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EVIDENCE FOR ELECTROGENIC ACCUMULATION OF MEFLOQUINE BY MALARIAL PARASITES

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Abstract—The uptake of mefloquine and chloroquine by *Plasmodium chabaudi*-infected mouse erythrocytes was measured in the presence and absence of ionophores and uncoupler in order to distinguish between the pH-dependent and pH-independent absorption of these drugs. Nigericin and CCCP (carbonylcyanide *m*-chlorophenylhydrazone) were used to relax the proton gradients and electrical potentials across the membranes. It was found that 40–60% of the mefloquine uptake, and 90% of the chloroquine uptake, was pH-dependent, the remainder being due to passive binding to cellular constituents. The distribution ratio of the pH-dependent uptake for mefloquine was about three times greater than for chloroquine. According to the lysosomotropic weak base hypothesis in which the neutral forms of weak bases are assumed to equilibrate across membranes, the mefloquine distribution should be smaller than the chloroquine distribution: since mefloquine is singly charged and chloroquine is doubly charged, the chloroquine distribution ratio should vary as the square of the mefloquine ratio. We interpret the greater uptake ratio of mefloquine to be evidence for the involvement of secondary active transport, with drug uptake being coupled to proton outflow by an antiporter protein. It is proposed that the uptake of mefloquine is electrogenic, with the proton gradient and the electrical potential both contributing to the driving force, but that the proton gradient alone is responsible for the chloroquine uptake.

Work from several laboratories has established that chloroquine and related weak base antimalarial agents are preferentially accumulated in the digestive food vacuoles of the malarial parasites [1–5]. Indications of this were first obtained by electron microscopy, which showed that chloroquine caused morphological changes of the food vacuoles [1, 2]. Homewood *et al.* [3] proposed that the food vacuoles are analogous to mammalian lysosomes, and that by virtue of their (assumed) acidic pH, they take up weak bases by the so-called "lysosomotropic effect" [6]. The validity of this proposal has since been strengthened by the experimental demonstration that the food vacuoles are indeed acidic, as shown by the fluorescence of entrapped fluoresceinated dextran particles [4, 5].

The mechanism originally proposed for the lysosomotropic effect was that weak bases cross the lysosomal membrane by passive diffusion of the deprotronated, electrically neutral form [6]. Reprotonation occurs upon contact with the acidic media on the inside, thereby trapping the weak base, since the membrane is assumed to be impermeable to the charged species. This hypothesis was put forward before the widespread occurrence of membrane transporter proteins was known [7, 8], but the equations generated by it are still in general use [4, 5, 9].

Schuldiner *et al.* [10] derived the thermodynamic relationship between vesicle pH and the equilibrium distribution ratio of weak bases, using the assumptions given above for explaining the lysosomotropic effect. Simple equations are obtained if the pK_as of the ionizable groups are appreciably above the pH of the medium. The distribution ratio for a monoprotonated compound is given by

$$\log(C^{+}_{in}/C^{+}_{out}) = -\Delta pH \qquad (1)$$

For a diprotonated compound, Eqn (2) applies:

$$\log(C^{2+}_{in}/C^{2+}_{out}) = -2\Delta pH \qquad (2)$$

C_{in} and C_{out} are the inside and outside weak base activities, or approximately, the free solution concentrations. ΔpH is the pH difference: pH (inside) −pH (outside).

It can be seen from Eqns (1) and (2) that the distribution ratio of a diprotonated compound should equal the square of the ratio of a monoprotonated compound:

$$(C^{2+}_{in}/C^{2+}_{out}) = (C^{+}_{in}/C^{+}_{out})^2. \qquad (3)$$

Reported measurements of mefloquine uptake by malarial parasites are much more limited than chloroquine studies. Fitch *et al.* [11] suggested that mefloquine uptake occurred in an energy independent manner, in contrast to chloroquine, for which the addition of glucose caused an enhanced uptake [12]. They also found a very considerable uptake of mefloquine by uninfected erythrocytes, again in contrast to chloroquine, which is taken up to only a small degree by uninfected cells. It has since been shown that a large amount of mefloquine can bind to red cell membranes and lipids, and also, to a lesser extent, to hemoglobin [13, 14]. Krogstad

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et al. [5] found that a portion of the mefloquine uptake by *P. falciparum* was prevented by the addition of NH_4Cl, indicating that part of the uptake occurs in a pH-dependent manner. They also observed changes in the pH of the food vacuole, which served to confirm that mefloquine, like chloroquine, is concentrated in the food vacuoles.

At pH 7.4, the ring nitrogen and the tertiary amine nitrogen of chloroquine are both protonated, giving a net charge of +2, but in the case of mefloquine the pK_a of the aromatic nitrogen is very low (below 2), so that only the secondary amine is protonated in the physiological pH range, yielding a net charge of +1 [15]. This difference can be used to probe the mechanism of drug uptake. In this paper, we report that in *P. chabaudi* the distribution ratio for the pH-dependent mefloquine uptake is considerably larger than the corresponding quantity for chloroquine. According to the weak base uptake hypothesis described above, the chloroquine distribution should be larger than the mefloquine distribution, varying as the square of the latter. Since the converse is actually observed, we suggest that there is a fundamental inadequacy in the passive diffusion model for weak base uptake of antimalarials. We make the alternative proposal that these antimalarial agents are accumulated into the food vacuoles by a secondary active transport process, involving the coupling of proton outflow to weak base inflow by a membrane bound antiporter protein [16].

MATERIALS AND METHODS

Erythrocyte preparation. A chloroquine sensitive cloned line of *P. chabaudi* (AS) kindly provided by Dr D. Walliker (University of Edinburgh) was used in this work. Normal and infected white mice were anesthetized and their blood collected by cardiac puncture, using ACD solution as an anticoagulant. Infected cells were obtained when the parasites were at the mature trophozoite stage. The erythrocytes were pelleted by centrifugation at 2000 *g* for 10 min at 4°, after which the plasma and buffy coat were carefully removed by aspiration. Leucocytes were removed by passage of a 50% suspension of the erythrocytes through a column of cellulose CF-11 [17]. The cells were washed three times with cold isotonic phosphate buffered saline, pH 7.4, and were finally suspended at a concentration of 20% in a buffer consisting of 68 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 50 mM NaH_2PO_4/Na_2HPO_4, and 5 mM glucose, pH 7.4 [12]. All subsequent procedures were carried out in the latter buffer.

Determination of drug uptake. All reactions were carried out at 25° in 1.5 ml plastic microcentrifuge tubes, unless otherwise noted. The final reaction volume was 1.0 ml in all cases. Infected or uninfected erythrocytes were suspended at a final hematocrit of 5%. The parasitemia of the infected cells was between 55 and 69%. Incubation was carried out for the specified length of time with [^{14}C]labeled chloroquine, mefloquine, or methylamine, together with other additives as required. The erythrocytes were rapidly spun down following incubation in a Tomy MC 15A microcentrifuge, and 0.8 ml of the supernatant was transferred to vials containing 50% (v/v) Triton X-100-toluene scintillation fluid for counting. A Beckman liquid scintillation counter, model LS-1801, was used for this purpose. All determinations were performed in duplicate and the results averaged.

The effect of ionophores and uncoupler was measured by pre-incubating the cells for 15 min, at 25°, in the presence of the selected agents, before the addition of chloroquine or mefloquine. An additional 15 min was allowed for equilibration of the antimalarial before centrifugation and determination of label remaining in the supernatant by scintillation counting. In some cases the cells were exposed to chloroquine or mefloquine 15 min before addition of the ionophores and uncoupler.

The concentration of labeled compound in the supernatant was calculated from the radioactivity by use of calibration curves prepared from solutions of known concentration. For methylamine and chloroquine, the calibration curves were linear and passed through the origin, so that no correction was necessary for binding to the plastic. Appreciable binding to the plastic tubes did occur with mefloquine; the degree of binding was a reproducible function of the solution concentration, and was saturable at higher concentrations. The amount bound was therefore determined as a function of the solution concentration, and the empirical calibration curve prepared in this manner was used to determine the amount of mefloquine bound to the tubes in the experiments involving erythrocytes.

The uptake is expressed as (excess nmols in pellet)/(μM in supernatant). The "excess nmols in pellet" equals the difference between the original (total) concentration and the concentration in the supernatant after centrifugation, times the total volume (1.0 ml). This gives the excess in the pellet over what would be present if the mean pellet concentration were the same as the supernatant concentration. In the case of mefloquine, the amount bound to the plastic tube is subtracted from the excess nmols. Division of the excess nmols by the volume of the compartment containing the excess would give the excess concentration in that compartment; the supernatant concentration must be added to this value in order to get the actual concentration in the compartment. Let M be the excess nmols in the pellet, and V the volume (in ml) of the compartment. C_{in} is the concentration in the compartment, and C_{out} is the supernatant concentration (μM). This gives

$$\frac{C_{in}}{C_{out}} = \frac{M/V + C_{out}}{C_{out}} = \frac{M}{C_{out}} \cdot \frac{1}{V} + 1. \qquad (4)$$

Since $C_{in}/C_{out} \gg 1$, the value of 1 on the right can be dropped without significant error. Thus M/C_{out}, which is the measured ratio, is directly proportional to C_{in}/C_{out}.

Reagents. The biochemical reagents, including the ionophores employed, were obtained from Sigma. [^{14}C]methylamine was from Amersham, and had a specific activity of 806 μCi/mg. [^{14}C]mefloquine (139 μCi/mg) and [^{14}C]chloroquine (5.1 μCi/mg) were generously provided by Dr R. Lasserre (Roche Far East Research Foundation, Hong Kong) and Dr

Coy D. Fitch (St Louis University), respectively. Nigericin and valinomycin solutions were prepared by dissolving the substances in a minimal volume of ethanol, which was then added to a larger volume of buffer to make the working solutions.

RESULTS

Time dependence

The uptake of chloroquine and mefloquine is plotted as a function of time in Fig. 1 for normal mouse red blood cells (RBC) and *P. chabaudi*-infected red cells (IRC). The time required for half maximal uptake of chloroquine by IRC was about 1 min, and 90% of maximal was reached within 5 min. Mefloquine uptake by IRC was more rapid than chloroquine uptake, requiring less than 1 min for half maximal uptake, and maximal uptake occurred in 5 min. The uptake of chloroquine by uninfected RBC was very low, but continued to increase over the time course of the experiment. For mefloquine, the uptake by RBC was large and was essentially complete by the time of the first measurement (~15 sec). The uptake by both infected and uninfected cells is much greater for mefloquine than for chloroquine, but infection increases the uptake of both drugs.

Effects of ionophores and uncoupler

In order to differentiate between the active uptake of chloroquine and mefloquine which is dependent upon the presence of a proton gradient, and passive uptake resulting from the binding to cell membranes or other cellular constituents, the uptake was measured in the absence and presence of ionophores

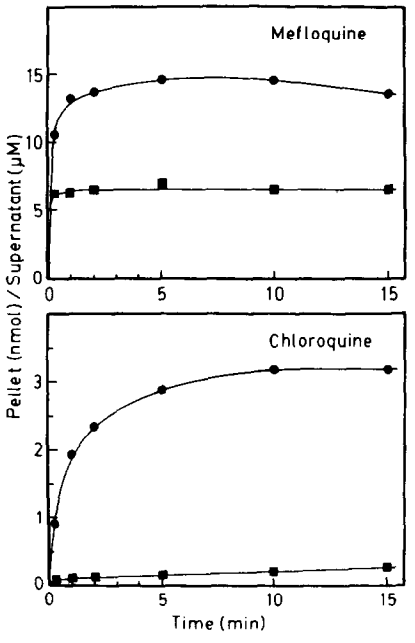

Fig. 1. Time dependence of uptake of mefloquine (upper figure) and chloroquine (lower figure) by *P. chabaudi*-infected (●) and uninfected (■) mouse red blood cells. The total concentration of mefloquine or chloroquine was 0.5 μM before centrifugation.

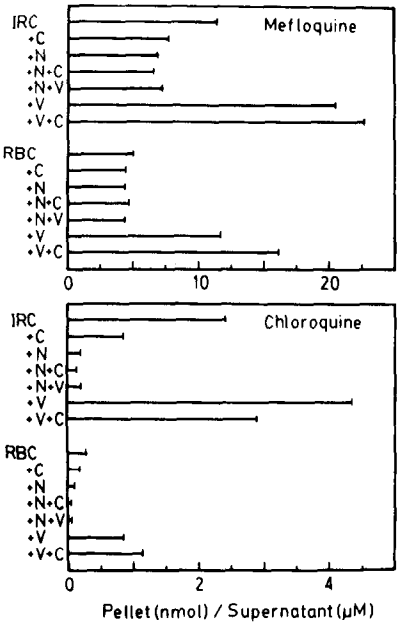

Fig. 2. Effect of ionophores on antimalarial uptake. IRC and RBC denote *P. chabaudi*-infected and uninfected red blood cells, respectively. Ionophores were added as indicated: C, CCCP; N, nigericin and V, valinomycin. The concentration of each ionophore was 2 μM in the mefloquine experiment (upper figure), and in the chloroquine experiment the concentrations were nigericin, 2 μM; CCCP, 5 μM; and valinomycin, 5 μM. The cells were preincubated for 15 min with the ionophores before the addition of mefloquine or chloroquine. This was followed by an additional 15 min incubation before centrifugation and determination of the antimalarial concentration remaining in the supernatant. In each case the volume of the reaction mixture was 1.0 ml, and the incubation temperature was 25°.

and uncoupler which relax proton gradients or increase cation permeability [18]. These include carbonylcyanide meta - chlorophenyl - hydrazone (CCCP), which increases proton permeability; valinomycin, which makes membranes permeable to K^+; and nigericin, which permits the electroneutral exchange of H^+ and K^+. The results of a survey of ionophore and uncoupler effects on the uptake of chloroquine and mefloquine are given in Fig. 2.

Qualitatively similar results were obtained in the presence of these agents for chloroquine and mefloquine, but there were significant quantitative differences between the two compounds. CCCP caused a partial decrease in antimalarial uptake, and a greater decrease was given by nigericin. Maximal diminution of uptake resulted when CCCP and nigericin were used together, indicating that this is the most effective combination for relaxing the pH and electrical potential gradients in the present system.

The large decrease in chloroquine uptake by IRC which results from the addition of nigericin and CCCP indicates that about 90% of the total is accumulated in a pH-dependent manner. For mefloquine, however, only 40–60% of the uptake is pH dependent, the remainder being passively bound. For the same final external solution concentration,

both the pH-dependent and pH-independent components of the mefloquine uptake are considerably greater than the corresponding amounts of chloroquine uptake.

Addition of valinomycin caused an appreciable increase in the uptake of mefloquine and chloroquine by both IRC and RBC. The enhancement effect of valinomycin was cancelled if nigericin was also added, but if valinomycin and CCCP were added together, an even greater enhancement was observed than when valinomycin was used alone in most cases (except for chloroquine/IRC). Increasing the valinomycin and CCCP concentrations to 20 μM did not change the results obtained.

Chloroquine and mefloquine release by ionophores and CCCP

For the experiments described in the preceeding section, the ionophores and uncoupler were added before the antimalarial agents, thereby preventing the pH-dependent uptake. We next ask whether molecules which have already been taken up as a result of a pH gradient are also retained in the cells by the pH gradient, or whether the accumulated molecules become bound to cellular constituents, and remain bound even after the gradient has been dissipated. The results in Fig. 3 show the effect of nigericin and CCCP added to the cells after 15 min exposure to chloroquine or mefloquine. For chloroquine, the addition of nigericin and CCCP caused the immediate release of essentially all of the pH-dependent uptake. Mefloquine was also released by the addition of nigericin and CCCP, but exited from the cells at a much slower rate than chloroquine, requiring 45 min for the uptake level to come down to that expected for pH-dependent accumulation. The mefloquine uptake in the buffer control experiment also decreased significantly with time, although not as rapidly as in the presence of nigericin and CCCP.

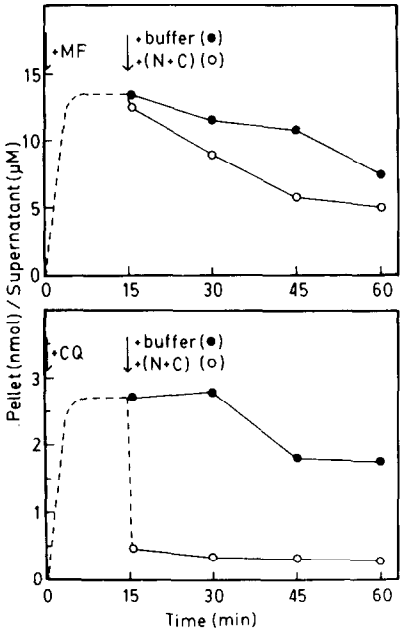

Fig. 3. Release of antimalarials by ionophores. Mefloquine (0.5 μM, upper figure) or chloroquine (0.5 μM, lower figure) was added at time zero. After 15 min nigericin (2 μM) and CCCP (5 μM) were added to half of the tubes (open circles), with an equivalent volume of buffer being added to the remaining tubes (filled circles), giving a final reaction volume of 1.0 ml. One pair of tubes was immediately centrifuged (~15 sec after buffer or ionophore addition), and the remainder were centrifuged at the times indicated.

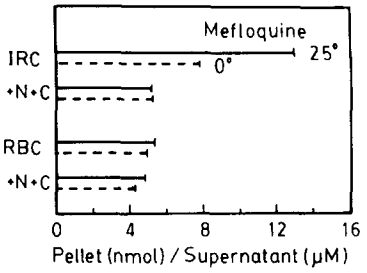

Fig. 4. Temperature dependence. Mefloquine (0.50 μM) was incubated for 15 min with infected (IRC) or uninfected (RBC) cells, at 0° or 25°, in the presence or absence of ionophores (2 μM nigericin and 5 μM CCCP). The cells were preincubated for 15 min at 0° or 25° with the ionophores before the addition of mefloquine. Solid lines, 25°; dashed lines, 0°.

Effect of temperature on mefloquine uptake

Parallel experiments were carried out at 0° and 25° with IRC and RBC, in the absence and presence of nigericin and CCCP, in order to determine the effect of temperature on the pH-dependent and independent uptake of mefloquine. The results are shown in Fig. 4. In the absence of ionophores, IRC took up 13 nmol/μM at 25°, but only 7.8 nmol/μM at 0°. In the presence of ionophores, by contrast, the uptake was the same at 0° and 25° (5.2 nmol/μM). Thus there is a 60% decrease in the pH-dependent uptake as a result of lowering the temperature from 25° to 0°; but the passive, pH-independent uptake is unaffected by the change in temperature. For RBC, lowering the temperature to 0° caused a small decrease in mefloquine uptake both with and without ionophores present. These results are consistent with the interpretation that the pH-dependent uptake of mefloquine is an active, carrier-mediated process, but that the pH-independent uptake occurs by a passive, unmediated mechanism.

The effect of temperature on chloroquine uptake was also studied. Lowering the temperature from 25° to 0° caused a 90% decrease in the uptake level, which is the fraction of total uptake shown earlier to be due to a pH gradient. This result is in agreement with the report of Fitch *et al.* [11], who also found that chloroquine uptake is reduced to a very low level at 0° in *P. berghei*.

Concentration dependence

The results presented thus far were obtained by working at a constant total antimalarial concentration of 0.5 μM. This resulted in a variable supernatant concentration, depending upon how much

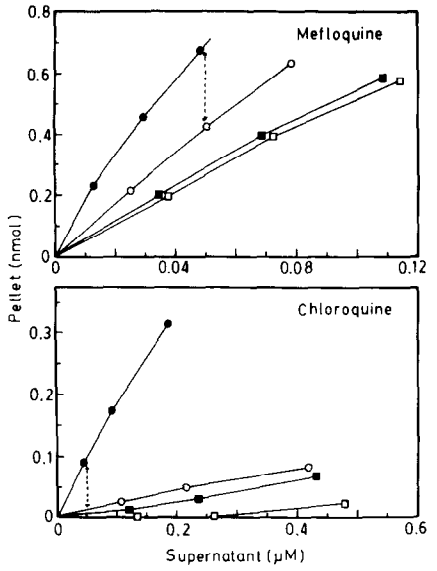

Fig. 5. Concentration dependence of antimalarial uptake. The excess nmols of mefloquine or chloroquine in the pelleted erythrocytes is plotted as a function of the concentration remaining in the supernatant after centrifugation. The total concentrations before centrifugation were 0.25, 0.50 and 0.75 μM for mefloquine, and 0.133, 0.266 and 0.5 μM for chloroquine. The reaction volume was 1.0 ml in each case; the hematocrit was 5%, and the parasitemia was 67%. The experimental protocol was as given in the legend to Fig. 2. Symbols: IRC (●); IRC plus nigericin (2 μM) and CCCP (5 μM) (○); RBC (■); RBC plus nigericin (2 μM) and CCCP (5 μM) (□). The vertical dashed lines indicate the concentration of supernatant (0.05 μM) at which values were taken for inclusion in Table 1.

Table 1. Uptake of antimalarials at constant external concentration

System†	Uptake (nmol/μM)* CQ	MF	MA‡
IRC	2.00	13.82	0.047
IRC + (N + C)	0.25	8.40	0.013
RBC	0.10	5.80	0.016
RBC + (N + C)	0.00	5.26	0.002
Differences§			
ΔIRC	1.75	5.42	0.034
ΔRBC	0.10	0.54	0.014
ΔIRC—ΔRBC	1.65	4.88	0.020

* The uptake is expressed as the excess nmols in the pellet/μM supernatant concentration. The uptake for mefloquine (MF) and chloroquine (CQ) was determined at a constant supernatant concentration of 0.05 μM from the data given in Fig. 5.

† IRC and RBC denote the infected and uninfected cells, respectively. Nigericin (N, 2 μM) and CCCP (C, 5 μM) were added as indicated. See legend to Fig. 5 for experimental details.

‡ Methylamine (MA). The concentration of methylamine was 0.75 μM in each tube before centrifugation, and the supernatant concentration after centrifugation was 0.71–0.75 μM.

§ ΔIRC and ΔRBC are the differences in uptake between that in the absence and presence of ionophores, and equal the pH-dependent portion of the uptake. ΔIRC—ΔRBC is the difference between the pH-dependent uptake by the infected and uninfected cells.

was taken up by the cells. The results were normalized to facilitate comparisons by dividing the nmols taken up by the actual final concentration in the supernatant. Quantitative comparisons were made, however, by making a series of measurements at different total concentrations, with and without ionophores, for RBC and IRC, from which data the uptake could be determined by interpolation for the same supernatant concentration. The results of measurements using mefloquine and chloroquine, made on the same batches of cells in order to permit reliable comparisons, are shown in Fig. 5. It can be seen that there is a slight downward curvature of uptake as a function of concentration, but the almost linear dependence validates the approximate normalization method used in the earlier experiments. Table 1 gives the numerical values obtained for the uptake at 0.05 μM supernatant concentration (indicated on Fig. 5 by vertical dashed lines).

Methylamine uptake

The uptake of methylamine by IRC and RBC was determined for comparison with the mefloquine and chloroquine results. The total methylamine concentration was 0.75 μM. The results obtained are given in the last column of Table 1, normalized to units of nmol/μM for comparison with the other values. The methylamine uptake is much smaller than the uptake of either chloroquine or mefloquine, as expected (see Discussion).

DISCUSSION

It can be seen from Table 1 and the various figures that only about 10% of the chloroquine is taken up by IRC in a pH-independent manner. This fraction is probably bound to the cellular membranes or to the hemozoin pigments [20–22]. A much larger proportion of the mefloquine uptake occurs in a passive manner, which is in keeping with its known lipophilicity, and its great tendency to bind to lipids and membranes [13, 14] as well as to hemin [13, 21].

Use of ionophores and uncoupler

The procedures involving the use of ionophores and uncoupler in this work were designed to permit the discrimination between pH-dependent and independent uptake of antimalarials without appreciably altering the state of the system as a whole. Ionophores have often been used with other membrane systems to achieve a similar purpose [18]. An alternative method which has been employed to abolish proton gradients involves the addition of a relatively high (10–50 mM) concentration of NH_4Cl [4, 5], but such concentrations are sufficient to affect significantly the osmolality of the solution and to alter the system in other unknown ways. Nigericin alone, which was nearly as effective as nigericin plus CCCP (Fig. 2), was previously used by Krogstad *et al.* [5]

to relax the pH gradient in isolated malarial parasites. The residual uptake in the presence of nigericin and CCCP is attributed to passive, pH-independent binding by cellular constituents. The uptake of chloroquine and mefloquine by uninfected RBC was also diminished by these ionophores, showing that a portion of the RBC uptake was also pH dependent. This is as expected, since the cytoplasmic pH of both infected and uninfected erythrocytes is acidic relative to the external medium [4, 9].

Valinomycin caused a marked increase in the uptake of both drugs. Since this increase occurred in the uninfected as well as in the infected erythrocytes, it must be primarily due to uptake by the erythrocytes themselves, rather than by the parasites. A plausible explanation for this effect is that valinomycin, which makes the membrane selectively permeable to K^+ ions, caused the establishment of an outwardly directed K^+ diffusion potential (inside negative), since the K^+ concentration is higher on the inside than the outside [23]. This diffusion potential may be directly responsible for driving the uptake of the antimalarials; alternatively, the diffusion potential may induce an electrically compensating inflow of protons, thereby lowering the internal pH, and the increased pH gradient which results is then responsible for the enhanced weak base uptake. The latter explanation is favoured by the observation that CCCP added in the presence of valinomycin causes an even greater weak base uptake; this is interpreted to mean that CCCP facilitates the compensating inflow of protons, thereby permitting a more rapid and greater build up of the proton gradient in exchange for the potassium gradient.

Temperature

Fitch *et al.* [11] found that mefloquine accumulation by *P. berghei* infected cells is the same at 2° as at 25°. These results appear to contradict our finding that incubation at 0° appreciably diminishes the uptake. The explanation may reside in the different procedures employed: in our work, 15 min incubation times were employed, but Fitch *et al.* used 1-hr incubation times. It can be seen in Fig. 3 that 1 hr of incubation with mefloquine (in the absence of ionophores) causes a decrease in the accumulated amount nearly to the passive uptake level. If only passive uptake was being observed in their experiments as a result of the long incubations, it is understandable that no effects of temperature or of glucose were observed.

Comparison of mefloquine and chloroquine uptake

Table 1 gives the uptake of mefloquine and chloroquine by IRC and RBC in the presence and absence of nigericin and CCCP. The decrease in uptake caused by the addition of nigericin and CCCP (ΔIRC and ΔRBC in the table) is identified as the pH dependent component. For ΔIRC, this component includes contributions from the host cells as well as from the parasite. Assuming that ΔRBC represents the uptake of the host component, the change in uptake due to the presence of the parasite will equal $\Delta IRC - \Delta RBC$. Most of the pH-dependent uptake of mefloquine and chloroquine by the parasite may be assumed to reside in the food vacuole, by virtue of the concentrative effect of the low pH within the vacuole. Thus the quantities $\Delta IRC - \Delta RBC$ in the last line of Table 1 are the appropriate values to use for comparison and for analysis by Eqs. (1)–(4). M/C_{out} in Eqn (4) may be equated to $\Delta IRC - \Delta RBC$, with V being the effective volume of the food vacuoles.

The pH-dependent component of the uptake is probably in a free solution state rather than a bound state, since it is released into the external solution by relaxing the pH gradient. The slower release of mefloquine than chloroquine (Fig. 3) may be related to the kinetic barrier provided by the erythrocyte membrane; this is consistent with the reported slow exchange of membrane-associated mefloquine with the external medium [14].

The pH-dependent uptake of mefloquine by the parasites (4.88 nmol/μM) is seen to be three-fold greater than the chloroquine uptake (1.65 nmol/μM). Both of these quantities are vastly larger than the uptake of methylamine (0.02 nmol/μM). The difference in uptake of methylamine and chloroquine is of the order of magnitude that one would predict from Eqns (3) and (4) by using a reasonable value for the food vacuole volume [4, 24], but the large uptake of mefloquine is clearly anomalous. According to the lysosomotropic weak base hypothesis, the pH-dependent mefloquine uptake should equal that of methylamine, since both are singly charged, and both should be less than that of chloroquine.

Two explanations may be offered for this anomaly. The first is that virtually all of the mefloquine which is taken up as a result of the pH gradient becomes bound after it is within the food vacuole, so that the activity within the vacuole is actually very low and is equal to the methylamine activity. This would require the binding of more than 99% of the pH-dependent mefloquine, in addition to the pH-independent mefloquine which is already bound. While it is true that increasing the internal mefloquine concentration by a pH-driven accumulation can be expected to cause a shifting of the association equilibria in the direction of a greater degree of binding, it is difficult to accept that nearly all of the mefloquine could be bound thus, but at the same time remain capable of being dissociated again and released to the medium at such time as the pH gradient is removed.

The alternative explanation which we would like to propose is that mefloquine accumulation occurs by an electrogenic secondary active transport process. This interpretation is developed in analogy to the mechanism by which catecholamines (epinephrine, norepinephrine and serotonin) are actively accumulated by the chromaffin granules of the adrenal medulla [25, 26]. Chromaffin granules are acidic membrane vesicles; the low pH is maintained by the presence of an inwardly pumping proton ATPase located in the chromaffin membrane [27, 28], and the catecholamines are secondarily pumped in by coupling to the proton gradient created by the ATPase. A membrane bound transporter protein mediates the coupled outflow of two protons for every catecholamine brought in [26, 29]. It is significant that these molecules cross the membrane in the positively charged, monoprotonated form, which is

also the predominant form in solution [30]. Since two positive charges (protons) move out for every one charge brought in, the process is electrogenic, with the electrical potential acting in concert with the pH gradient to provide the force for pumping in the drug. The equation describing the equilibrium distribution of the monoprotonated drug, assuming perfect coupling of proton and drug transport, is similar to Eqn (2), but with an additional term which is due to the electrical component of the driving force [25, 26].

$$\log (C_{in}/C_{out}) = -2\Delta pH + (F/2.3RT)\Delta\psi \quad (5)$$

F is the Faraday constant, R and T are the gas law constant and absolute temperature, respectively and $\Delta\psi$ is the electrical potential difference across the membrane. It can be seen from the equation that the electrical term will cause C_{in}/C_{out} to be larger than expected on the basis of the pH gradient alone since $\Delta\psi$ will tend to be positive, as a result of the action of the proton pumping ATPase in the membrane.

Mefloquine bears a single positive charge, and therefore it is possible that it is pumped into the malarial food vacuole in a similar manner to that by which catecholamines are accumulated by chromaffin granules, the equilibrium distribution ratio being given by Eqn (5). This proposal entails the assumption that the vacuolar membrane contains a proton pumping ATPase which creates and maintains the low pH of the internal medium, and also a transporter protein capable of catalyzing the antiport movement of protons and mefloquine. Both of these assumptions are plausible. Krogstad *et al.* [5] showed that addition of ATP to a free parasite preparation caused an immediate drop in the food vacuole pH, which implied the presence of a proton pumping ATPase. As for the second assumption, it is now well known that biological membranes in general contain many proteins which catalyze the active or passive transport of charged or uncharged molecules [7, 8], and a proton or ion gradient is often used to provide the required energy for active transport [16]. It is also known that transporter proteins often have a rather broad substrate specificity, so that it is not unlikely that one of the transporters of the food vacuole membrane which has a normal metabolic role could also function to transport mefloquine.

If mefloquine is accumulated in an active transport process by antiport with protons, it is possible that chloroquine is taken up in a similar way. Chloroquine might be carried by the same or a different transporter as mefloquine. If the outflow of two protons is coupled to the inflow of one chloroquine, the process will be electrically neutral, since two charges move in both directions (assuming that chloroquine is transported in the doubly charged form that is predominant in solution). In this case the driving force for chloroquine uptake is provided by the pH gradient alone and is given by the first term on the right side of Eqn (5), or equivalently, by Eqn (2). Thus we find that this particular model for proton/chloroquine antiport yields the same distribution ratio dependence on ΔpH as the lysosomotropic weak base hypothesis, although the mechanisms assumed in the two cases are very different.

If Eqn (2) describes the uptake of chloroquine (regardless of the mechanism assumed) and Eqn (5) applies to mefloquine, then it is possible to calculate the magnitude of the membrane potential ($\Delta\psi$) across the food vacuole membrane which would account for the greater degree of mefloquine uptake. The 2ΔpH term appears in both equations and therefore cancels. This leaves

$$\log(C_{in}/C_{out})_{MF} - \log(C_{in}/C_{out})_{CQ} = (F/2.3RT)\Delta\psi \quad (6)$$

The values given in Table 1 equal M/C_{out} as defined in Eqn (4), but these can be used in place of C_{in}/C_{out} in Eqn (6), since the volume term cancels in the logarithmic operation. Using the quantities given in the last line of Table 1, a value of 28 mV for $\Delta\psi$ (positive inside) is obtained.

Membrane transport

Antimalarials must pass through four membranes before arriving in the food vacuole: the erythrocyte, the parasitophorous and parasite plasma membranes as well as that of the food vacuole itself. The kinetics of chloroquine transport across the plasma membrane of uninfected human erythrocytes was studied by Yayon and Ginsburg [31], who concluded that the membrane contains a simple symmetric carrier which permits chloroquine to equilibrate across the membrane according to its concentration gradient. No active accumulation of chloroquine into the uninfected erythrocytes was found in that study, although it was reported in a more recent paper on a similar system that a small uptake of chloroquine and methylamine did occur [24]. Fitch *et al.* [12] found that mouse erythrocytes take up a small amount of chloroquine, and our finding is in agreement with that report. We obtained a relatively small amount of pH-dependent uptake by the uninfected erythrocytes, but also found that the magnitude of this uptake could be greatly increased by adding valinomycin, which appears to indirectly cause the pH gradient across this membrane to increase. Thus the transport across the mouse erythrocyte membrane is coupled to the proton gradient, and the transporter presumed to be present is not of the simple symmetric carrier type which has been described for human erythrocytes [31].

Passage of the antimalarials across the parasitophorous membrane and the parasite plasma membrane is probably also assisted by the presence of transporter proteins, but the nature of these transporters is unknown. Warhurst [32] has postulated the existence of a "permease" in the parasite membrane which causes chloroquine to be actively accumulated in the parasite cytoplasm under the influence of the transmembrane proton gradient. It is difficult to understand how this could occur, however, since the cytoplasm of the parasite is alkaline relative to the erythrocyte cytoplasm [32], and hence the pH gradient would tend to exclude weak bases from the parasite cytoplasm. On the other hand, a membrane potential ($\Delta\psi$) could exist across these membranes and be used to drive antimalarial accumulation, similarly to the mechanism reported for the uptake of the cationic drug gentamicin by *Staphylococcus aureus* [33]. An alternative to be considered is that a simple transporter is present which permits the equilibration

of the charged forms of the antimalarials across these membranes.

Based upon the results presented in this paper, we propose that the transport of mefloquine and chloroquine across the food vacuole membrane occurs by antiport with protons, the process being catalyzed by a suitable transporter protein. We suggest that proton and electrical potential gradients contribute in an additive manner to the uptake of mefloquine, but that the proton gradient alone is responsible for the chloroquine accumulation. The greater driving force experienced by mefloquine accounts for its quantitatively greater degree of uptake as compared to chloroquine. The presence of an H^+-ATPase in the food vacuole membrane is deemed essential for maintaining the low pH which is needed for the normal digestive functions, and which incidentally also provides the energy for antimalarial weak base accumulation.

Mechanism of toxicity

It appears unlikely, from the evidence presented here, that the action of these antimalarials is simply or solely a lysosomotropic effect in which the pH of the food vacuoles is increased, thereby moving the pH away from the optimum values for the digestive enzymes [34]. Any pH change which does occur in the intact, viable host–parasite system will tend to be compensated for the action of the food vacuole ATPase, as has recently been demonstrated [24]. Krogstad and Schlesinger [35] have noted, however, that chloroquine can increase the pH of acid vesicles much more than can be accounted for by its properties as a weak base. This may be due to effects such as proton pump inhibition or vesicle disruption.

The possibility should be considered that chloroquine and related antimalarials may have multiple sites of action, with the observed toxicity being the result of cumulative effects on several enzymes or membrane transporters. The concentrations of antimalarials which are achieved in the food vacuole may be in the millimolar range [24, 36] and are orders of magnitude higher than the dissociation constants of typical competitive enzyme inhibitors, but are in the same range as the inhibitor constants reported for the relatively nonspecific inhibitory actions of local anesthetics on a variety of enzymes [37, 38], including phospholipases which have been postulated as a target for chloroquine [39].

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