Antimalarials Increase Vesicle pH in *Plasmodium falciparum*

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ABSTRACT The asexual erythrocytic stage of the malarial parasite ingests and degrades the hemoglobin of its host red cell. To study this process, we labeled the cytoplasm of uninfected red cells with fluorescein-dextran, infected those cells with trophozoite- and schizont-rich cultures of *Plasmodium falciparum*, and harvested them 110–120 h later in the trophozoite stage. After lysis of the red cell cytoplasm with digitonin, the only fluorescence remaining was in small (0.5–0.9 μm) vesicles similar to the parasite’s food vacuole. As measured by spectrofluorimetry, the pH of these vesicles was acid (initial pH 5.2–5.4), and they responded to MgATP with acidification and to weak bases such as NH₄Cl with alkalinization. These three properties are similar to those obtained with human fibroblasts and suggest that the endocytic vesicles of plasmodia are similar to those of mammalian cells. Each of the antimalarials tested (chloroquine, quinine, and mefloquine) as well as NH₄Cl inhibited parasite growth at concentrations virtually identical to those that increased parasite vesicle pH. These results suggest two conclusions: (a) The increases in vesicle pH that we have observed in our digitonin-treated parasite preparation occur at similar concentrations of weak bases and antimalarials in cultures of parasitized erythrocytes, and (b) *P. falciparum* parasites are exquisitely dependent on vesicle pH during their asexual erythrocytic cycle, perhaps for processes analogous to endocytosis and proteolysis in mammalian cells, and that antimalarials and NH₄Cl may act by interfering with these events.

More than a decade ago, Homewood et al. suggested that chloroquine might act against plasmodia by increasing the pH of acid vesicles in the parasite so that acid proteases of the parasite could no longer degrade hemoglobin (1). This hypothesis is based on at least three implicit assumptions: (a) The parasite has acid vesicles similar to the lysosomes of mammalian cells, (b) these vesicles are part of a parasite system for the endocytosis and degradation of hemoglobin, and (c) hemoglobin degradation is essential for parasite survival. Because it has not been possible to examine these assumptions or Homewood’s hypothesis, this construct has not been tested previously.

Meanwhile, our understanding of endocytosis and the associated vesicle system of mammalian cells has progressed substantially (2–4). Perhaps most relevant to *P. falciparum* has been the development of methods to measure the pH of small cytoplasmic vesicles. Ohkuma and Poole were first to use fluorescein linked to dextran to measure the pH of the vesicle’s interior (5). They were able to demonstrate a baseline vesicle pH of 4.8–5.2 that was increased by the addition of weak bases to the medium. Given the acid pH optima of lysosomal hydrolases (6, 7), this provides an explanation for the observation that weak bases inhibit lysosomal proteolysis in situ (8, 9). The distribution of weak bases into acid compartments is due to the protonation of the base in the acid vesicle and the relative impermeability of the protonated base (10, 11). The action of weak bases (including chloroquine) on the pH of mammalian lysosomes can be predicted from their pKs and the number of protons that can be bound.

In contrast to mammalian cells, the intracellular vesicles of the malaria parasite are less well characterized. The demonstration of acid proteases in the parasite that can degrade hemoglobin (12) and the accumulation of ferriprotoporphyrin IX in the parasite (13) are consistent with the vesicular degradation of hemoglobin by the parasite. The earlier observation of Aikawa (14) that chloroquine is concentrated in the parasites’ vesicles is consistent with an acid vesicle pH. Despite these observations, Homewood’s hypothesis has remained untested; and it has, therefore, been unclear whether antimalarials increase the pH of parasite vesicles at concentrations similar to those that inhibit parasite growth in vitro and produce a clinical response in vivo.

In these studies, we used fluorescein-dextran (FD)¹ to mea-

¹ Abbreviation used in this paper: FD, fluorescein-dextran.

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sure the pH of vesicles within *P. falciparum*. The parasite was allowed to take up the FD from the erythrocyte cytoplasm, and the remaining cytoplasmic FD was removed before measurement of the vesicle pH. Thus, these studies have delineated a system of acid vesicles in *P. falciparum* that has a number of properties in common with the lysosome/endosome system of mammalian cells. We have also used this preparation to characterize the effect of antimalarials (chloroquine, quinine, and mefloquine) and NH4Cl on vesicle pH. Using the in vitro culture system (15), we have correlated the effect of antimalarials and NH4Cl on parasite growth with the ability of these weak bases to increase vesicle pH.

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MATERIALS AND METHODS

Parasite Strains and Culture Conditions: The *Plasmodium* strains of *P. falciparum* were kindly provided by Drs. Phuc Nguyen-Dinh and C. C. Campbell of the Center for Disease Control. Parasites were grown in the medium devised by Trager and Jensen (15) using modular incubation chambers (Billups-Rothenberg, Inc., Del-Mar, CA) containing 3% oxygen and 3% carbon dioxide, balance nitrogen (16). Because parasite mass contained at least 3% parasite contamination, uptake of FD by unidentified cell types and effects of NH4Cl and known antimalarials on parasite growth were defined by the concentrations of these compounds necessary to inhibit [3H]hypoxanthine uptake by 50% using a modification of the method of Desjardins et al. (18, 19). The method of Litchfield and Wilcoxon (20) was used to calculate the 50% inhibitory concentrations (ED50).

FD Labeling of Erythrocytes: FD was prepared using a modification of the procedure of deBeer and Granath (21), purified by passage over a Sephadex G-25 column, eluted with 10 mM ammonium acetate (pH 7.0), and dialyzed exhaustively against distilled water. FD labeling was performed by exposing fresh (<4-c-d-old) red cells to a hypotonic solution (1 part packed cells/2.25 parts hypotonic labeling solution, vol/vol) with 10 mg/ml FD, 5 mM HEPES, 11 mM glucose, and 2 mM MgATP. The MgATP concentration is based on the studies of Olson and Kilejian who found that 2 mM MgATP was necessary for the invasion of red cell ghosts by *P. falciparum* merozoites (22). After 10 min at 30°C, an equal volume of hypotonic salt solution that had been prewarmed to 37°C (equal to the volume of the hypotonic FD labeling solution and containing 280 mM NaCl, 40 mM KCl, and 11 mM glucose) was added to permit resealing (23), and the cells were washed three times in culture medium. This procedure produced significant hemolysis (it reduced the packed cell volume from 0.5 to 0.3) and labeled 75-80% of the remaining red cells (by visual inspection using fluorescence microscopy).

Labeling of Parasite Vesicles: Synchronous cultures of *P. falciparum* parasitemia of 0.5-0.7% were produced by adding the trophozoite- and schizont-rich supernatant from a gelatin cut (24) to uninfected FD-labeled cells. After 110-120 h of incubation in culture medium at 37°C with one or more medium changes per day, these cultures contained primarily trophozoites and reached parasitemias of 20-35%. Those parasites in fluorescent red cells contained one or two fluorescent vesicles that were 0.3-0.5 μm in diameter (Fig. 1C). To remove the FD remaining in the red cell cytoplasm of parasitized (and unparasitized) red cells, the cells were sedimented by centrifugation at 800 g for 5 min and resuspended in a medium containing 110 mM KCl, 30 mM NaCl, 2 mM MgATP, 11 mM glucose, 5 mM HEPES (pH 7.1), and 200 μg/ml digitonin. After a 2-min incubation (on ice), an equal volume of the same medium was added with 1 mg/ml bovine serum albumin (BSA) and the digitonin deleted and the parasites collected by centrifugation at 800 g for 7 min at 4°C. The parasites were then washed three times by centrifugation at 800 g for 7 min in the medium containing BSA but not digitonin and subjected to a final wash in medium alone without BSA or digitonin. The final parasite pellet was suspended in the same medium that was used for the final wash. After treatment with digitonin (200 μg/ml) to remove FD in the red cell cytoplasm, the only visible fluorescence associated with the parasites was in small intracellular vesicles (0.5-0.9 μm) (Fig. 1D). Both digitonin lysis and spectrophotometry (see below) were performed in a buffer containing 110 mM KCl, 30 mM NaCl, 11 mM glucose, and 5 mM HEPES. Similar results were also obtained with a modification of Trager's medium (25) containing 60 mM NaCl, 60 mM KCl, 1 mM NaH2PO4, 10 mM K2HPO4, 0.2 mM MnSO4, 1.8 mM sodium acetate, 11 mM dextrose, 33 mM nicotinamide, 2 mM MgCl2, 2 mM potassium ATP, and 10 mg/ml BSA at pH 7.1. ATP was excluded from the digitonin lysis and washing media when the calibration curves of vesicle pH were performed or when the effect of subsequent ATP addition was to be tested. This had no effect on the size or fluorescence of the parasite vesicles other than resulting in a higher vesicle pH.

Labeling of Fibroblast Lysosomes: Normal human fibroblasts were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The day before lysosomal pH measurement, the cells were transferred to medium containing 5 mg/ml FD and incubated in this medium for 24 h. After thorough washing with medium to remove extracellular FD and an additional 20-60-min incubation at 37°C, the cells were ready for lysosomal pH measurements. The cells had been grown on a 2 x 1-cm glass plate which was then placed in a lucite holder that held it in position for fluorescence measurements according to the method of Okum and Poole (5). When fibroblasts were plated in Hanks' salt solution buffered with 5 mM HEPES (pH 7.2), the pH of their lysosomes was 4.8-5.0 and was stable for several hours.

Measurement of Vesicle pH by Spectrofluorimetry: The pH of parasite vesicles and fibroblast lysosomes was determined using a modification of the method of Okum and Poole (5). This technique relies upon the characteristic excitation spectrum of fluorescein which has a maximum at 495 nm that is pH sensitive and another maximum at 450 nm that is independent of environmental pH. The ratio of the intensity of the light emitted at 516 nm with excitation at 495 nm to that emitted at 450 nm is a pH-dependent quantity that is independent of fluorescein concentration and therefore allows measurement of vesicle pH without having to estimate the dye concentration within the vesicles. Vesicular pH was determined by calibrating the F(495/450) ratio for parasite vesicles or fibroblast lysosomes. Vesicle pH was recalibrated with medium pH by placing the cells in a medium with 110 mM KCl containing 20 μg/ml (2.8 x 10^-6 M) nigericin. Nigericin is a K+ and H+ specific ionophore that rapidly equilibrates vesicular and medium pH under these conditions (26). A standard curve was determined for parasite vesicle pH by varying the medium pH with concentrated acetic acid or NaOH (Fig. 2). This curve and the similar calibration curve obtained with human fibroblasts (not shown) allowed determination of vesicle pH independent of the total medium FD present during the labeling studied. The parasite calibration curve was repeated six times under various conditions (KCl 140 to 60 mM and various parasitemias) and was not found to change by >0.05 pH units from one experiment to another. This curve was similar to the F(495/450) vs pH curve obtained from FD in solution (Fig. 2).

Effect of NH4Cl on Antimalarial Uptake: In these experiments, red cell suspensions with parasitemias of 25-30% were washed and suspended in culture medium at a hematocrit of 10%. These suspensions were then mixed in culture medium at a hematocrit of 10%. These suspensions were then mixed with equal volumes of culture medium containing either 2 mM MgCl2, 2 mM potassium ATP, and 10 mg/ml BSA at pH 7.1. ATP was excluded from the digitonin lysis and washing media when the calibration curves of vesicle pH were performed or when the effect of subsequent ATP addition was to be tested. This had no effect on the size or fluorescence of the parasite vesicles other than resulting in a higher vesicle pH.

Antimalarials and Vesicle pH in P. falciparum

RESULTS

Examination of FD-Labeled Parasites and Parasite Vesicles

After 110-120 h of incubation, cultures of FD-labeled red cells that had been incubated with the trophozoite- and schizont-rich supernatant from a gelatin cut (24) achieved parasitemias of 20-35%. By phase microscopy these parasites contained 25-30% merozoites (22).
Figure 1 Phase-contrast and fluorescent microscopy of FD-labeled parasitized red cells. Phase microscopy revealed parasites within intact red cells before treatment with digitonin (left side, A) and loss of the surrounding host cell cytoplasm after digitonin lysis (B). Using fluorescence microscopy, uninfected FD-labeled cells were diffusely fluorescent (right side, C). Parasitized FD-labeled red cells were also fluorescent, although the area occupied by the parasites was clear except for the parasite vesicles (0.3–0.5 μm in diameter) (left side, C). No fluorescent vesicles were observed in unlabeled parasitized red cells if FD was added to the culture medium. After digitonin lysis (using 200 μg/ml of digitonin in lysis buffer), the only visible fluorescence was in small (0.5–0.9 μm) vesicles within the parasites (D). The fluorescence photomicrographs were obtained with a Zeiss Universal epifluorescence microscope using monochromatic laser excitation at 476 nm. All micrographs were taken at the same magnification. C and A, and D and B represent the same fields photographed sequentially with fluorescence and phase illumination. Bar in D, 5 μm.

Fluorescence (0.3–0.5 μm in diameter) within the parasites (Fig. 1 C). These fluorescent structures were observed to coincide with the parasites’ vacuoles and appeared to represent FD that had been taken up from the red cell cytoplasm by endocytosis. To remove the FD that remained in the cytoplasm of parasitized and unparasitized red cells, the culture was exposed to 200 μg/ml digitonin as described in Materials and Methods. After this treatment, the parasites were observed by phase microscopy to be denuded of the red cell cytoplasm and membranes. However, the parasites were still actively motile and retained the morphological features (by phase contrast microscopy, Fig. 1 B) that characterized them before digitonin treatment. More importantly, by fluorescence microscopy the cytoplasmic red cell fluorescence was entirely gone after digitonin lysis (Fig. 1 D). The only FD remaining was confined to small areas within the parasites (0.5–0.9 μm in diameter). These appeared to be the same structures (vesicles) noted in the intact parasitized red cell, although their average diameter was slightly greater.

Measurement of Parasite Vesicle pH

Suspensions of the digitonin-treated parasites were placed in 110 mM KCl, 30 mM NaCl, 11 mM glucose, and 5 mM HEPES at 37°C to study the pH of the FD-labeled vesicles by determining the F(495/450) ratio. The pH of the intravesicular environment was calibrated by adding 20 μg/ml nigericin.
pH in digitonin-treated parasites. The rapid increase of vesicle diameter and the rise in the vesicle pH in the subsequent experiments. The close correspondence between the F(495/450) ratio in FD-labeled parasites and in solution (Fig. 2) confirms that this technique gives an accurate determination of vesicle pH in parasites prepared by this method. Parasites suspended in buffer without nigericin had an initial vesicle pH of 5.2-5.4. If ATP was omitted from the medium the vesicle pH typically rose substantially over several hours, sometimes becoming equal to the medium pH. The increase in vesicle diameter and the rise in the vesicle pH (in the absence of ATP) suggests that the parasites' vesicles were altered by the removal of the red cell cytoplasm. The addition of MgATP to a suspension of digitonin-treated parasites resulted in a rapid decrease in the parasite vesicle pH. In the example shown (Fig. 3), the vesicle pH rose to ~7.2 and fell to ~5.8 upon addition of MgATP. This acidification did not occur if the ATP or MgCl2 was added alone. The maximum acidification was observed with 2 mM MgATP, and the lower pH was typically stable for 2 h.

**Effect of Antimalarials on Parasite Vesicle pH**

If the digitonin-treated parasites were suspended in buffer containing MgATP, vesicle pH began at 5.2-5.4 with only a slight increase observed over a 60-min period. Under these conditions it was possible to test the effects of weak bases, including antimalarials, on the parasite vesicle pH. We have determined the effect of NH4Cl and three important antimalarials (chloroquine, quinine, and mefloquine) on the vesicle pH in digitonin-treated parasites. The rapid increase of vesicle pH after the addition of 50 mM NH4Cl is shown in Fig. 3. The slower increase of vesicle pH after the addition of 100 nM chloroquine and the pH of untreated (control) vesicles are shown in Fig. 4. The time course of the pH increase was such that the vesicle pH reached a new stable value within 5-10 min. The ability of each of these compounds to increase parasite vesicle pH is shown in Fig. 5B. Each point shown in Fig. 5B was determined from several pairs of curves similar to those in Fig. 4 for chloroquine. The change of vesicle pH was determined in each case from the difference between the experimental and control vesicle pH after the pH of the experimental vesicle preparation had stabilized. Similar results were also observed with quinine (at 100-1,000 nM) and mefloquine.

**Fluorescence and Vesicle pH**

In the presence of 2.8 x 10^{-3} M nigericin, which eliminates H+ gradients across membranes (26), concentrated acetic acid and NaOH were used to alter the medium pH of Indochina I parasite suspensions derived from FD-labeled red cells. The relative fluorescence intensity observed at 516 nm, after excitation at wavelengths of 495 and 450 nm, was measured with an SPF-500 Aminco spectrophotometer using suspensions containing ~10^7 parasites/ml. This figure contains data points obtained from two different experiments, and the line was determined using a least squares fit of the experimental points. In addition, the F(495/450) ratio as a function of pH for FD in solution (1 #g/ml) is shown (O) in a medium containing 110 mM KCl, 30 mM NaCl, and 5 mM HEPES, and adjusted to the indicated pH values with acetic acid or NaOH.
Figure 5. Effects of antimalarials and NH₄Cl on vesicle pH in fibroblasts and P. falciparum and on parasite growth. For A fibroblasts were loaded with FD as described in the Materials and Methods section. The only visible fluorescence was in 0.3-0.5-μm vesicles, pH values for fibroblasts were determined from a standard curve derived using nigericin (26). The pHs of the vesicles in the Indochina I parasite suspensions (B) were extrapolated from the standard curve determined in Fig. 2 after the pH had stabilized as shown in Fig. 4. The increase in pH (ΔpH) was determined by comparison with an untreated control cuvette containing another aliquot of the same parasite suspension to which no drug had been added. In each panel, the curves shown are for NH₄Cl (O), quinine (●), chloroquine (△), and mefloquine (●●), respectively. (C) The effects of these drugs on [³H]hypoxanthine uptake per well in culture medium (18, 19). Identical results were obtained using both normal (not labeled with FD) and FD-labeled red cells in the hypoxanthine assay.

Effect of Antimalarials on Lysosomal pH

Although NH₄Cl and chloroquine are well known as weak bases that can increase lysosomal pH, the effect of quinine has been reported only once (28); and the effect of mefloquine on lysosomal pH has not been reported previously. The addition of these weak bases to fibroblasts loaded with FD resulted in a rapid increase in vesicle pH (a new stable value was achieved in 5–10 min). The ability of each of these compounds to raise lysosomal pH as a function of their concentration in the medium is shown in Fig. 5A.

Importance of Acid Vesicles in the Uptake of Antimalarials

To determine whether the concentration of antimalarials by the parasite (which has been associated with their antiplasmodial activity by a number of investigators) (29, 30) was dependent on acid vesicle pH, we tested the effect of alkalinizing the parasite vesicle on the uptake of [³H]chloroquine and [¹⁴C]mefloquine by parasitized red cells. The addition of 50 mM NH₄Cl to the incubation mixture raises the pH of the acid vesicles of the digitonin-treated parasites (Figs. 3 and 5) to 6.4–7.2. When applied to parasitized red cells 50 mM NH₄Cl reduces the uptake of [³H]chloroquine by 84% and that of [¹⁴C]mefloquine by 79% (Table I). In contrast, the addition of 50 mM NaCl to the medium did not affect either [³H]chloroquine or [¹⁴C]mefloquine uptake (data not shown).

Effect of Weak Bases on Parasite Growth

Although the susceptibility of P. falciparum to chloroquine, quinine, and mefloquine is well known, the effect of NH₄Cl on parasite growth has not been tested previously. In these studies, we have taken the opportunity to define the susceptibility of the Indochina I strain of P. falciparum to all four weak bases (NH₄Cl and the three antimalarials) in the in vitro culture system (15). The purpose of these studies was to determine if the known antimalarials (chloroquine, mefloquine, and quinine) and especially the simple weak base NH₄Cl inhibit parasite growth at concentrations that were observed to increase vesicle pH. Using [³H]hypoxanthine uptake as described in the Materials and Methods section, we determined the concentration of each drug that reduced the [³H]hypoxanthine uptake to 50% of the control level. The concentrations of these compounds that inhibited parasite growth by 50% were: 3.1 mM NH₄Cl, 415 nM quinine, 194 nM chloroquine, and 10 nM mefloquine (Fig. 5C). Chloroquine was more active against the chloroquine-susceptible FCC₂ strain (ED₅₀ 6 nM) (data not shown).
DISCUSSION

These studies establish several points fundamental to Homewood’s hypothesis (1) on the mechanism of antimalarial action as applied to \textit{P. falciparum}. (a) The erythrocytic form of \textit{P. falciparum} takes up FD from the cytoplasm of the red cell which is incorporated into small parasite vesicles; (b) consistent with the results of Yayon et al. (31), these vesicles display an acid pH as determined by spectrofluorimetry using the method first employed by Ohkuma and Poole (5); and (c) the addition of MgATP produces an acidification of the vesicle interior. From these observations we conclude that \textit{P. falciparum} does indeed have acid vesicles that have a pH very similar to that of lysosomes in mammalian cells. Furthermore, FD (and presumably hemoglobin) enters these vesicles by a process that appears to be endocytosis although there may be differences in the details of this process in the parasite. Finally, these intraparasite vesicles appear to contain an ATP-dependent acidification mechanism like that of the endosomes and lysosomes of mammalian cells (32, 33).

Our data show that NH\textsubscript{4}Cl, chloroquine, quinine, and mefloquine act as weak bases and raise the pH of lysosomes in cultured fibroblasts. In the fibroblast lysosomes the effectiveness of NH\textsubscript{4}Cl and chloroquine is similar to what has been reported previously (5). The approximately 100-fold decrease in the chloroquine concentration required to raise the lysosomal pH (compared with NH\textsubscript{4}Cl) is in large part due to the fact that chloroquine is a diprotic weak base (34). Similarly, quinine and mefloquine (which are also diprotic bases) both show greater activity than NH\textsubscript{4}Cl in raising lysosomal pH. The minor differences among these compounds results from the differences in their pKs.

Given the above observations, it would be most surprising if the weak bases NH\textsubscript{4}Cl, chloroquine, and mefloquine did not affect the pH of parasite vesicles. The effect of these compounds on vesicle pH in digitonin-treated malaria parasites is slightly more complicated than that seen in fibroblasts. We presume from their initial pH response to MgATP, and the effect of NH\textsubscript{4}Cl on the pH, that the parasite’s vesicles are in general similar to mammalian lysosomes. Despite this, it must be kept in mind that after digitonin treatment the parasite vesicles appear swollen and may have been altered in some unknown way which affects their response to antimalarials. When we compare the effect of NH\textsubscript{4}Cl, chloroquine, or quinoline on vesicle pH, the parasite and the fibroblast curves are quite similar (Fig. 5). Given the differences between the preparation of parasites and fibroblasts for these measurements, it would be difficult to support a distinction between the vesicles of \textit{P. falciparum} and fibroblasts on the basis of the response of the vesicles’ internal pH to NH\textsubscript{4}Cl, chloroquine, and quinoline. However, the effect of mefloquine is markedly different in parasites and fibroblasts. In the parasites, mefloquine is effective at raising the vesicle pH at concentrations ~200-fold lower than those required in the fibroblasts. The importance of this observation becomes clear when it is realized that the strain of \textit{P. falciparum} used for these determinations is resistant to chloroquine. However, this particular strain retains susceptibility to mefloquine (ED\textsubscript{50} 10 nM). Presumably, the ability of mefloquine to raise the pH of lysosomes in fibroblasts is a reflection of its chemical properties as a weak base. However, the effect of this compound on the parasite vesicles must reflect properties of the parasite preparation that are not shared with the fibroblast.

We have also determined the effect of each of these compounds on \textit{P. falciparum} growth in vitro. In general, the concentrations that were found to inhibit growth are identical with the concentrations of these compounds that raised the pH of the parasites’ vesicles. This comparison can be made more obvious by noting that the concentrations of the known antimalarials (1–5 \textmu}M quinine, 0.1–1.0 \textmu}M chloroquine, 5–10 nM mefloquine, and 1–5 mM NH\textsubscript{4}Cl), which increase vesicle pH by 0.3–0.5 pH units, are also the concentrations of these agents that inhibit parasite growth by 50–90%. It is especially interesting that NH\textsubscript{4}Cl is an effective antimalarial at concentrations that increase the pH of the parasites’ vesicles.

Since it appears that chloroquine is behaving like a weak base, it should be concentrated in the parasite vesicles in proportion to the pH gradient across the vesicle membranes. We have studied the uptake of [\textsuperscript{3}H]chloroquine by parasitized red cells in vitro and found that 84% of the uptake can be inhibited by 50 mM NH\textsubscript{4}Cl. This concentration of NH\textsubscript{4}Cl increases the pH of digitonin-treated parasite vesicles to 6.4–7.2, decreasing the H\textsuperscript{+} gradient for the concentration of chloroquine inside the vesicles by ~10-fold, which could produce the dramatic decrease of the chloroquine uptake we have observed. We have also studied the uptake of [\textsuperscript{14}C]mefloquine and found it to be 79% inhibited by 50 mM NH\textsubscript{4}Cl. These observations are consistent with the interpretation that the concentration of chloroquine (and mefloquine) by parasitized red cells is dependent upon the acid pH of the parasites’ vesicles, and that increasing the vesicle pH with 50 mM NH\textsubscript{4}Cl eliminated the bulk of the chloroquine (and mefloquine) uptake.

In addition to the weak base hypothesis formulated by Homewood et al. (1), there are two other clearly described hypotheses of chloroquine action against \textit{P. falciparum}. The first involves chloroquine’s ability to intercalate into the DNA helix and thus, inhibit the synthesis of DNA by the parasite (35, 36). However, the concentrations of chloroquine and mefloquine that inhibit the growth of susceptible parasites (5–20 nM) are considerably less than those that have been reported to inhibit DNA synthesis (1–2 mM) (37–39). In addition to this quantitative argument, we have also observed that NH\textsubscript{4}Cl has antimalarial activity (it inhibits parasite growth at the same concentration that increases parasite vesicle pH). To our knowledge NH\textsubscript{4}Cl has not been proposed to intercalate into nucleic acids and inhibit nucleic acid synthesis. The single property, of which we are aware, that is common to these four compounds is their ability to raise the pH of acid vesicles because they are weak bases with pKs from 7–11. In addition to Homewood’s hypothesis (1), the other proposed mechanism of chloroquine action that has been clearly stated involves ferriprotoporphyrin IX. This compound is produced as a result of hemoglobin degradation and is thought to accumulate in the parasites’ vesicles (13). It can also form a complex with chloroquine that is toxic to the parasite and may be the active species in chloroquine action (40, 41). Because of our observations with NH\textsubscript{4}Cl, we do not favor either of these hypotheses. Rather, we prefer to focus on the striking correlation between the ability of these compounds to raise the pH in parasite vesicles and their activity to inhibit parasite growth.

There are two additional issues regarding Homewood’s hypothesis and our observations that need to be mentioned.
First, we now know that there are several biological consequences of increasing the pH of acid intracellular vesicles in addition to the inhibition of the catabolic functions of lysosomes. Endosomes and coated vesicles are acid compartments that participate extensively in the intracellular traffic of membranes and proteins (3). Included among the potential effects of raising their pH is the inhibition of movement and processing of receptors and ligands (for example the targeting of newly synthesized lysosomal enzymes) (4). The intra-erythrocytic asexual cycle of the parasite must involve extensive cellular re-organization and membrane movement in which the acid vesicles of the parasite might play an important role. As pointed out by Homewood et al. (1) increasing the pH of the parasite’s acid vesicles may also inhibit the parasite’s ability to degrade hemoglobin. Which of these potential consequences of increasing parasite vesicle pH is important in antimalarial activity is presently unclear. The second issue concerns the mechanism by which NH4Cl, chloroquine, quinine, and mefloquine increase the pH of the parasites’ acid vesicles. The accepted mechanism of weak base action on acid intracellular vesicles depends on the pKs of the weak base, the pH difference between the vesicle and the medium, the number of protons the weak base will bind (in the pH range of interest), and a differential membrane permeability of the neutral and protonated forms of the weak base (10). Within the accuracy of our measurements NH4Cl, quinine, and chloroquine have a similar effect on fibroblast and Indochina I vesicles that is consistent with the acid vesicles of \textit{P. falciparum} having the same ability to acidify their interior as the lysosomes of fibroblasts. The importance of these observations is demonstrated by contrasting the concentration of mefloquine that produces a 0.3 pH unit increase in fibroblast vesicles (5 μM) with the concentration that results in the same pH increase in the vesicles of Indochina I (1–10 nM). Increasing vesicle pH at such low concentrations of mefloquine is not consistent with the normal mechanism of weak base action and implies an additional interaction between the parasite vesicle and this drug. Despite this, the inhibition of parasite growth by mefloquine is still closely correlated with the increase in parasite vesicle pH. The potential mechanisms for increasing vesicle pH at nanomolar concentrations of mefloquine include excessive (greater than that which can be caused by the pH gradient maintained by the vesicle) concentration of the drug in the vesicle, a more direct effect on the vesicle’s proton pump, or the permeability of the vesicle membrane.

Yayon et al. (42) reported observations on the uptake of chloroquine by parasitized erythrocytes which are consistent with our results on chloroquine and mefloquine uptake and inhibition by high concentrations of weak bases that raise lysosomal pH. They also used the distribution of labeled chloroquine and methylamine to calculate the pH of the parasite vesicles. Those calculations did not consider the depletion of the labeled weak base from the incubation media as a possible consequence of parasite uptake. Their data indicates that such depletion is occurring and this will result in higher added concentrations of chloroquine or methylamine being required to increase the vesicle pH. In our studies we know that the media concentration of chloroquine or mefloquine was not significantly decreased during the course of our measurements of vesicle pH or chloroquine or mefloquine uptake.

In conclusion, these studies have demonstrated that \textit{P. falciparum} contains acid cytoplasmic vesicles similar to mammalian lysosomes. Macromolecules (such as FD and hemoglobin) enter these vesicles by a process similar to endocytosis. These vesicles can be stimulated to acidify their interior by the addition of MgATP and have their internal pH increased by the addition of weak bases to the medium. NH4Cl, quinine, chloroquine, and mefloquine increase the vesicle pH (as expected); but they also inhibit parasite growth at the same concentrations that increase the vesicle pH. In particular, the effects of NH4Cl are striking because they demonstrate the importance of raising vesicle pH in addition to other activities that have been proposed in the case of chloroquine (36–41). Furthermore, the concentration of mefloquine that raises vesicle pH and inhibits parasite growth is anomalously low. The antimalarials are concentrated by parasitized red cells, and the pH gradient across the parasite’s acid vesicles appears to account for virtually all of the parasite specific uptake of chloroquine and mefloquine. These observations appear to verify most of Homewood’s hypothesis (1). However, it is now clear that raising the pH of acid intracellular vesicles (lysosomes, endosomes, or coated vesicles) can have a number of consequences in addition to the inhibition of proteolysis.

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