Biochemical Pharmacology, Vol. 35, No. 21, pp. 3805–3812, 1986.
Printed in Great Britain.

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

UPTAKE OF [^{3}H]CHLOROQUINE BY DRUG-SENSITIVE AND -RESISTANT STRAINS OF THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM**

TIMOTHY G. GEARY,† JAMES B. JENSEN and H. GINSBURG‡

Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, U.S.A., and ‡ Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel

(*Received* 30 *October* 1984; *accepted* 24 *February* 1986)

Abstract—Chloroquine accumulation by human erythrocytes infected with nine different strains of the malarial parasite *Plasmodium falciparum*, which varied by ≥ 20-fold sensitivity to the drug, was measured as a function of time and drug concentration. Although the kinetics of uptake were clearly quite complex in this system, at least two general phases were observed, an extremely rapid short phase (< 30 sec), followed by a slower phase leading to steady state within 60 min. The concentration of chloroquine in the parasite food vacuole quickly exceeded 1 mM at 10^{-6} M external drug concentration. Minor alkalinization of this organelle was observed during the first 30 sec; this pH was reduced progressively over time in a concentration-dependent manner. The rate of pH reduction was highest in the drug-sensitive strains. Neither the rate of chloroquine accumulation nor intracellular chloroquine concentrations at steady state could adequately differentiate sensitive from resistant strains. Higher intracellular drug concentrations were required to kill resistant versus sensitive strains, suggesting that a change in sensitivity to chloroquine of an intracellular effector is the mechanism of resistance. The rapid rate and extensive accumulation of chloroquine, and the lack of significant alkalinization, indicate that a new theory of the mechanism of antimalarial action of the drug is required.

Two major hypotheses have been advanced to explain the mechanisms of action of quinoline-containing antimalarials such as chloroquine (CQ) in killing malaria parasites. One is that these drugs act as lysosomotropic agents, accumulating by virtue of their properties as weak bases into acidic intracellular compartments of the parasite, such as the food vacuole, and there inhibiting the function of acid hydrolases by alkalinization [1]. The second suggests that CQ forms complexes with ferriprotoporphyrin IX (FP), a putative product of the digestion of hemoglobin by the parasite, and that these complexes account for the accumulation of the drugs inside parasitized erythrocytes and, by damaging crucial parasite membranes, their lethal effects [2].

Experimental evidence discounting the latter possibility has been accumulating [3]. Thus, it has been clearly demonstrated recently that CQ accumulation by intraerythrocytic *Plasmodium falciparum* is driven by a proton gradient [4], and, in agreement with a previous report, that the drug is specifically localized in the food vacuole [5], where it can reach millimolar concentrations [6]. Nonetheless, the alkalinization caused by CQ in this organelle is slight [6], unlike the situation observed in mammalian lysosomes [7], and the small extent of the pH change is incompatible with the classic lysosomotropic hypothesis [8].

Previous investigations of CQ accumulation by *P. falciparum* [2, 9] were hampered by the use of a low specific activity [^{14}C]chloroquine preparation, the use of *P. falciparum*-infected owl monkey erythrocytes instead of human red cells, the use of an experimental medium which could not maintain parasite viability, and the testing of only one drug-sensitive and one drug-resistant strain. The availability of a variety of strains of *P. falciparum* with different, well-characterized CQ sensitivities in cultured human erythrocytes and the development of a high specific activity preparation of [^{3}H]chloroquine allowed us to characterize CQ uptake by this parasite, to determine strain variation in food vacuole pH and its influence upon CQ accumulation, and to define the relationship between external CQ concentrations required for cell killing and food vacuole CQ concentrations and pH.

MATERIALS AND METHODS

Parasites. Cultures of *P. falciparum* were maintained in candle jars [10]. Strains used included the CQ-sensitive isolates FCC_1, FCN, $FCMSU_1$/Sudan [11], Honduras 1/CDC and FCR_8, and the CQ-resistant strains FCR_1 (Vietnam Oak Knoll strain),

* Supported in part by grants from the World Bank/UNDP/WHO Special Programme for Research and Training in Tropical Diseases. We gratefully acknowledge the contributions of Dr. Crist Filer of the New England Nuclear Corp. in the preparation of [^{3}H]chloroquine. This is article number 11459 of the Michigan Agricultural Experiment Station.

† To whom correspondence should be sent. Current address: The Upjohn Co., 7923-25-5, Kalamazoo, MI 49001, U.S.A.

Vietnam Smith (VNS), FCR_{3TC} [12] and FCR_7. Drug sensitivities of some of these strains have been described previously [13].

Chloroquine IC_{50} determinations. Parasite killing was monitored by measuring drug-induced reduction in [^{3}H]hypoxanthine incorporation as described [14]. Values for IC_{50} were estimated by graphic extrapolation. Chloroquine diphosphate was purchased from the Sigma Chemical Co., St. Louis, MO.

Uptake of [^{3}H]chloroquine and [^{14}C]methylamine by uninfected human erythrocytes. Blood was drawn from healthy volunteers in citrate–phosphate–dextrose anticoagulant. Erythrocytes were washed three times (10:1, v/v with phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, 4°, pH 7.4; PBS) and then were incubated for 1 hr at 37° at 10% hematocrit (ht) in either PBS (pH 7.4) containing 10 mM glucose or RPMI 1640 (Grand Island Biological Co., Long Island, NY) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 25 mM $NaHCO_3$ (Sigma Chemical Co.), adjusted to pH 7.4. Using [^{14}C]inulin exclusion, the determination of ht was shown to underestimate the true cell volume by 2.0 ± 0.1% (N = 10; data not shown). Data as presented are not corrected for this underestimate. The cells were then washed once with the same medium and resuspended to the desired ht. Incubations were carried out at 37° in medium containing either 10 nM to 10 μM [^{3}H]CQ ([N-*ethyl*-^{3}H]chloroquine, 73 Ci/mmole. New England Nuclear Corp., Boston, MA) at 0.1 to 1.0 μCi/ml, or [^{14}C]methylamine (New England Nuclear Corp., 46 mCi/mmole) at 0.1 to 1.0 μCi/ml, or in medium containing both compounds. Total incubation volume was 0.1 to 0.2 ml in 1.5 ml screw-capped microfuge tubes (Sarstedt, Inc., Princeton, NJ). Exposure was terminated by centrifugation (5 sec at 10,000 rpm in a Beckman Microfuge 11), and radioactivity was determined in an aliquot of the supernatant fraction (Beckman LS 7500 Liquid Scintillation Spectrometer) with quench correction. No binding of [^{3}H]CQ to the materials used in these experiments was detected, as expected [15].

Given the ht and initial and final label concentrations, the concentration of drug associated with the cellular compartment is easily calculated. Values for λ, the distribution ratio, are defined as the ratio of intracellular concentration/extracellular concentration at any time.

Uptake of [^{3}H]CQ by erythrocytes infected with P. falciparum. Experiments were carried out essentially as described above. However, due to the ability of malaria parasites to accumulate large amounts of CQ, the ht was reduced to ~1%. Parasites synchronized by sorbitol lysis [16] were used at the trophozoite/schizont stage, since these are the most sensitive to CQ [17]. Parasitemias varied from 7 to 16%. Concentration of [^{3}H]CQ in parasitized erythrocytes was determined by subtracting the component due to drug uptake by uninfected erythrocytes.

Determination of erythrocyte pH. As a dibasic amine, CQ is expected to distribute according to the square of the proton gradient across any membrane [18]. Thus, [CQ]in/[CQ]out = $[H^+]^2$in/$[H^+]^2$out. Knowing the extracellular pH and [CQ] inside red cells and in the extracellular medium at any given time, it is thus easy to determine intracellular pH. Methylamine, which is monobasic, accumulates proportionately to the proton gradient, i.e. [MA]in/[MA]out × $[H^+]$in/$[H^+]$out, and the intracellular pH is again readily determined.

Determination of food vacuole pH. Since we have shown that the distribution of CQ across infected red cell membranes is determined by the relationship shown above [6], the distribution into the parasite food vacuole can be represented by the equation:

$$\frac{[CQ]_{fv}}{[CQ]_o} = \frac{[H^+]_e^2}{[H^+]_o^2} \cdot \frac{[H^+]_p^2}{[H^+]_e^2} \cdot \frac{[H^+]_{fv}^2}{[H^+]_p^2}$$

where o = extracellular medium, e = erythrocyte cytosol, p = parasite cytosol and fv = food vacuole. Hence, $\lambda_{fv} = [H^+]^2_{fv}/[H^+]^2_o$ and $pH_{fv} = 7.4 - \frac{1}{2}\log \lambda_{fv}$ (where the extracellular medium is at pH 7.4).

Determination of [CQ] in parasite compartments. It has been shown previously that the pH of the parasite cytosol, infected erythrocyte cytosol, and uninfected erythrocyte cytosol are essentially equal [4]. Hence,

$$[CQ]_p = [CQ]_c \cdot V_c + [CQ]_{fv} \cdot V_{fv}$$

Where V = relative volume, p = parasite (total), c = host cell and parasite cytosol, and fv = food vacuole. Obviously, $V_p = V_c + V_{fv} = 1$. The volumes of these compartments were determined from electron micrographs as described [4]; V_{fv} was estimated to be 3.2% of the volume of the infected cell. To date, there is no evidence showing differences in V_{fv} among strains of *P. falciparum*, nor does V_{fv} change significantly during short exposures to CQ [4]. Dividing both sides of the equation by $[CQ]_o$, we find

$$\lambda_p = \lambda_c \cdot V_c + \lambda_{fv} \cdot V_{fv}$$

Since $V_c = 0.968$ and values for λ_c are ~3 [4], the product of these terms is always much smaller than λ_p and thus

$$\lambda_{fv} = \lambda_p / V_{fv}, \text{ or } [CQ]_{fv} = [CQ]_p / V_{fv}$$

RESULTS

The IC_{50} values of the nine strains of *P. falciparum* varied by about 20-fold (Table 1).

Equilibrium was reached for CQ and methylamine uptake into uninfected erythrocytes by 5 min. At this time the mean value for λCQ was 3.12 ± 0.19 and for λ methylamine, 1.62 ± 0.24; (λ methylamine)^2 was 2.62, not significantly different than λCQ. No significant differences were observed in λ values for CQ or methylamine with different ht (10–20%), temperature (25 vs 37°), or in PBS-glucose vs RPMI 1640. Incubation times of ≥60 min at ht > 25% resulted in higher λ values for both compounds. Intracellular pH calculated from λCQ was 7.15 ± 0.015, not significantly different than that obtained from λ methylamine (7.19 ± 0.04).

The time course of [^{3}H]CQ accumulation by the FCN strain is shown in Fig. 1. The complex kinetics can be arbitrarily separated into a short period of

Table 1. Sensitivity of the different strains of *P. falciparum* to chloroquine

Strain	IC_{50}* (M)
FCN	$1.5 \cdot 10^{-8}$
FCR_8	$1.6 \cdot 10^{-8}$
FCC_1	$2.4 \cdot 10^{-8}$
Honduras 1/CDC	$3.0 \cdot 10^{-8}$
$FCMSU_1$/Sudan	$3.7 \cdot 10^{-8}$
VNS	$1.8 \cdot 10^{-7}$
FCR_7	$2.5 \cdot 10^{-7}$
FCR_{3IC}	$2.7 \cdot 10^{-7}$
FCR_1	$2.9 \cdot 10^{-7}$

Cultures were tested at 1% ht, 0.2–0.3% initial parasitemia (synchronous schizonts). Incubations were performed for 48 hr in candle jars.

* IC_{50} values represent the molar concentration resulting in 50% decrease in [^{3}H]hypoxanthine incorporation compared to drug-free controls. Each value is the mean of six observations. S.E. were < than 5% of the means.

very rapid uptake (< 30 sec) followed by a long, slower phase. Steady state was attained by 60 min. Incubation times of ~3 hr at initial $[CQ]_o$ of 10^{-6} M sometimes resulted in a gradual decrease in intracellular [CQ]. A comparison of this process in the FCN and VNS strains (Table 2) shows that the accumulative process was similar in them and that there was less than a 3-fold difference in intracellular [CQ] attained between them, despite the fact that they differ by > 10-fold in sensitivity.

Values of λ_p for the FCN strains (Fig. 2) show that, during the slow phase, the rate of CQ uptake relative to the initial $[CQ]_o$ increased as initial $[CQ]_o$ increased. At 10^{-6} M, λ_p reached about 50% of the steady-state level in <30 sec, but at 10^{-8} M, less than 1% of the steady-state λ_p value was attained during this time.

However, the absolute rate of CQ accumulation from 5 to 60 min was increased with increasing initial $[CQ]_o$ (Table 3). These rates showed considerable

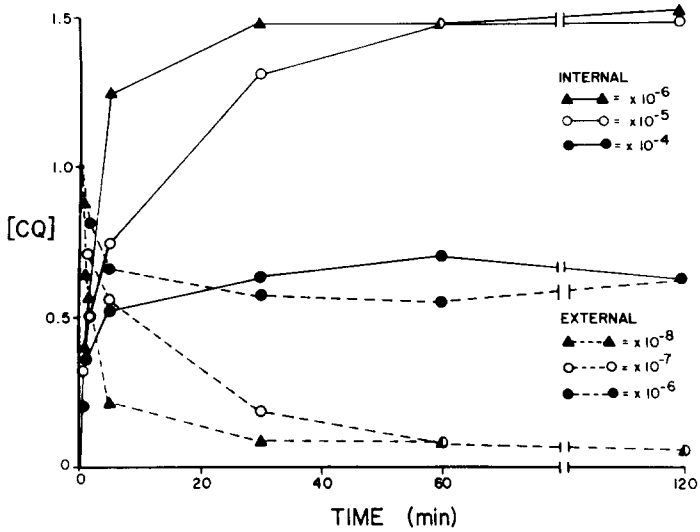

Fig. 1. Time course of CQ uptake as a function of drug concentration. Strain: FCN. Hematocrit: 0.86%. Parasitemia: 7.3%, trophozoites and schizonts. Infected cells were incubated at 37° in the presence of CQ at 10^{-6} M, 10^{-7} M and 10^{-8} M labeled with 1 μCi/ml [^{3}H]CQ. At the indicated time points, triplicate aliquots were taken and rapidly spun down. Supernatant fractions were sampled for radioactivity, and CQ concentrations in the extracellular medium (- - - -) and in infected cells (——) were calculated.

Table 2. Intracellular [CQ]: Dependence on time and initial [CQ]

Strain	Initial concn. (M)	Time 10 sec	30 sec	60 sec	5 min	30 min	60 min	120 min
				Intracellular concentration ($\times 10^{-4}$ M)				
FCN	10^{-6}	2.7	3.0	3.7	5.2	6.0	7.0	6.1
VNS	10^{-6}	1.6	2.2	2.2	2.6	3.2	3.7	3.8
				Intracellular concentration ($\times 10^{-5}$ M)				
FNC	10^{-7}	3.1	4.1	6.0	7.4	11.3	15.1	15.0
VNS	10^{-7}	2.0	2.1	2.2	2.7	4.5	5.5	6.2
				Intracellular concentration ($\times 10^{-6}$ M)				
FCN	10^{-8}	3.6	4.8	5.9	12.5	14.9	15.1	15.2
VNS	10^{-8}	2.9	3.4	3.7	5.7	11.5	12.1	12.2

[^{3}H]CQ uptake was assayed, and drug concentration in infected cells was calculated as described in Materials and Methods. All values are means of triplicate observations in two to three experiments and are corrected for parasitemia.

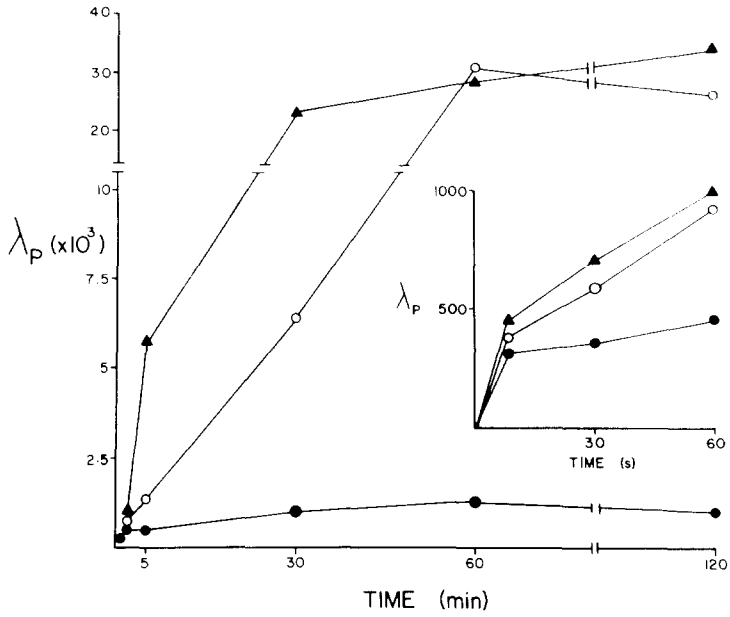

Fig. 2. CQ distribution ratios (λ_p) in infected cells as a function of incubation time and initial $[CQ]_o$. Data from Fig. 1 were used. The cellular CQ concentration of infected cells was divided by the extracellular concentration for each time point. Initial $[CQ]_o$ were: 10^{-6} M (●); 10^{-7} M (○) and 10^{-8} M (▲). Inset: details of short time points.

inter-strain variation but were not correlated with sensitivity (10^{-7} M rate vs IC_{50}: $r = -0.04$).

Intracellular [CQ] at 60 min at various $[CQ]_o$ likewise varied considerably among these strains (Table 3). Again, intracellular [CQ] values at steady state were not correlated with sensitivity (intracellular [CQ] at 60 min at 10^{-7} M initial $[CQ]_o$ vs IC_{50}: $r = -0.17$).

These data were divided by V_{fv} to give $[CQ]_{fv}$ at steady state. Values for $[CQ]_{fv}$ at initial $[CQ]_o = IC_{50}$ for any strain can be interpolated from graphs of $[CQ]_{fv}$ vs initial $[CQ]_o$. Values of $[CQ]_{fv}$ at IC_{50} external concentrations at steady state were higher for CQ-resistant than for CQ-sensitive strains (Fig. 3). Furthermore, this figure shows that $[CQ]_{fv}$ must be in the millimolar range to kill parasites.

The time-dependent changes in pH_{fv} as a function of initial $[CQ]_o$ are shown in Fig. 4. Uptake of CQ rapidly alkalinized the food vacuole, an effect which was generally more pronounced at higher drug concentrations. At low [CQ], the vacuolar pH decreased over time until a constant level was attained. This

Table 3. Accumulation and intracellular concentration of CQ in erythrocytes infected with different parasite strains

Strain	Intracellular [CQ]* at 60 min, Initial $[CQ]_o$ (M) 10^{-6}‡	10^{-7}§	10^{-8}‖	Rate of accumulation†, Initial $[CQ]_o$ (M) 10^{-6}	10^{-7}	10^{-8}
FCN	7.0	15.1	15.1	31.6	14.1	2.7
FCR_1	4.1	6.7	7.1	12.2	4.4	0.2
FCC_1	4.7	5.2	5.4	54.5	2.2	0.1
Honduras 1/CDC	6.2	6.9	ND¶	9.8	5.7	ND
$FCMSU_1$/Sudan	2.3	4.5	9.4	23.1	8.5	1.9
VNS	2.7	5.5	ND	10.2	5.1	ND
FCR_2	3.7	5.1	6.8	17.1	3.6	0.2
FCR_{3IC}	2.0	4.7	7.2	12.7	3.5	1.0
FCR_3	4.2	10.1	11.2	43.5	12.4	0.8

* Intracellular [CQ] was determined 60 min after initiation of uptake. Values are means of triplicate observations in two to three experiments. S.E. were < 10% of these means.

† Rates are expressed as 10^{-7} moles/l infected cells/min, measured from 5 to 60 min.

‡ Values are $\times 10^{-4}$ M.

§ Values are $\times 10^{-5}$ M.

‖ Values are $\times 10^{-6}$ M.

¶ Not determined.

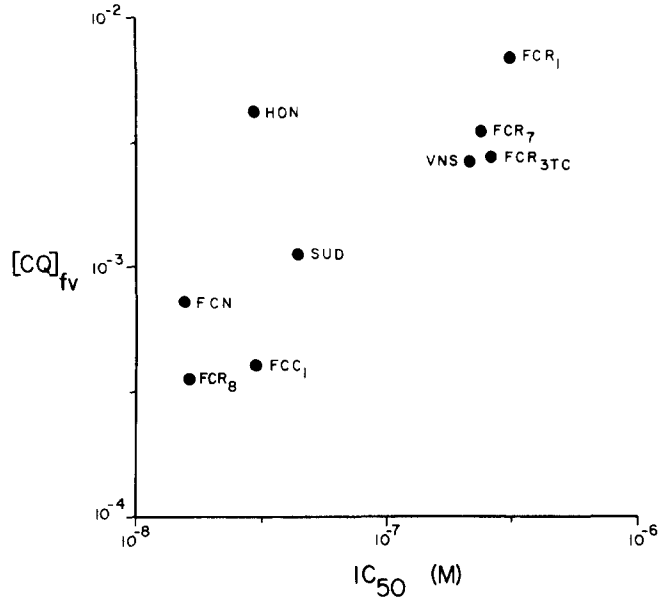

Fig. 3. Steady-state vacuolar drug concentrations achieved at $[CQ]_o = IC_{50}$. Steady-state distribution of CQ into infected cells at different initial $[CQ]_o$ were determined experimentally. Intracellular drug concentrations at $[CQ]_o = IC_{50}$ were found by interpolation of graphs of data presented in Tables 1 and 4, and vacuolar concentrations were calculated as detailed in the text.

occurred more rapidly at 10^{-8} M CQ than at 10^{-7} M, and the vacuole was only partially reacidified at 10^{-6} M. Reacidification was generally faster in CQ-sensitive strains and, at steady state, their vacuolar pH was nearly always lower than that observed in the CQ-resistant isolates.

Plotting pH_{fv} at steady-state vs initial $[CQ]_o = IC_{50}$ for these strains shows that CQ-resistant isolates had significantly higher food vacuole pH values at drug concentrations associated with lethality (Fig. 5).

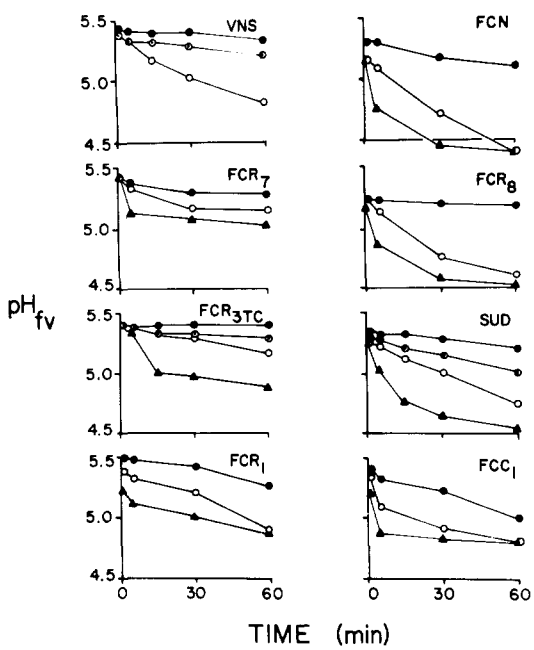

Fig. 4. Time and $[CQ]_o$ dependence of vacuolar pH in various strains of *P. falciparum*. The λ values in infected erythrocytes were determined experimentally as a function of time and [CQ] as described in Materials and Methods. Vacuolar pH was calculated as described in text. Initial CQ concentrations (M) were: (●) 10^{-6}; (⊙) $3.2 \cdot 10^{-7}$; (○) $1 \cdot 10^{-7}$; and (▲) $1 \cdot 10^{-8}$.

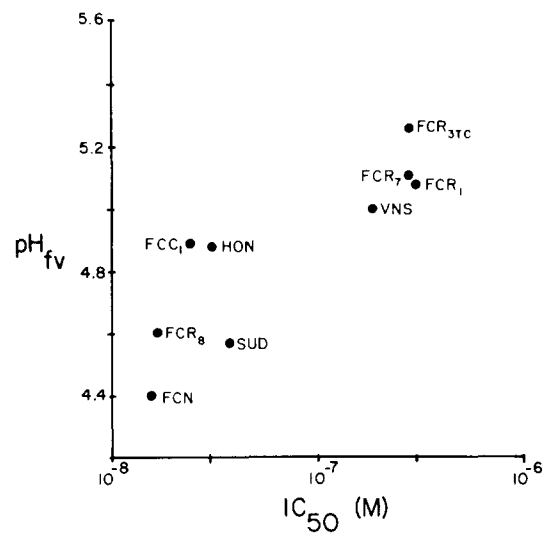

Fig. 5. Steady-state vacuolar pH achieved when parasites were exposed to drug levels of their respective IC_{50}. Vacuolar distribution ratios of drug were determined at various initial drug concentrations, and those achieved at steady state at the IC_{50} of the respective strains were obtained by interpolation. The vacuolar pH values were calculated as described in the text.

DISCUSSION

The susceptibility of malaria parasites to CQ is related to their ability to extensively accumulate the drug at low external concentrations. The development of techniques for cultivation of *P. falciparum* [19] and the recent availability of high specific activity [^{3}H]CQ made it possible to critically analyze the dynamics of drug uptake in strains which vary by ~20-fold in CQ sensitivity. Data on the extent and rate of drug accumulation as a function of CQ sensitivity can be used to evaluate current theories on the mechanism of CQ accumulation, on its mechanism of action, and on drug resistance.

The first major conclusion to be reached is that CQ resistance in *P. falciparum* is not mediated by reduced uptake of the drug, contrary to previous reports [2, 20]. Neither intracellular [CQ] at steady state nor the rate of CQ accumulation is correlated with drug sensitivity. Furthermore, if reduced uptake was the mechanism of resistance, one would expect that the intracellular [CQ] attained at $[CQ]_o = IC_{50}$ would be identical for all strains. Resistant strains would reach this toxic intracellular [CQ] only at higher $[CQ]_o$ compared to sensitive strains. We now show, however, that higher intracellular [CQ] are needed to kill resistant strains. A decrease in sensitivity to CQ of an intracellular effector or "receptor", the nature of which remains speculative (see below), is required to explain resistance.

The major prevailing hypothesis advanced to explain the selective antimalarial action of CQ is that FP, thought to be released from hemoglobin during the digestion of host cell cytoplasm by the parasite, becomes complexed with CQ and thus mediates both the accumulation and toxic effects of the drug [20]. FP, which is thought to be present in low amounts in a transiently free state, is highly lytic to malaria parasites and other cells [20]. Normally FP is thought to be sequestered into the malaria pigment, thus protecting the cell from lysis; CQ–FP complexes could remain lytic and would not be inactivated by incorporation into pigment [20]. That this theory is inconsistent with a wide variety of experimental observations has been discussed elsewhere [3]. Here we show an extremely rapid initial rate of CQ accumulation by *P. falciparum*, inexplicable by FP-mediated uptake. Within 30 sec at external [CQ] of 10^{-6} M, intracellular [CQ] reaches $2–3 \times 10^{-4}$ M. If this is concentrated in the food vacuole, as has been shown [4–6] and where free FP would have to be located, the food vacuole [CQ] would be above 10^{-3} M. Since CQ–FP complexes have a 1:2 stoichiometry [21, 22], extremely high free FP concentrations would have to be available in this short span. Such concentrations are well over those reported to completely lyse cells [23–25], and it is important to consider that free FP in any amount has never been detected in malaria parasites [26, 27]. Clearly then, CQ uptake into *P. falciparum* does not involve FP.

Distribution ratios (or their equivalent) have been commonly used in studies of CQ accumulation by malaria parasites. The values we report are much higher than previously found (cf Refs. 2 and 20). These discrepancies are most likely due to the fact that our studies, unlike those done previously, used a culture system and medium which completely maintain viability. It is evident that data based on actual CQ concentrations are more meaningful for drug toxicity, but those based on λ are not less instructive since they reflect the pH dependence of drug accumulation (see below).

There is little doubt that CQ accumulates by virtue of its weak base properties in cells in general, and in malaria-infected erythrocytes in particular, along proton gradients [4, 6]. This has been confirmed here in normal erythrocytes using monobasic methylamine and dibasic CQ. The cellular pH derived from these experiments agrees with previous publications (cf. Refs. 28 and 29), the distribution ratio of CQ being equal to the square of that of methylamine. It was shown previously that these probes distributed into parasites as expected when it was assumed that they accumulate into the acidic food vacuole [6], and that modulation of the H^+-gradient reversibly and predictably affected CQ accumulation in these cells [4, 6]. Therefore, this process should be analyzed using an H^+-gradient driving force as the underlying mechanism.

The lysosomotopic hypothesis suggests that the selective accumulation of CQ in acidic parasite organelles results in alkalinization and consequent loss of activity of pH-dependent hydrolases [1]. These effects are typically observed in mammalian lysosomes exposed to high $[CQ]_o$ [7, 8, 30–32]. Our data are compatible with the proposal that CQ accumulation is based on pH gradients and that this dibasic drug is selectively concentrated in acidic organelles. However, the initial food vacuole alkalinization observed in *P. falciparum* due to CQ accumulation was gradually reversed by all strains tested, so that at steady state, pH of food vacuole was at most 0.4 pH units higher than in untreated cells. This agrees with an earlier observation [6], in which it was also shown that the high $[CQ]_o$ needed to alkalinize mammalian lysosomes will also alkalinize the food vacuole of *P. falciparum*. Furthermore, a much higher pH in the food vacuole was caused by NH_4Cl at concentrations which had minimal effects on viability [6]. The present observation that CQ-sensitive strains were better able to compensate for CQ-induced alkalinization than resistant strains also argues against the lysosomotropic hypothesis. That the pH of the food vacuole associated with steady-state intracellular [CQ] at $[CQ]_o = IC_{50}$ was higher in resistant vs sensitive strains is most likely simply due to the higher intracellular [CQ] needed to kill the resistant strains. These data also may suggest that pH-dependent CQ toxicity could develop in the resistant strains. For instance, a hemoglobin digesting acid peptidase of parasite origin (maximal activity *in vitro* at pH 3.5) was recently identified in *P. falciparum* [33], which logically is necessary for parasite feeding. It is inhibited by millimolar [CQ] when pH is elevated. Thus, the concerted effect of high [CQ] with slight alkalinization could block vacuolar digestion of host cell stroma with consequent parasite starvation. Peptidases of various strains may be differentially susceptible to the drug, a prediction which is amenable to experimental verification.

The driving force for the initial phase of CQ accumulation is clearly the pH gradient across the food vacuole membrane, which must depend on the balance between metabolically-dependent H^+ uptake and simultaneous H^+ efflux along the concentration gradient. Comparing the food vacuole pH to [CQ] attained in this organelle by 30 sec, it seems that enough drug accumulates therein to completely titrate the free protons. That almost identical λ values during the first 30 sec are found at over 100-fold variation in $[CQ]_o$ suggests the presence of an efficient buffering system. Acidic peptides derived from the digestion of host cell cytoplasm (e.g. Hb) could provide the required buffer capacity. A Donnan equilibrium system, characteristic of mammalian lysosomes [34], could also contribute to the buffering effect.

Initial CQ accumulation as the protonated species would reduce the H^+ gradient and thus H^+ efflux, but with further energy-dependent H^+ uptake, the food vacuole would be reacidified. Accumulation of protonated CQ would continue as free protons were made available in the food vacuole until steady state was achieved (about 60 min in these experiments). The rate of CQ accumulation up to this point must be proportional to, but lower than, net H^+ uptake in the food vacuole. At $[CQ]_o = 10^{-6}$ M, the rate of uptake of the drug is likely to reflect the maximal rate of H^+ uptake, since H^+ efflux would be minimized due to the alkalinization observed. At low $[CQ]_o$, reacidification of the vacuole was achieved more rapidly, most likely due to the fact that less drug is available to sequester H^+ injected by a metabolically-driven pump. The present results are compatible with previous studies in which CQ uptake by parasitized erythrocytes was reduced by inhibitors of energy production or withdrawal of metabolic substrates required for proton pumping [35] as well as by protonophores which dissipate transmembrane H^+-gradients [36]. Although the latter could also act through the inhibition of mitochondrial energy production [37], their immediate effect [4] suggests the vacuolar membrane as their primary site of action.

If protonated drug cannot diffuse back across the vacuolar membrane, concentration-independent λ values are expected [28]. That this was not observed could be due to some back-flux of protonated CQ along its huge concentration gradient, resulting in proton shuttling and hence reduction in net H^+ uptake and reacidification. High vacuolar concentrations of protonated CQ could permeabilize the membrane to this species and induce an H^+-leak with similar results.

Although our data are incompatible with the two current hypotheses on the mechanism of action of CQ, they can be interpreted to support a novel alternative. It has been shown recently that CQ inhibits the feeding process in malaria parasites, and it was suggested that this is caused by inhibition of food vacuole phospholipase(s) necessary to liberate host cell cytoplasm from digestive vesicles [38, 39]. CQ and similar drugs inhibit lysosomal phospholipases [40, 41]. The inhibition is mediated by the binding of CQ to phospholipid substrates; neutral phospholipids bind CQ and acidic species bind even more, while cholesterol decreases the interaction [42–44]. Generally, drug concentrations in the range of 0.1 to 1.0 mM are required to inhibit phospholipases. We have now shown that such CQ concentrations are attained in the food vacuole, where the target enzyme is presumed to occur. Thus, studies on the pharmacology of CQ and similar drugs must focus on their activities at millimolar concentrations and not on the fact that $[CQ]_o$ as low as 10^{-8} can be toxic; phospholipase inhibition is a valid direction for further research.

REFERENCES

1. C. A. Homewood, D. C. Warhurst, N. Peters and V. C. Baggaley, *Nature, Lond.* **235**, 50 (1972).
2. C. D. Fitch, in *Malaria and the Red Cell*, p. 222. Pitman, London (Ciba Foundation Symposium 94) (1983).
3. D. J. Krogstad and P. H. Schlesinger, *Biochem. Pharmac.* **35**, 547 (1986).
4. A. Yayon, Z. I. Cabantchik and H. Ginsburg, *EMBO J.* **3**, 2695 (1984).
5. M. Aikawa, *Am. J. Path.* **67**, 277 (1972).
6. A. Yayon, Z. I. Cabantchik and H. Ginsburg, *Proc. natn. Acad. Sci. U.S.A.* **82**, 2784 (1985).
7. O. Ohkuma and B. Poole, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3327 (1978).
8. C. de Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof, *Biochem. Pharmac.* **23**, 2495 (1974).
9. C. D. Fitch, *Science* **169**, 289 (1970).
10. J. B. Jensen and W. Trager, *J. Parasit.* **63**, 883 (1977).
11. A. A. Divo, J. A. Vande Waa, J. R. Campbell and J. B. Jensen, *J. Parasit.* **71**, 504 (1985).
12. J. B. Jensen, T. C. Capps and J. M. Carlin, *Am. J. trop. Med. Hyg.* **30**, 523 (1981).
13. T. G. Geary and J. B. Jensen, *J. Parasit.* **69**, 97 (1983).
14. T. G. Geary, A. A. Divo and J. B. Jensen, *J. Parasit.* **69**, 577 (1983).
15. T. G. Geary, M. A. Akood and J. B. Jensen, *Am. J. trop. Med. Hyg.* **32**, 19 (1983).
16. C. Lambros and J. P. Vanderberg, *J. Parasit.* **65**, 418 (1979).
17. A. Yayon, J. A. Vande Waa, M. Yayon, T. G. Geary and J. B. Jensen, *J. Protozool.* **30**, 642 (1983).
18. S. Schuldiner, H. Rottenberg and M. Avron, *Eur. J. Biochem.* **25**, 64 (1972).
19. W. Trager and J. B. Jensen, *Science* **193**, 673 (1976).
20. C. D. Fitch, *Proc. natn. Acad. Sci. U.S.A.* **64**, 1181 (1969).
21. A. C. Chou, R. Chevli and C. D. Fitch, *Biochemistry* **19**, 1543 (1980).
22. S. Moreau, B. Perly and J. Biguet, *Biochimie* **64**, 1015 (1982).
23. A. C. Chou and C. D. Fitch, *J. clin. Invest.* **66**, 856 (1980).
24. A. U. Orjih, H. S. Banyal, R. Chevli and C. D. Fitch, *Science* **214**, 667 (1981).
25. C. D. Fitch, R. Chevli, H. S. Banyal, G. Phillips, M. A. Pfaller and D. J. Krogstad, *Antimicrob. Agents Chemother.* **21**, 819 (1982).
26. K. A. Yamada and I. W. Sherman, *Expl. Parasit.* **48**, 61 (1979).
27. A. Yayon, E. R. Bauminger, S. Ofer and H. Ginsburg, *J. biol. Chem.* **259**, 8163 (1984).
28. A. Roos and W. F. Boron, *Physiol. Rev.* **6**, 269 (1981).
29. C. J. Deutsch, A. Holian, S. K. Holian, R. P. Daniels and D. F. Wilson, *J. cell. Physiol.* **99**, 79 (1979).
30. M. Wibo and B. Poole, *J. Cell Biol.* **63**, 430 (1974).
31. A. C. Allison and M. R. Young, *Life Sci.* **3**, 1407 (1964).

32. J. T. Dingle and A. J. Barrett, *Proc. Roy. Soc. B* **173**, 85 (1969).
33. F. N. Gyang, B. Poole and W. Trager, *Molec. biochem. Parasit.* **5**, 263 (1982).
34. D-J. Reijngoud and J. M. Tager, *Biochim. biophys. Acta* **472**, 419 (1977).
35. C. D. Fitch, N. G. Ynis, R. Chevli and J. Gonzales, *J. clin. Invest.* **54**, 24 (1974).
36. C. O. Diribe and D. C. Warhurst, *Trans. R. Soc. trop. Med. Hyg.* **74**, 675 (1980).
37. H. Ginsburg, A. A. Divo, T. G. Geary, M. T. Boland and J. B. Jensen, *J. Protozool.* **33**, 121 (1986).
38. A. Yayon and H. Ginsburg, *Cell Biol. Int. Rep.* **7**, 895 (1983).
39. A. Yayon, R. Timberg, S. Friedman and H. Ginsburg, *J. Protozool.* **31**, 367 (1984).
40. Y. Matsuzawa and K. Y. Hostetler, *J. biol. Chem.* **255**, 5190 (1980).
41. C. A. Dise, J. W. Burch and D. B. P. Goodman, *J. biol. Chem.* **257**, 4701 (1982).
42. A. Harder, S. Kovatchev and H. de Buch, *Hoppe-Seyler's Z. physiol. Chem.* **361**, 1847 (1980).
43. A. Harder, K-H. Hille and H. Debuch, *Hoppe-Seyler's Z. physiol. Chem.* **364**, 997 (1983).
44. J. K. Seydel and O. Wassermann, *Biochem. Pharmac.* **25**, 2357 (1976).