2.1	4.5×10^{-7}
H/10 min + oxine	7.9	1.7×10^{-6}
P/30 min	9.3	2.0×10^{-6}
P/30 min + oxine	23.0	5.0×10^{-6}
H/30 min	4.0	8.5×10^{-7}
H/30 min + oxine	8.4	1.8×10^{-6}
P/6 hr	14.0	3.0×10^{-6}
P/6 hr + oxine	51.2	1.1×10^{-5}
H/6 hr	9.1	2.0×10^{-6}
H/6 hr + oxine	21.4	4.6×10^{-6}

In 1 min, 6.6% of the iron was taken up in parasitized red cells, in the absence of chelator. In 6 hr this increased to 14%. Addition of oxine increased the uptake values compared to those without chelator, i.e, 7.1% in 1 min and 51.2% in 6 hr. Fifty per cent uptake occurred at 5 hr, 45 min. A similar trend was seen in normal red cells from the same donor, i.e., 1.5% in 1 min without oxine compared to 9.1% in 6 hr. Addition of oxine resulted in a 1-min uptake of approximately 9.1% and a 6-hr uptake of 21.4%.

These results suggest that the chelator facilitates the uptake of metal, and the amount of complex the infected cells take up is significantly higher than that by uninfected cells. One might argue that the medium used was unphysiological for long-term uptake experiments (since parasites will not continuously grow in buffered glucose and normal saline), but these results were corroborated by a similar study in the presence and absence of oxine. The later experiment was done in complete culture medium; cation was added last and rapidly mixed to minimize the chance that phosphate buffer could precipitate it, rendering it unavailable for uptake and, therefore, biasing the results.

Uptake of the radiolabeled cation-chelator complex in the erythrocytes versus parasite. In an effort to determine whether radiolabeled cation-chelator complexes were taken up to a greater degree in the parasite compartment or the erythrocyte compartment, tubes containing a larger number of parasitized red cells (total volume of 4.5 ml) were treated as in the small tube experiments. After 1 hr of incubation at room temperature, parasites were separated from the host cell by saponin lysis (24). The free parasites were washed three or four times and centrifuged as a pellet, and each fraction was counted.

The results of this study (Table 6) suggest that ^{59}Fe allowed to complex with oxine is rapidly taken up by the parasite fraction of parasitized red cells. In two trials, 30.0% of the ^{59}Fe:oxine complex was found in the parasite pellet and 44.8% in the red cell lysate with the remainder in the supernatant. This agrees with electron microscopic observations of the rapid onset of widespread structural changes in the parasite which begins within 5 min when plasmodia are subjected to low doses

TABLE 6

Uptake of radiolabled cation-chelator complex in red cell versus parasite

Cation-chelator complex	Uptake					
	Parasite pellet		RBC lysate		Supernatant	
	%	μmol	%	μmol	%	μmol
^{59}Fe:oxine[a]	30.0	8.7×10^{-5}	44.8	1.3×10^{-4}	28.6	8.3×10^{-5}
^{59}Fe:iodochlorhydroxyquin[b]	14.0	6.0×10^{-5}	2.8	1.2×10^{-5}	74.4	3.2×10^{-4}
^{59}Fe:KAN-322[c]	65.1	2.8×10^{-4}	3.5	1.5×10^{-5}	15.6	6.7×10^{-5}

[a] ^{59}Fe (6.4×10^{-6} M) complexed with 7.6×10^{-6} M oxine in a total volume of 4.5 ml with a specific activity of 5.3×10^{8} dpm/μmol. The parasite pellet and red cell lysate contained 2.3 mg and 21.8 mg of protein, respectively.

[b] ^{59}Fe (9.6×10^{-6} M) complexed with 7.3×10^{-7} M iodochlorhydroxyquin in a total volume of 4.5 ml with a specific activity of 2.8×10^{8} dpm/μmol. The parasite pellet and red cell lysate contained 9.0 mg and 25.6 mg of protein, respectively.

[c] ^{59}Fe (9.6×10^{-6} M) complexed with 7.8×10^{-6} M KAN-322 in a total volume of 4.5 ml with a specific activity of 5.6×10^{7} dpm/μmol. The parasite pellet and red cell lysate contained 5.4 mg and 39.7 mg of protein, respectively.

of oxine (6.89×10^{-7} M) in culture.[1] An insignificant amount of the rhodotorulic acid:iron complex is found in either the parasite (0.04%) or the erythrocyte (0.27%), which is compatible with the hypotheses that this compound works by a different cytotoxic mechanism.

In agreement with the uptake over 6 hr, the ^{59}Fe:iodochlorhydroxyquin complex is not readily taken up by the parasitized erythrocyte. Within 1 hr, approximately only 16% of the drug is taken into the parasitized cell and most of this appears to be in the parasite compartment. In contrast, the non-iodinated drug KAN-322, which probably acts entirely through chelation, enters the parasitized cell easily. Approximately 65% appears to be in the parasite pellet and very little is in the red cell lysate. This is considerably more than is taken up in a similar length of time in the small tubes (Table 4). Only about 35% of KAN-322 entered parasitized erythrocytes in 6 hr and 28% in 30 min when the experiment was completed in small tubes. As yet there is no explanation for this discrepancy between the small and large volume experiments. In all instances, the isotope was accounted for in the three compartments.

Discussion

Trivalent cations such as Fe^{3+} form saturated 3:1 complexes with oxine (6). These are lipid soluble and rapidly penetrate cell membranes (28). In this study, evidence was demonstrated that a metal such as ^{59}Fe, when complexed with 8-hydroxyquinoline (oxine), is capable of rapidly partitioning itself in parasitized and nonparasitized erythrocytes. Our experiments suggest that ^{59}Fe is capable of penetrating both the erythrocyte and the parasite when complexed with appropriate chelators such as 8-hydroxyquinoline. In addition, the rate of this entry is also much greater in erythrocytes parasitized with mature schizonts of *P. falciparum* than is seen in normal erythrocytes from the same donor. The presence of the chelating agent facilitates the entry of cations such as iron over 6 hr. This agrees with the dramatic electron microscopic changes observed within 5 min when low doses of oxine are added to cation-containing culture medium *in vitro* and the metabolic derangements and with rapid plasmodiacidal effects described in our earlier reports (1–5). The exact fate of these complexes is unknown, but metalloprotein biosynthetic oxidase enzymes (1–4) and ribonucleic acid metabolism (2, 8, 29, 30) in the parasite have both been suggested as possible primary targets, and glycolysis as a secondary target (1, 2, 6).

The pharmacological action of 8-hydroxyquinoline and its analogues (KAN-322), therefore, could be explained in large part by extracellular formation of chelator:metal complexes which rapidly penetrate the cell and exert their activity. ^{59}Fe:iodochlorhydroxyquin is also capable of entering parasitized cells to a greater extent than it enters normal cells and also greater than iron alone enters parasitized cells (Tables 3 and 5). The rate of entry, however, of ^{59}Fe:iodochlorhydroxyquin is not as great as for ^{59}Fe:oxine or ^{59}Fe:KAN-322 (which may be partly due to the lower concentration of iodochlorhydroxyquin used). Significantly more KAN-322 (Table 6) also reaches the parasite pellet than does oxine. This may be a result of the long aliphatic group on carbon number 7 of the quinoline nucleus of KAN-322 which is not present on unsubstituted 8-hydroxyquinoline. Conversely, iodochlorhydroxyquin may owe much of its activity to slow liberation of iodine, as originally proposed, and a lesser degree to chelation, whereas KAN-322 and oxine appear to act entirely by chelation. The attributed mechanism of the iodinated oxines, used for years in the therapy of amebiasis and still employed to some extent as luminal amebicides, is, presumably, by the steady evolution of inorganic iodine (3, 4). Increases in the plasma iodine content do occur following the administration of diiodohydroxyquin and iodochlorhydroxyquin (for references see Ref. 27), but there is also substantial absorption of the drug from the gastrointestinal tract (31). From the data presented here, these drugs also depend on chelation for some of their activity as was predicted years ago by Burckhalter *et al.* (32) and Thompson *et al.* (33). In agreement with this, diiodohydroxyquin has been reported to enhance zinc absorption, functioning as a zinc ionophore in the zinc deficiency disorder acrodermatitis enteropathica (34).

The rapid entry of chelators, such as 8-hydroxyquinoline, and an appropriate concentration of metal ions is compatible with a theory originally proposed by Albert (6) and later by Scheibel (15). Albert (6) presented evidence that a toxic complex is produced with certain nontoxic metals which in turn produce a variety of lethal subcellular reactions that lead to the death of the parasite. Scheibel (15) later suggested that the free chelator, liberated within the cell from 2:1 complexes, could inactivate biosynthetic metalloprotein oxidase enzymes and contribute to the action of toxic 1:1 complexes arising from the parent lipophilic 2:1 complex which had penetrated the cell.

The proportion of chelator versus metal present in the system may regulate the rates of entry of these complexes in the *P. falciparum*-infected erythrocytes. Varying the concentration of chelator to metal would determine whether 1:1, 2:1, or 3:1 complexes predominate in the incubation medium. This may not have significant effects on malaria *in vitro* cultures since there is no "concentration quenching" in the killing of parasites

[1] Dr. S. Langreth and Dr. L. W. Scheibel, unpublished observations.

concomitant with increasing levels of chelator as has been reported in bacterial systems. Since there are a variety of other ions in the medium, we believe a more accurate assessment of uptake could be made by saturating labeled metal with oxine chelator before adding it to the system (i.e., employing 3:1 chelator-metal complexes with a slight excess of chelator).

Rhodotorulic acid and for the most part mycobactin P (Table 2) do not kill *P. falciparum* by the mechanism described above, (i.e. ultimate production of intercellular toxic complex.) They, like desferroxamine, may indeed act in large part through iron deprivation.

An understanding of the mechanism of action of these agents would assist in the rational design of new antimalarials and of other antimicrobial agents. In the search for new agents to be used for treatment of tetracycline resistant mycoplasma, Timmerman et al (35, 36) demonstrated that non-lethal amounts of copper are transported across cytoplasmic membranes of *Mycoplasma* by chelators and inhibit the pathogen.

Studies are underway to further explain this mechanism and to assess other agents with antimalarial activity.

Addendum. Ginsburg *et al.* [*Biochim. Biophys. Acta* **886**:337–344 (1986)] reported that the chelator, dipicholinic acid, inhibits intracellular development of *P. falciparum*. In addition, penetration of the chelator is significantly higher in trophozoite- and schizont-infected erythrocytes than in normal red cells, in agreement with these findings.

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