Biochemical Pharmacology, Vol. 35, No. 14, pp. 2435–2442, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

DIGESTION OF THE HOST ERYTHROCYTE BY MALARIA PARASITES IS THE PRIMARY TARGET FOR QUINOLINE-CONTAINING ANTIMALARIALS*

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(*Received* 4 *November* 1985; *accepted* 31 *January* 1986)

Abstract—Intraerythrocytic malaria parasites feed on their host cell cytosol. We show that human red blood cells infected with the malaria parasite *Plasmodium falciparum*, produce free amino acids the composition of which resembles that of globin, the most abundant red blood cell protein. The rate of amino acid production is almost equal to the rate of efflux of these acids from the infected cell. Production of amino acids increases with parasite age: the rates of production at the young ring and the mature trophozoite stages were 3.3 and 13.5 nmol/10^8 infected cells per min at 37°, respectively, compared with 0.04 nmol/10^8 cells per min in uninfected cells. The quinoline-containing antimalarial drugs, chloroquine, quinine and mefloquine, inhibit amino acid production at the same concentrations at which they inhibit parasite growth, but have no effect on the endogenous parasite protein degradation. We suggest that parasite feeding on host cell cytosol is the primary target for the antimalarial action of these drugs.

Chloroquine accumulation, the rate of amino acid production by infected cells and the inhibitory effect of the drug, were determined simultaneously at the different stages of parasite development. At all stages the rate of amino acid production and chloroquine accumulation were directly related and both were inversely related to the inhibitory efficiency of the drug. The lysosomotropic agents methylamine and NH_4Cl at millimolar concentrations also inhibit amino acid production, suggesting that the process is pH dependent and localized in the vacuole. Host cytosol degradation and drug accumulation both take place in the parasite food vacuole. Our observations imply that the metabolically dependent acidification of this parasite organelle is involved in both processes.

Intraerythrocytic malarial parasites digest their host cell cytosol [1, 2] producing amino acids needed for protein anabolism, the developing parasite possessing a very limited ability to synthesize amino acids [3]. However, only a small portion of these amino acids is utilized by the parasite, the remainder being released from the infected cell [4–8]. Parasites also incorporate into their proteins exogenously supplied amino acids but the pattern of this incorporation is complex [9] suggesting that the intracellular pool of an individual amino acid is not directly correlated to its prevalence in hemoglobin [3]. The composition of the amino acids mixture released from the infected cell is very similar to the constitution of globin [6, 7], hemoglobin being the most abundant protein in red blood cells (RBC). In most *Plasmodium* species investigated, proteases of different pH optima, substrate specificities and susceptibilities to different inhibitors are detectable [10–17]. The acid proteases may occur in acidic compartments where host proteins are degraded [18], while the neutral and alkaline peptidases are thought to be confined to the parasite cytoplasm [8].

In *Plasmodium falciparum* infected human RBCs, the antimalarial drug chloroquine accumulates to millimolar levels in the parasite acidic central food vacuole by virtue of its weak base properties [18, 19]. The drug also causes the accumulation of undigested endocytic vesicles in the food vacuole [20], suggesting that the feeding process is a major target for the antimalarial effect of this drug and that parasite acidic hydrolases are involved in drug action, as originally outlined by Homewood *et al.* [21]. Parasite proteases are inhibited *in vitro* by millimolar concentrations of chloroquine and other quinoline antimalarials [13, 15, 22] but these drugs do not inhibit amino acid production in mouse RBCs infected with *P. berghei* or *P. yoelii nigeriensis* [22]. In the present study we demonstrate the *P. falciparum* grown in culture, produces appreciable amounts of amino acids as a result of host cytosol digestion and that this process is efficiently inhibited by therapeutic concentrations of chloroquine, quinine and mefloquine.

MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: Human blood—Hadassah Hospital Blood Bank; outdated human plasma—Shaarei Zedek Hospital, Jerusalem; RPMI-1640—GIBCO, Grand Island, NY; chloroquine di-phosphate (CQ), quinine, gelatin (300 bloom), fluorescamine—Sigma Chemical Co., St. Louis, MO; 1-nitroso-2-naphtol—Fluka; mefloquine (α-2 piperidyl-2,8-bis(trifluoro-

* This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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methyl)-4-quinoline methanol) was generously provided by C. J. Canfield, Walter Reed Army Research Institute, Washington, DC. Labeled amino acids were from Amersham and [*N*-ethyl-^{3}H(N)]chloroquine was from New England Nuclear. All other reagents were of the best available grade and all solutions were prepared in glass distilled water.

Cultivation and synchronization of parasites. The FCR_3 strain of *P. falciparum* was cultivated in RPMI-1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, 50 μM hypoxanthine, 20 mM glucose and 10% (v/v) heat inactivated A^+ or AB^+ human plasma. Fresh A^+ or O^+ erythrocytes were used at 2.5% hematocrit in culture flasks (Nunc); medium was changed daily and flasks were gassed with a mixture of 90% N_2, 5% CO_2 and 5% O_2. Parasitemia, the percentage of infected erythrocytes, was determined by microscopic inspection of Giemsa stained thin blood smears.

Cultures were synchronized in isotonic mannitol [23]; when tight synchronization was required, two such treatments were done 30 hr apart. Infected erythrocytes were isolated by Percoll-sorbitol gradient fractionation [24] for the young stages and by gelatin flotation [25] for mature parasite stages.

Determination of amino acid production by infected RBCs. Mannitol-synchronized cultures were washed twice with 100 vol. of phosphate-buffered saline (PBS) supplemented with 10 mM glucose (PBS-G), 37°, to remove the amino acid rich growth medium. When the effect of glucose deprivation on amino acid production was to be tested, cultures were washed in PBS alone. Cells were resuspended in fresh buffer of the same composition to 5% hematocrit, in the presence or absence of drugs, and 150 μl samples were aliquoted at different time intervals. An equal volume of cold trichloroacetic acid (TCA) was added immediately; the mixtures were left for 24 hr at 4° and then centrifuged. The clear supernatant (250 μl) was taken for the determination of amino acid content by the fluorescamine method [26]. RPMI-1640 (6.7 mM total amino acids) or an amino acid mixture similar in composition to globin was used as calibration standard. Fluorescence was determined with either a Perkin-Elmer MPF-4 or a Spex Fluorolog II. Alternatively, amino acid production was assessed by the fluorometric determination of tyrosine [27]. The amino acid production by uninfected cells from the same culture was similarly tested. In some experiments the extracellular medium was sampled separately on parallel aliquots to assess the role of permeability barriers in the egress of intracellularly produced amino acids. To test the effect of drugs on amino acid production, they were added to the desired concentration at the onset of incubation.

The amount of amino acids was calculated in nanomoles per 10^8 cells for each sample. The contribution of uninfected cells was subtracted. The results divided by the degree of parasitemia, yielded the amount of amino acid per infected cell. Rates of amino acid production were calculated from these values and from the elapsed time between consecutive samples.

Estimation of parasite protein turnover. Parasites in culture were metabolically labeled with [^{3}H]isoleucine or [^{3}H]tyrosine (both at 100 Ci/mmol) presented at 0.5 μCi/ml in the culture medium for 18–20 hr. Cells were washed 5–7 times in PBS-G, 37°, to remove most of the soluble radiolabel, resuspended in the same buffer at 5% hematocrit and incubated in the absence or the presence of drug at 37°. Aliquots were taken at different time intervals, mixed with cold TCA and centrifuged. After neutralization of the supernatant, radioactivity was determined by scintillation counting. The total radioactivity in the suspension, essentially representing labeled parasite proteins, was determined in aliquots taken directly from the suspension. The supernatant counts, as percentage of the total counts of the respective sample, represent the extent of endogenous parasite protein degradation.

Accumulation of chloroquine in infected cells. Cells were suspended in PBS-G containing 1 μM CQ labeled with [^{3}H]CQ (40–70 Ci/mmol), and incubated for 30 min at 37°. (By this time intracellular CQ reaches a steady-state level [19].) After a quick spin, the radioactivity of the supernatant was determined. The ratio of intracellular to extracellular drug concentration (distribution ratio) was calculated from the decrease of label in the extracellular medium, divided by the volume of infected cells, the latter obtained from the hematocrit and the parasitemia. As uninfected cells accumulate relatively little CQ one may assume that most of the drug is taken up by infected cells [18, 19].

RESULTS

Production of amino acids by infected RBCs

The trophozoite stage of parasite development was chosen for the establishment of the experimental protocol as it is most active metabolically [28–31], contains a discrete food vacuole [20], and would be expected to have a high rate of amino acid production. As seen in Fig. 1, infected RBCs (IRBC) produce considerably more amino acids than their uninfected counterparts (NRBC). The initial higher production in the latter, while very reproducible, remains unexplained at present. Rates of production were estimated from the steady-state part of the production vs time curve. The initial endogenous levels of amino acids in IRBCs averaged 510 ± 135 nmol/10^8 cells compared with 102 ± 16 nmol/10^8 NRBCs. A similar situation is found in avian erythrocytes infected with *P. lophurae* [4, 5]. In several experiments the amino acid content of the extracellular space was determined and compared with the total amino acid content of the system. Net increase in amino acid was identical in both procedures, and for technical reasons the determination of total content was adopted. In the representative experiment shown in Fig. 1, the rates of production in the infected culture (20% parasitemia) and in NRBCs were 2.71 and 0.037 nmol/10^8 cells/min, respectively. After correction for the contribution of NRBCs in the infected culture and normalizing for parasitemia, the rate of production of the infected cells was found to be 13.5 nmol/10^8 IRBC/min, i.e. 355-fold higher than that of NRBC. Small fluctuations in the rates observed in different cultures, probably reflected slight differences in para-

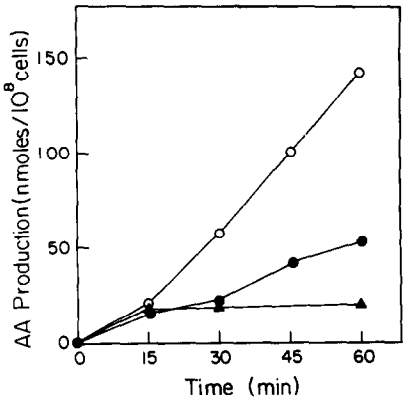

Fig. 1. Amino acid production by normal and trophozoite-infected red blood cells. Uninfected cells and infected cultures were washed twice in 100 vol. of PBS-G, suspended to 5% hematocrit in the same buffer and incubated at 37°. At various time intervals, the content of free amino acids was determined by the fluorescamine method, as detailed in Materials and Methods. A culture containing infected cells harboring trophozoites at 20% parasitemia, was obtained by mannitol synchronization: ▲, uninfected cells; ○, infected culture; ●, infected culture without glucose.

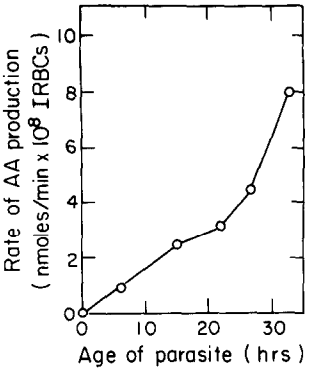

Fig. 3. Rate of amino acid production by infected cells as a function of parasite age. Parasites were synchronized by two mannitol treatments, 30 hr apart, and were subjected to Percoll-sorbitol fractionation 5 hr after the second mannitol treatment. The fraction containing early rings at 90% parasitemia was returned to culture conditions. After 2, 10, 19, 22 and 27 hr, the rate of amino acid production in PBS-G at 37° (1% hematocrit) was determined by the fluorescamine method.

site age or in the metabolic status of the specific culture. It was further established that, up to about 15% hematocrit, the amount of amino acid produced was directly proportional to the hematocrit of the infected culture in the assay system (Fig. 2). The leveling-off beyond this cell concentration probably resulted from the high rate of glucose utilization by the infected cells [29, 32], since amino acid production fell sharply in the absence of glucose (Fig. 1). The latter result indicates that amino acid production is an energy consuming process. When trophozoite-infected RBCs enriched by gelatin flotation were mixed at different ratios with NRBCs, it was found that the rate of amino acid production was directly proportional to the parasitemia (data not shown). Similar rates of amino acid production were observed in PBS-G and in a medium containing all the salt ingredients of the RPMI-1640 medium supplemented with 10 mM glucose.

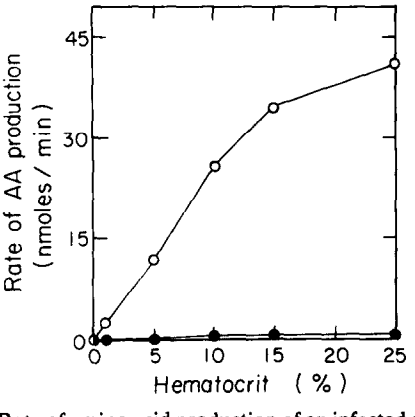

Fig. 2. Rate of amino acid production of an infected culture as a function of hematocrit. A trophozoite-containing culture at 15% parasitemia and uninfected cells were washed, suspended in PBS-G at the indicated hematocrits and incubated at 37°. The steady-state rate of amino acid production was determined by the fluorescamine method. The precise cell concentration was determined either by centrifugation in microcapillaries or by means of a cell counter: ●, uninfected cells; ○, infected culture.

Amino acid production as a function of parasite age

By means of a recently developed Percoll-sorbitol gradient method [24] for enrichment with young parasite stages, the rate of amino acid production was determined as a function of parasite development. As shown in Fig. 3, the rate of production increases slowly during the ring stage (0–24 hr after invasion of the RBC). It then becomes faster as the parasite differentiates into the trophozoite stage, which is characterized by vigorous energy metabolism as well as protein and nucleic acid anabolism [30, 33].

Effects of antimalarial drugs on amino acid production in IRBCs

Figure 4 depicts two separate experiments testing the effect of CQ on the rate of amino acid production by IRBCs at the trophozoite stage. Amino acid production is inhibited at concentrations partially coinciding with those which effectively inhibit the growth of this parasite strain in culture [34].

The extent of inhibition at a given CQ concentration was found to be related to the rate of production of amino acids in the absence of CQ, i.e. cultures producing amino acids at higher rates were also more susceptible to the inhibitory action of the drug. Figure 5 shows results obtained from several similar experiments and presented as rate of amino acid production (in the absence of drug) against the CQ concentration required for 50% inhibition (ED_{50}). The data indicate an inverse linear relationship between these two parameters. A similar behavior was obtained with younger ring stage parasites (data not shown).

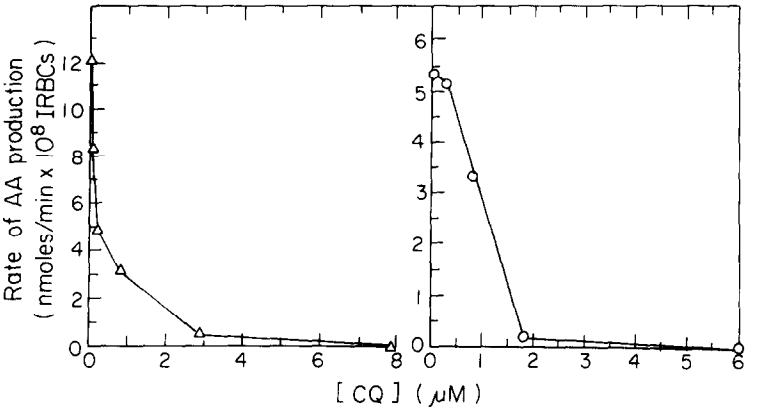

Fig. 4. Effect of chloroquine on the rate of amino acid production in trophozoite-infected cells. Cultures were synchronized by the mannitol method and, at the trophozoite stage, were suspended in PBS-G to 5% hematocrit and 15% parasitemia. The rate of amino acid production in the presence of increasing CQ concentrations was determined at 37° by the fluorescamine method. Results obtained with two different cultures are depicted.

The ability of IRBCs to accumulate CQ, and the rate of amino acid production were tested in parallel on the same culture. As previously shown [18, 19], IRBCs accumulate this drug to very high levels. The extent of accumulation was found to be directly correlated with the rate of amino acid production in absence of the drug (Fig. 6). The drug distribution coefficient was inversely correlated with the inhibitory effect of the drug throughout the life cycle of the parasite (Fig. 7).

Two other quinolines, quinine and mefloquine, were also found to inhibit amino acid production by IRBCs. As shown in Fig. 8, both drugs were effective in their respective *in vitro* growth inhibition concentration ranges [34].

The effect of the lysosomotropic agents NH_4Cl and methylamine on amino acid production in IRBCs was also tested. The first compound gave 72% and the second 85% inhibition at 1 mM, a concentration which equally affects the proteolytic digestion of endocytosed proteins in macrophages [35].

Effects of drugs on parasite protein turnover

Antimalarial drugs containing quinoline interact with neutral and model lipid membranes [37], and in so doing they cause the release of lysosomal enzymes into the cytoplasm [38, 39]. In order to test whether their antimalarial activity involves a similar mech-

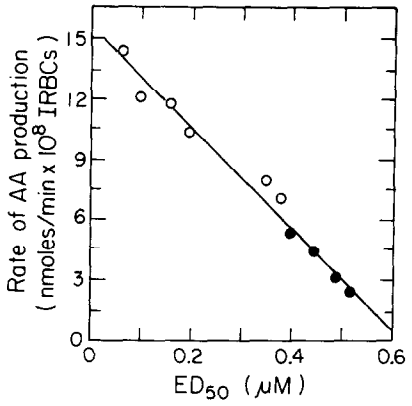

Fig. 5. Correlation between the rate of amino acid production and the inhibitory effect of chloroquine. The rates of amino acid production by trophozoite (○) and ring (●) infected cells were determined by the fluorescamine method in the absence and in the presence of increasing CQ concentrations. The concentration of CQ needed for 50% inhibition of amino acid production (ED_{50}) was found by interpolation. The ordinate represents the rate of production in the absence of CQ. Coefficient of correlation—0.965.

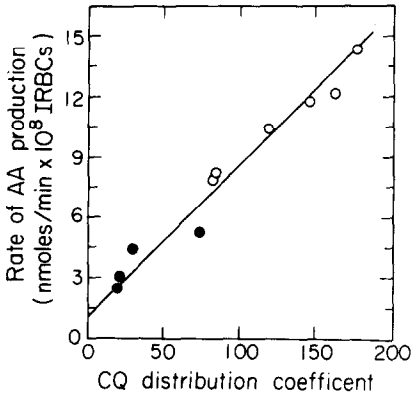

Fig. 6. Correlation between the rate of amino acid production and chloroquine accumulation. The rates of amino acid production were determined in trophozoite (○) and ring (●) infected cells. The uptake of CQ was determined in parallel in the same cultures by incubating the cells at 1% hematocrit for 30 min in PBS-G containing 1 μM CQ labeled with [^{3}H]CQ. The distribution ratio of CQ ($[CQ]_{in}/[CQ]_{out}$) was determined after 5 and 30 min as described in Materials and Methods. As there was no difference between the values obtained at these sampling times, the distribution ratios represent steady-state levels of CQ accumulation. Coefficient of correlation—0.969.

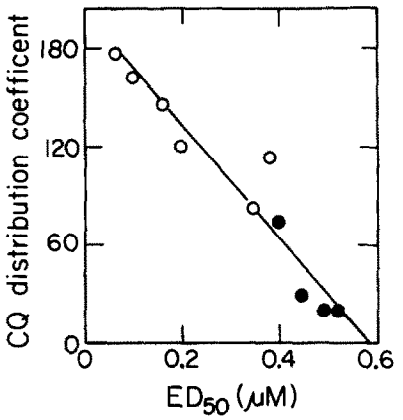

Fig. 7. Correlation between chloroquine accumulation in IRBCs and its inhibition of amino acid production. The rates of amino acid production by IRBCs harboring ring (●) and trophozoite (○) stages, as affected by CQ, was determined in parallel with CQ accumulation. The ED_{50} values were determined by interpolation and the CQ distribution ratios were calculated from the amount of drug associated with a given volume of infected cells divided by the extracellular drug concentration at steady-state. Coefficient of correlation—0.956.

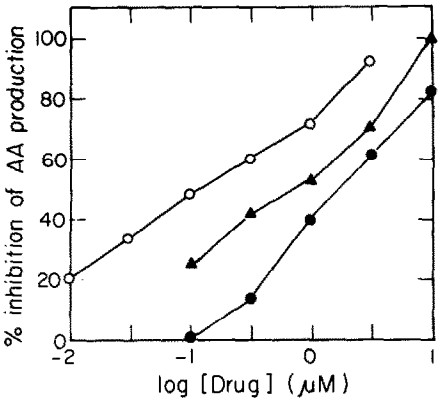

Fig. 8. Effect of quinoline-containing antimalarials on amino acid production by IRBCs. Mannitol-synchronized cultures at the trophozoite stage (15% parasitemia) were suspended to 5% hematocrit in PBS-G containing increasing concentrations of CQ (▲), quinine (●), and mefloquine (○), and the rate of amino acid production at 37° was determined by the fluorescamine method.

anism, parasites were metabolically labeled with either [^{3}H]isoleucine or [^{3}H]tyrosine, and the egress of label in the absence or the presence of the various drugs was measured as a function of incubation time at 37° in PBS-G. Drug concentrations used were 5 times higher than those needed for complete inhibition of amino acid production. No drug effect could be detected (results not shown).

The egress from [^{3}H]tyrosine labeled cells of labeled and total tyrosine was measured in the absence and the presence of 1 μm CQ. As shown in Table 1, the efflux of label remained unaffected, while that of unlabeled tyrosine was inhibited by 50% in the presence of CQ. Furthermore, the data indicate that protein turnover is relatively small as compared with host protein degradation: only 0.018% of the total labeled protein egressing per min.

Composition of free amino acids in IRBCs

To identify the origin of amino acids produced by the parasite, IRBCs harboring mature stages were concentrated to higher than 90% parasitemia by gelatin flotation [25], returned to culture conditions for 1 hr, washed twice in 100 vol. of PBS-G and incubated in the same buffer at 50% hematocrit for 30 min at 37°. Cells were denatured in TCA and the free amino acids were analyzed in an LKB amino acid analyzer. The concentration of each amino acid relative to the total was calculated and the results were plotted against the relative content of the acid in human hemoglobin [40] as shown in Fig. 9. The direct correlation obtained (correlation coefficient of 0.867) suggests that the major protein degraded by the parasite is indeed hemoglobin. (Attempts to find similar relationship with the amino acid composition of two other types of proteins, resulted in much smaller correlation coefficients: 0.554 for serum albumin and 0.587 for carbonic anhydrase.) The slight deviations from the straight line probably result from

Table 1. Effect of chloroquine on parasite endogenous protein and host cytosol degradation

Treatment	Rate of TYR production nmol/(10^8 IRBCs/min)	Inhibition %	Rate of [^{3}]TYR release %/min
Control	1.00 ± 0.08	0	0.018 ± 0.002
1 μM CQ	0.50 ± 0.05	50	0.018 ± 0.002

Synchronized cultures at the ring stage (20% parasitemia) were radiolabeled with [^{3}H]tyrosine (0.5 μCi/ml) until they reached the trophozoite stage. Free label was discarded by several washes in PSB-G preheated to 37° until the supernatant contained only background count levels. Cells were suspended in PBS-G to 5% hematocrit and incubated at 37° in the absence or the presence of 1 μM CQ. At various time intervals aliquots were taken, and the labeled tyrosine in the medium and the total tyrosine in the suspensions, were determined by scintillation counting and by nitrosonaphtol fluorescence, respectively. The rate of appearance of free label was normalized to the total amount of label in the infected culture, and the rate of total tyrosine production was related to the number of infected cells. Uninfected RBCs produce negligible amounts of tyrosine. The difference in tyrosine production between control and CQ-treated IRBCs, was significant to a level of $P < 0.005$.

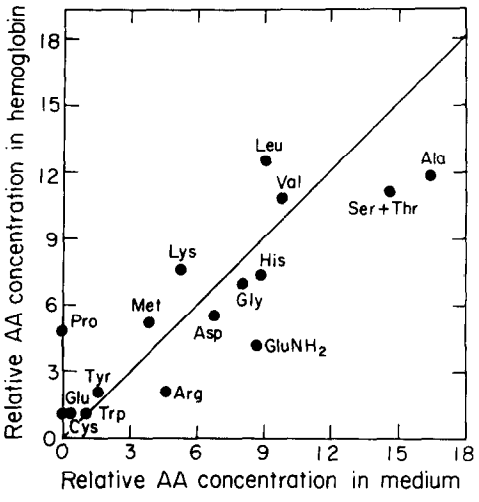

Fig. 9. Correlation between the amino acid composition of human globin and the composition of amino acids released by IRBCs. Trophozoite and schizont infected cells were isolated by gelatin flotation and returned to culture conditions for 1 hr. After two washes in 100 vol. of PBS-G, cells were resuspended in the same medium to 50% hematocrit and incubated at 37° for 30 min. Concentrated TCA was then added to a final concentration of 5% (w/v), and after 18 hr at 4°, the precipitate was pelleted by centrifugation and the amino acid composition of the supernatant was determined with an LKB amino acid analyzer. The relative concentration (%) of individual amino acid was calculated and related to its respective prevalence (%) in human globin. Coefficient of correlation—0.867.

the differential rates of acid incorporation by the parasite and efflux from the infected cells. Interestingly, negligible levels of isoleucine could be detected in these experiments, consistent with the scarcity of this acid in human hemoglobin [40] and with the absolute dependence of the parasite on the supply of isoleucine in the growth medium [9].

DISCUSSION

As found in avian and rodent RBCs infected with their respective species of malaria parasites [4–6, 8], human RBCs infected with *P. falciparum* can produce amino acids at a rate some hundredfold higher than do uninfected RBCs. The production rate increases from 0.04 nmol/10^8 cells per min in uninfected cells, to a mean of 3.3 at the ring stage, reaching a maximum of 13.5 nmol/10^8 IRBCs per min at the mature trophozoite stage. (More mature stages were not investigated because of their demonstrable extracellular proteolytic activities [41, 42].) The production rate increases in parallel with other metabolic activities as the parasite develops and the biomass increases. Some of this correspondence may be related to the fact that hemoglobin degradation occurs in acidic food vacuoles [18, 20] and that the maintenance of acidity depends on an active proton pump which requires ATP [43]. The supply of the latter undoubtedly depends on the oxidative capacity of the cell, which increases with parasite development [29].

Normal RBCs contain 18 mM of hemoglobin monomers [44], each of which is composed of 143 amino acid residues, and the parasite degrades approximately 30% of its host cell cytosol [45] which consists essentially of hemoglobin. Hence, a mere integration of the rate over the first 30–32 hr of the parasite life cycle would suffice to account for the observed extent of degradation, most of it occurring at the trophozoite stage. Interestingly, it was found that *P. knowlesi* trophozoites produce amino acids at a rate similar to that found for *P. falciparum* in this work, resulting in 28% degradation of their host cell hemoglobin within 8 hr [46]. This observation also implies that host cell digestion is minimal at the schizont and the segmentor stages.

Although amino acid production in IRBCs is an energy consuming process, most of the amino acids are not utilized by the parasite and leave the infected cell. Efflux is mostly mediated by the permeability pathways which are induced by the parasite in the host cell membrane [47]. Indeed, the normal permeability of RBCs to amino acids [48] cannot cope with such high efflux rates. The permeability of the parasite membranes to amino acids is probably equally high, since the rate of egress matches the rate of production. What could be the reason for such high rate of host cell digestion when most amino acids leave the cell? The parasite, like any other organism, needs a balanced intracellular composition of amino acids to optimally synthesize its proteins. The achievement of such a balance would depend on several interdependent factors which control the intracellular level of each amino acid: the rate of amino acid production and consumption, the established concentration gradients and the relative permeability of each individual acid. Arginine and lysine, for example, cannot easily translocate across the permeabilized membrane of IRBCs [47]. Thus their adequate supply would essentially depend on degradation. Commensurately, an acid which is relatively abundant in hemoglobin but which is very permeable, would leak rapidly out of the cell and consequently may become rate limiting in parasite protein synthesis, therefore imposing a high rate of host cell degradation. This is probably why a specific assortment of amino acids is required for the cultivation of malaria parasites [9]. Clarification of this problem would require the establishment of the fate of each individual amino acid.

The relationship between degradation of host cytosol and the susceptibility to the antimalarial drug CQ, has been known for quite some time [48, 49]. Recently, by means of ultrastructural analysis of CQ-treated IRBCs, we have shown that parasite feeding is impaired by the drug [20]. In the present work we found that CQ inhibits the production of amino acids in IRBCs at concentrations which are also arresting the growth of FCR_3, a relatively CQ-resistant parasite strain: The ED_{50} for growth arrest in culture is 0.3 μM and maximal inhibition of amino acid production was usually observed below 1 μM. We suggest that the primary antimalarial activity of the drug involves the inhibition of the vital process of host cytosol degradation. Similar experiments should be performed on CQ-sensitive strains in order to assess the generality of this observation.

The dependence of CQ accumulation in IRBCs on parasite energy metabolism is well documented [50–52]. The direct relationship observed between CQ accumulation in IRBCs and the uninhibited rate of amino acid production, points to the dependence of both processes on the food vacuole pH. Since the food vacuole is analogous to a phagolysosome, its acidic pH is probably also achieved by ATP-dependent proton pumping [43, 53]. An acidic pH is a requisite for optimal activity of vacuolar acidic hydrolases involved in hemoglobin degradation [15] and for maximal accumulation of CQ [18]. Therefore, a more acid pH will result in a greater susceptibility of amino acid production to CQ. For these reasons there exists the inverse relationship between amino acid production or CQ accumulation and the extent of inhibition (ED_{50}) of the former process by the drug. As a corollary, it would appear that the lesser susceptibility of ring stage parasites to CQ can be caused by its smaller ability to concentrate the drug or by its smaller dependence on amino acid production. The fact that the hydrolytic activity requires an acid pH is exemplified by the inhibitory effects of the acidotropic agents NH_4Cl and methylamine, through these agents were effective at a thousand-fold higher concentration compared to CQ. These compounds are known to accumulate in acidic cellular compartments and consequently to increase their pH [36]. This result implies that CQ exerts its effect through specific interactions with the hydrolytic processes and not via the alkalinization of the food vacuole [18]. Indeed, calculation of the CQ concentration in the food vacuole, shows that it reaches millimolar levels, which could not affect the pH but would suffice for the inhibition of parasite acidic proteases [15].

Ferriprotoporphyrin IX (FP) is another product of hemoglobin digestion which is sequestered in the malarial pigment. Since it has been suggested that FP is the intracellular receptor for CQ [54], the simultaneous measurement of amino acid production and CQ accumulation in IRBCs, can be used to test this hypothesis. From the rate of amino acid production and the stoichiometry of 1 FP molecule per 143 amino acid residues in a hemoglobin monomer, a rate of production of 0.09 nmole FP/min per 10^8 IRBCs was calculated. This might be an underestimate, if the globin is not fully degraded and is partially sequestered in the malarial pigment [55]. From the CQ distribution data we calculated that the same number of IRBCs accumulate within less than 5 min 0.13–0.63 nmoles of CQ. Hence, the rate of hemoglobin digestion could apparently provide enough FP to ligate CQ intracellularly. However, there are several arguments which seem to preclude this possibility.

(1) CQ binds to free but not to FP complexed in pigment. It is most unlikely that any significant amount of FP is free, for the following reason: FP is produced at a rate which would increase its vacuolar concentration by 280 μM in 1 min. Yet, much lower concentrations of FP are sufficient to lyse cellular membranes [57, 58]. Thus, FP must be rapidly and efficiently sequestered into the pigment to avoid damage to the vacuolar membrane and the concentration of free FP should be much lower than that needed to account for CQ accumulation by ligation to FP.

(2) It was previously demonstrated that acidification of the extracellular medium [18] or alkalinization of the food vacuole by the addition of NH_4Cl [59], can quantitatively release CQ which preaccumulated in IRBCs. These observations are perfectly consistent with a mechanism of pH-driven CQ accumulation and these pH changes would not be expected to affect the postulated high affinity intracellular binding of CQ to FP [54].

(3) Ultrastructural studies of CQ-treated IRBCs did not reveal any damage to the food vacuole membrane [20] as would be expected from the known toxicity of FP-CQ complexes for membranes. Thus, although at present one cannot exclude involvement of the latter in the antimalarial action of CQ, all circumstantial evidence seem to point against it.

Inhibition of amino acid production in IRBCs was also observed with two other quinoline-containing drugs, quinine and mefloquine. The first was somewhat less, and the second significantly more effective than CQ in inhibiting amino acid production, in parallel with their inhibition of parasite growth in culture [34]. These drugs are monovalent weak bases and are therefore expected to accumulate in the food vacuole to a much lesser extent than CQ [60]. This would mean that the processes that are inhibited are much more susceptible to these drugs than to CQ. In accord with this possibility, an acid protease isolated from *P. falciparum* is more sensitive to quinine and mefloquine than to CQ [15]. In addition, mefloquine could dissipate the transvacuolar pH gradient acting as an uncoupler due to its amphipatic character [37]. Such mechanism would be compatible with the finding that mefloquine reduces the inhibitory effect of CQ on parasite growth [61] because by dissipating the pH gradient it reduces the driving force for CQ accumulation in the food vacuole. It also agrees with the lack of cross resistance between CQ and mefloquine [34].

Finally, none of the drugs tested had any effect on the degradation of endogenous parasite proteins. Such an effect could have resulted, if drug accumulation in lysosomes would have caused their osmotic rupture with the consequent release of lysosomal hydrolases into the cytosol. The latter effects have been described for liver [38] and retina cells [39], using relatively high CQ concentrations. This result constitutes a further indirect evidence to implicate parasite feeding as the primary target for antimalarial action of quinoline containing drugs.

In conclusion, we suggest that the primary antimalarial effect of quinoline containing drugs is the feeding process of the parasite. Drugs accumulate in the acidic food vacuole by virtue of their weak base properties and reach concentrations known to inhibit acidic phospholipases [62] and proteases [15] which are essential for the degradation of host cell cytosol. Inhibition of feeding eventually leads to the impairment of parasite growth and propagation.

Acknowledgements—We wish to thank Professor W. D. Stein for critical reading of the manuscript, Mrs C. Stein for excellent editorial assistance, Mr and Mrs S. Neidorf and Mr and Mrs I. Scheinman for their contribution towards the purchasing of the Spex fluorometer.

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