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# Protonophoric effects of antimalarial drugs and alkylamines in *Escherichia coli* membranes

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**Inside-out vesicles of *Escherichia coli* whose lumen was acidified by substrate oxidation, were used to study the mode of pH gradient dissipation by quinoline-containing antimalarial drugs and alkylamines. The pH was dissipated by micromolar drug concentrations, the dibasic chloroquine being most potent, followed by the monobasic mefloquine, quinine and the dibasic 7*H*-quinoline. The time dependence of pH dissipation as a function of membrane potential suggests that the monoprotonated forms of the drugs are able to cross the bacterial membrane. Alkylamines were able to dissipate the pH gradient in the 0.01–5 mM range, their rank order of potency being related to their hydrophobicity. Tertiary amines were less effective than less hydrophobic primary primary amines, implying an effect of molecular volume of their diffusion across the membrane. Both sets of results suggest that amphiphilic weak bases can cross membranes in their free-base form, become protonated in an acid environment and diffuse in this form along their concentration gradient and aided by the membrane potential, thereby dissipating the pH gradient.**

## Introduction

Quinoline-containing antimalarial drugs, such as chloroquine and quinine, are weak bases which accumulate to very high levels in malaria-infected red blood cells [1]. Accumulation occurs into the demonstrably acidic compartments of the parasite, e.g., food vacuole [2] and lysosomes, driven by the pH gradient between the compartment and the extracellular medium. A major controversy exists among investigators of the mode of action of these drugs. Some contend that the drug acts directly on vacuolar acid hydrolases responsible for the degradation of ingested host cell cytosol, with no apparent alkalinization of the acidic compartment [3]. Others suggest that the drug raises the pH in the acidic compartment, which has apparently a low buffer capacity compared to that of somatic mammalian cells, thereby shifting it from the optimum needed for hydrolase action [4]. This would explain not only the mechanism of drug action but also its relative specificity for malarial parasites.

Several major questions arise from these observations: (a) Why are antimalarial drugs more efficient than other weak bases in raising vacuolar pH? (b) What is the mechanism of pH alteration: is it by proton trapping, as suggested for ammonia, or by back-flux of protonated drug, as could be the case for some hydrophobic weak bases? (c) Is the effect of the drug exerted on the pH observed at therapeutic drug concentrations?

To answer these questions, inside-out vesicles of *E. coli*, whose lumen can be acidified by substrate oxidation [5], have been used as a simple two-compartment system (the malaria-infected cell consists of four compartments). Evidence is presented to show that antimalarial drugs at substantially higher than therapeutic concentrations dissipate the pH gradient across the vesicle membrane by back-flux of their protonated species.

## Materials and Methods

*E. coli* ML 308-225 was grown at 37°C on a minimal medium [6]. Cells were washed in phosphate buffer (120 mM potassium phosphate (pH 7.8)) and resuspended in the same buffer containing 2.5 mM $MgCl_2$. Vesicles were prepared either by French press (300 atm) or by mild sonication in a Brown sonicator, level 3, six times for 10 s in the cold. Unbroken cells and debris were pelleted at 10000 × *g* for 10 min and the everted vesicles in the supernate were collected by two successive washes

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; AO, acridine orange; CQ, chloroquinone; HQ, 7-*H*-quinoline; Q, quinine; MQ, mefloquine.

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in the same buffer at 100000 × *g* for 1 h and kept at −70°C until used.

Vesicles were dispersed to 0.1 mg protein/ml in either choline buffer (140 mM choline chloride/2.5 mM $MgCl_2$/10 mM Hepes (pH 7.5–8)) or in phosphate buffer, as indicated for the individual experiments, containing 0.25–1.0 μM acridine orange (AO), maintained at 23°C. Acidification of the vesicular lumen was started by the addition of either lithium-D-lactate or sodium succinate (final concentration 15 mM) or MgATP (0.5 mM), and monitored by measuring AO fluorescence in a SPEX Fluorolog II (SPEX Industries, Metuchen, NJ), computer-driven fluorometer (excitation, 495 nm; emission, 525 nm). Dissipation of the pH gradient with $NH_4Cl$ (final concentration 20 mM) or with CCCP (final concentration 5 μM), yielded a fluorescence signal intensity equal to that measured before acidification, indicating no substantial binding of AO to the membranes. Test compounds were added in small aliquots from stock solutions each time after a new steady-state fluorescence was observed.

Although AO quenching can be used for precise quantitation of ΔpH [7], in the present work we were interested in estimating the change in the pH gradient. pH-dependent dye accumulation is given by: $\log(A_i/A_o) + \log(V_o/V_i) = pH_o - pH_i$ [8], where $A_i$ and $A_o$ are the intravesicular and external amounts of dye, respectively, and $V_i$ and $V_o$ are the vesicular and external volumes, respectively. Equal amounts of vesicles were used in all experiments (i.e., $\log(V_i/V_o)$ is constant). The relative change in the pH gradient dΔpH, as induced by the drugs, was calculated from the logarithm of $Q/(100 - Q)$, where $Q$ is the percentage of fluorescence quenched and setting the value in the absence of drug arbitrarily to zero.

## Results and Discussion

Rupture of *E. coli* cells by shearing forces, either by French press or by mild sonication, yields small vesicles whose membrane is inverted relative to the membrane of intact cells and which are able of respiration and ATP-dependent generation of ΔpH (interior acid) and Δψ (interior positive) (see Ref. 5 for review). As expected, these vesicles accumulate large amounts of the weak base fluorophore AO, as measured by the fluorescence quenching of intravesicular probe, when supplied with either D-lactate (Fig. 1), succinate or ATP (not shown). AO accumulation is totally reversed by the addition of the protonophore CCCP, which demonstrably dissipated the pH gradient by shuttling protons down their concentration gradient, or by $NH_4Cl$, which presumably penetrates as an $NH_3$ neutral species and traps protons inside, thereby raising the intravesicular pH. It has been previously suggested that ammonia dissipates ΔpH across chloroplast membranes due to

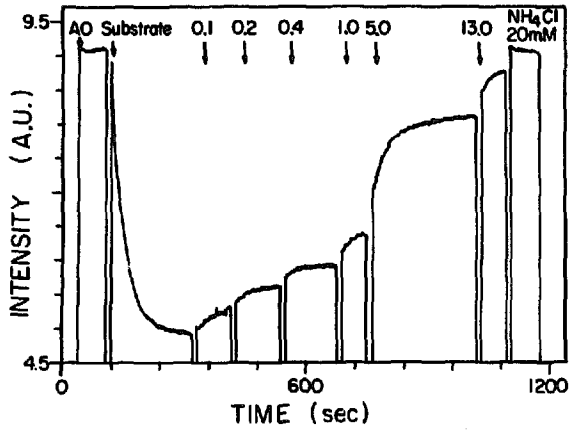


Fig. 1. Alkalinization of vesicular lumen by chloroquine. Everted *E. coli* vesicles (0.1 mg/ml) were mixed in a choline chloride medium (23°C) containing 1 μM AO. Lithium D-lactate (15 mM) was added and the vesicles were allowed to acidify their lumen, as evidenced from the quenching of the fluorescence signal. Aliquots of CQ were added to the final indicated concentrations and the release of AO, indicating alkalinization of the vesicular lumen, was monitored with time. $NH_4Cl$ (20 mM) was added to achieve full alkalinization.

efflux of $NH_4^+$ [9]. If this were also true for *E. coli* membranes, one should have observed in the presence of $NH_4Cl$ a decrease in Δψ and hence in the total protonmotive force, $\Delta\tilde{\mu}_{H^+}$. However, no such change is observed in everted *E. coli* vesicles [5], implying infinitesimally small back-flux of $NH_4^+$ in this system.

Titration of substrate-acidified vesicles with the quinoline-containing antimalarial drugs, chloroquine (CQ), 7*H*-quinoline (7HQ), quinine (Q) or mefloquine (MQ), results in a time- and dose-dependent release of preaccumulated AO, caused by the alkalinization of the vesicular lumen (Fig. 1). Although not shown, it is important to note that no change in the rate of D-lactate oxidation is observed over the range of drug concentrations used. From the steady-state effect of drug on vesicle pH shown in Fig. 2, it is clear that CQ is the most effective ΔpH dissipator, followed by MQ, Q, and 7HQ. CQ is dibasic, with $pK_a$ values of 8.1 (the ring nitrogen) and 10.2; both Q ($pK_a = 8.4$, on the quinuclidine nitrogen) and MQ ($pK_a = 8.6$, on the piperidine nitrogen) are monobasic. Under the conditions of the present experiments, a pH gradient of approx. 2 pH units is generated [5] and the dibasic CQ is expected to accumulate in the acid interior of the vesicles 104-fold, while Q and MQ should accumulate 100-fold [10]. At a luminar pH of 5.8, most of the CQ is in its diprotonated form ($CQH_2^{2+}$) and the relative concentration of monoprotonated form ($CQH^+$) is 0.5%. Only the un-ionized form of CQ can readily dissolve into, and hence translocate across, phospholipid membranes; $CQH^+$ can bind to phosphatidylcholine membranes to a minimal extent [11], but does not intercalate into them [12,13]. However, CQ binds much more avidly to negatively charged phospholipids [14], suggesting that, given the lipid com-

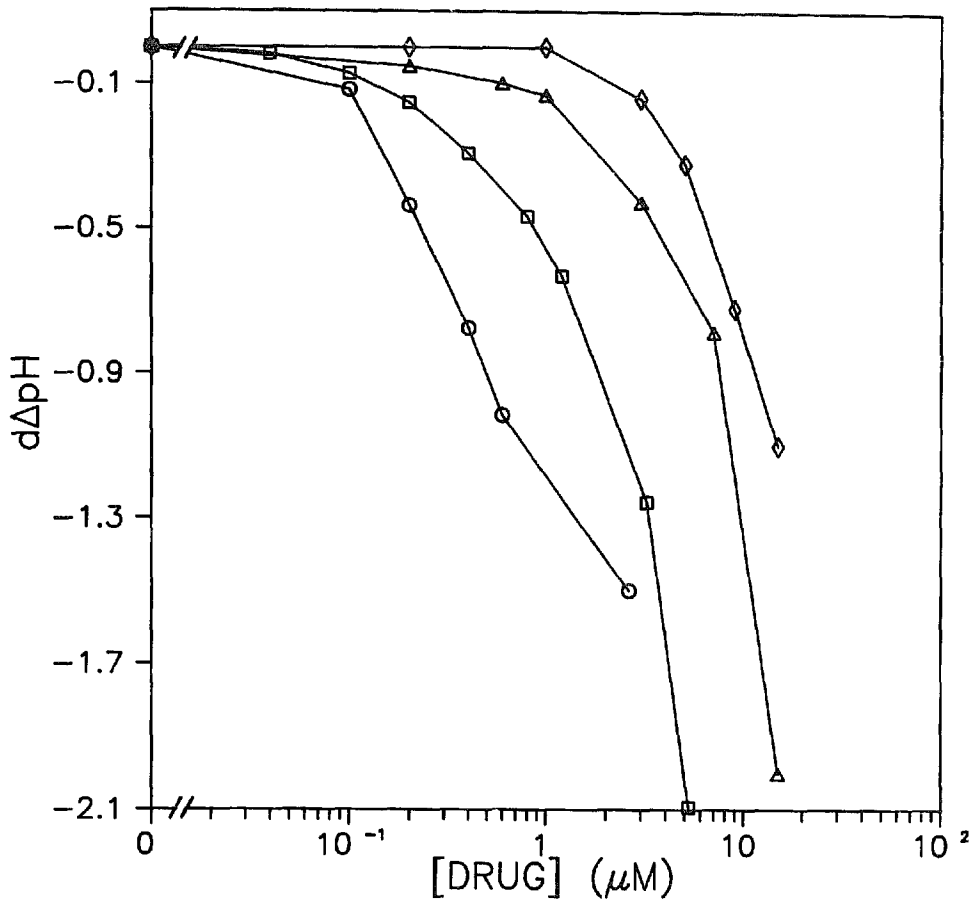


Fig. 2. Concentration-dependent alkalinization of vesicular lumen by antimalarial drugs. Acidified vesicles in choline chloride medium were titrated with different drugs and the change in the pH gradient was calculated as described in Materials and Methods and plotted against drug concentration. CQ, ○; MQ, □; Q, △; 7HQ, ◇.

position of the bacterial membrane, $CQH^+$ could translocate across the membrane. Hence, the effect of CQ on ΔpH could be due to both proton titration and proton shuttling. Since *E. coli* membranes are permeable to $Cl^-$ ions, it is unlikely that at $[Cl^-]_o = 145$ mM used in this experiment, any appreciable $\Delta\psi$ could have developed due to the efflux of protonated drug. Hence, the observed steady-state drug-induced alkalinization presumably reflects a decrease in $\Delta\tilde{\mu}_{H^+}$. Since the magnitude of $\Delta\tilde{\mu}_{H^+}$ is determined by the efficiency of the proton pump and the back-flux of the protons through the membrane, drug-mediated proton shuttling could obviously account for much of the observed alkalinization.

The downward curving of the dose–response profile (Fig. 2) could be explained as follows. At low concentrations, D-lactate-driven $H^+$ uptake can compensate for both $H^+$ titration and drug-mediated $H^+$ efflux. As the drug level is increased, the concentration of the putative proton-translocating species increases (In the case of CQ and 7HQ, higher pH also results in larger concentrations of $CQH^+$ or $7HQH^+$) and the resulting efflux swamps $H^+$ uptake. The dose–response curve then represents a simple titration curve.

The dual effect of CQ offers an explanation as to why in macrophages the drug causes vacuolization of lysosomes at low concentrations, the effect of disappearing at higher levels [15]: at first, the drug accumulates in the acidic lysosomes, driving water into them and raising their pH. At higher [CQ], the pH gradient is dissipated by the protonophoric effect, the driving force for further CQ accumulation is lost and the lysosomes shrink.

Comparing the alkalinizing effect of CQ and 7HQ, it is obvious that the latter is less effective. This difference seems clear if one considers that the substitution of Cl with H decreases considerably the hydrophobicity of 7HQ [16] and hence its partitioning (either as a free base or in its monoprotonated form) into the membrane. Indeed, not only did 7HQ have a smaller overall pH-dissipating effect, but the effect was achieved at a lower rate.

In terms of extravesicular concentrations, both Q and MQ are less effective than CQ in dissipating pH. However, considering their calculated luminar concentration, their pH-dissipating effect is considerably more potent. Compared to the effect of $NH_4Cl$ and methylamine, which are also monobasic but acting in

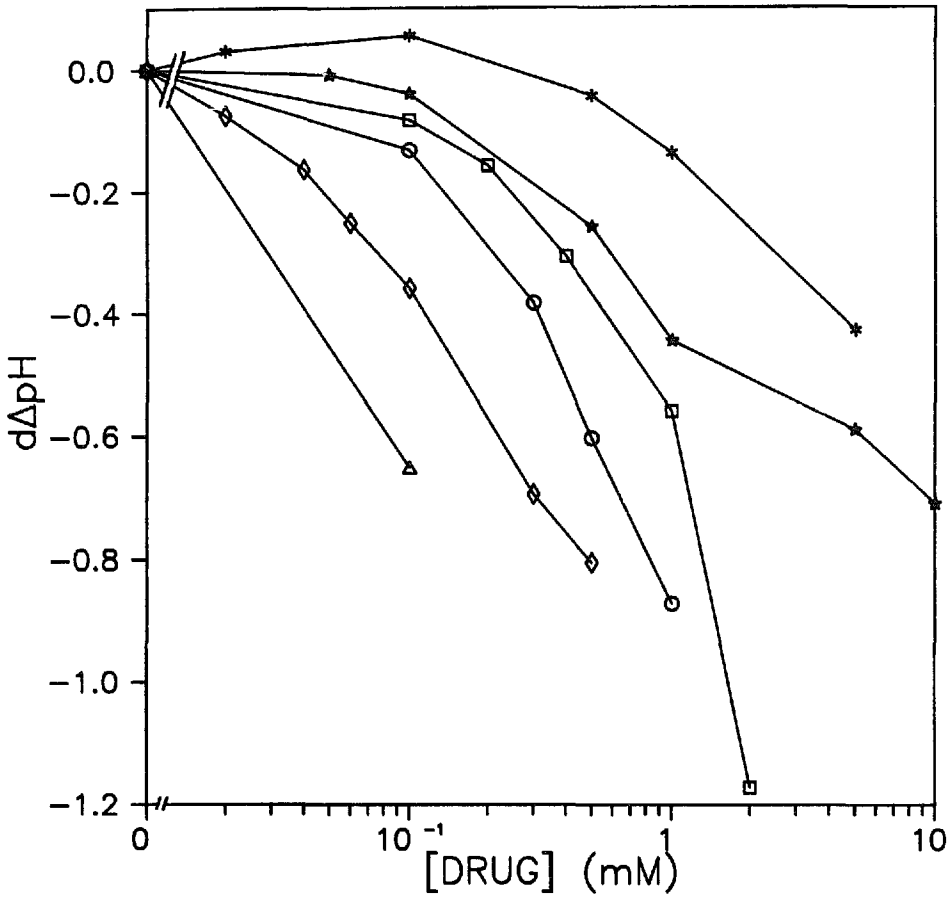


Fig. 3. Concentration-dependent alkalinization of vesicular lumen with alkyl amines. Acidified vesicles in choline chloride medium were titrated with different alkylamines and the change in the pH gradient was calculated as described in Materials and Methods and plotted against drug concentration. Propylamine, □; butaylamine, ○; triethylamine ★; tributylamine, *; heptylamine, ◇; octylamine, ◇.

the millimolar range (not shown), Q and MQ are effective in the micromolar range, suggesting that their protonated forms translocate across the membrane much faster than those of ammonia or methylamine. This suggestion is consistent with the following considerations: both compounds intercalate into phospholipid monolayers [13], implying that they can dissolve into bilayers in their protonated form, a prerequisite for diffusion across the membrane [17]. This effect probably stems from the fact that their quinoline ring is not charged above pH 4–4.5, rendering them more hydrophobic; indeed, the partitioning of Q into octanol is 400-times larger than that of CQ at pH 4.5, and 30-times larger at pH 7.4 [18]. The trifluoromethyl moieties of MQ certainly increase its hydrophobicity as compared with Q, and hence its greater potency in pH dissipation.

The hydrophobic effect in proton shuttling is best exemplified by the results obtained on pH dissipation with a series of primary and tertiary alkylamines varying in their alkyl chain length. The octanol partitioning of the amines in this study, given as log $P_{octanol}$, are [19]: octyl, 2.9; heptyl, 2.02; tributyl, 1.52; triethyl, 1.44; butyl, 0.81 and propyl, 0.37. Solubility in the membrane has been previously suggested to be the major determinant in the diffusion of alkylamines into egg phosphatidylcholine liposomes [20]. As seen from Fig. 3, this is also their rank order in alkalinizing the lumen of everted *E. coli* membrane vesicles, except for the tertiary amines. All tested compounds are monobasic, with $pK_a$ values around 10.5, and are expected to accumulate to the same extent along the pH gradient and hence to have a similar effect on $\Delta$pH if they were acting only by proton titration. The fact that the more hydrophobic congeners of this series of compounds are more effective in dissipating $\Delta$pH must therefore be related to the ability of their free base and protonated species to translocate across the membrane. Although it has been contended that most probably only the neutral amine partitions into membranes [20], a rough calculation based on the permeability coefficient of alkylamines (extrapolated to octylamine) and the rate of proton pumping in everted *E. coli* vesicles [7], shows that less than 0.05% of the total octylamine flux has to be in the protonated form in order to account for its effect on pH dissipation. Obviously, since the permeability coefficient of alkylamines decreases approx. 8-fold with decreasing number of $CH_2$ groups [20], the lower efficiency of the shorter alkylamines in dissipating $\Delta$pH

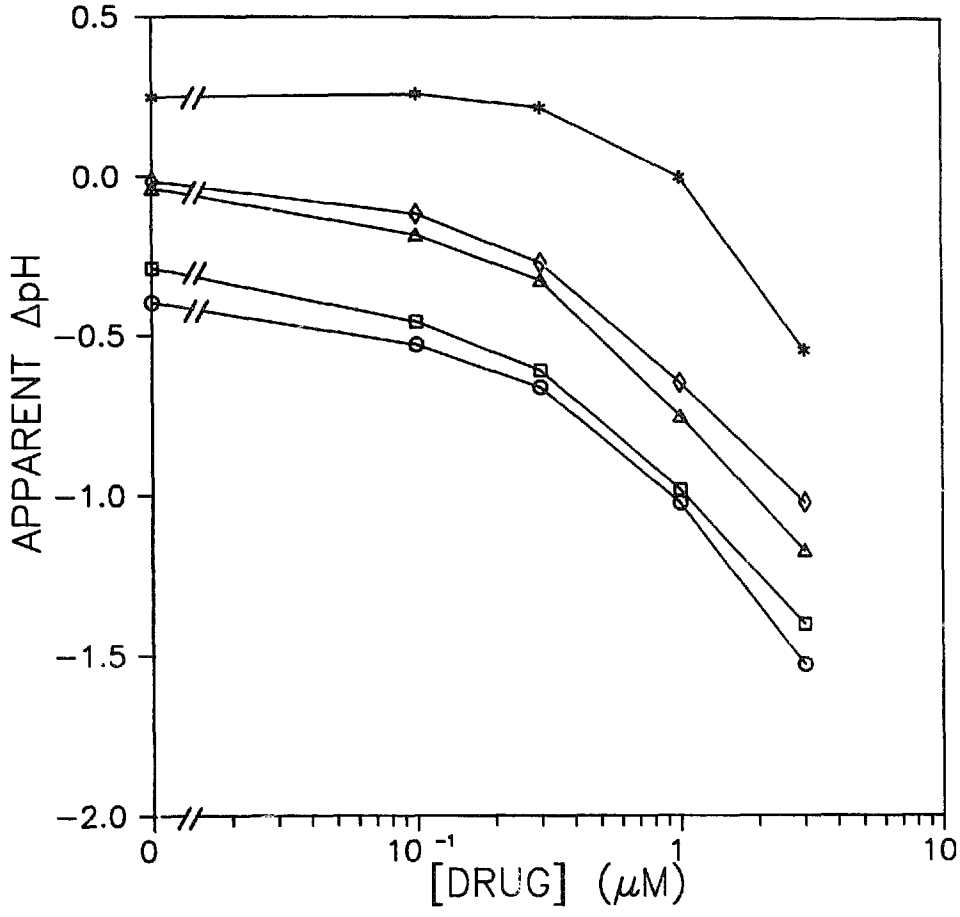


Fig. 4.Concentration-dependent alkalinization of vesicular lumen by chloroquine in media containing KCl. Apparent $\Delta pH = \log(Q \cdot V_o/((100-Q) \cdot V_i))$, as described in Materials and Methods. Vesicles were suspended in potassium phosphate buffer in which part of the phosphate was substituted with Cl, keeping osmotic concentration constant, and acidified with lithium D-lactate. This was followed by titration with CQ and the apparent pH gradient was calculated as described in Materials and Methods. Cl concentrations (mM) were: ○, 0; □, 16; △, 32; ◇, 62; *, 140.

is self-evident. The suggested protonophoric effect of alkylamines agrees with the suggestion of Ohkuma and Poole [15] that isobutylamine does not cause osmotically driven vacuolization of macrophage lysosomes due to the high permeability of its protonated form. The lesser efficacy of the teriary amines may be expected due to their bulkier volume, a property which reduces simple diffusion across membranes [17]. Hence, their influence on luminar pH was observed at relatively higher concentrations, similar to those of $NH_4Cl$ (not shown).

A corollary of the suggested protonophoric effect, i.e., shuttling of protons by protonated drug species, is an expected dependence of the rate of alkalinization on the membrane potential. *kc* has been altered in two ways (1) Addition of valinomycin in potassium phosphate medium reduces the proton-pump-generated $\Delta\psi$ (potassium concentrations are equal in and outside the vesicle) with concomitant increase in $\Delta pH$ [21]. (2) Substitution of phosphate by chloride in the potassium phosphate medium reduces $\Delta\psi$ because of the greater permeability of $Cl^-$ [5]. Under both conditions $\Delta pH$ increases since $\Delta\tilde{\mu}_{H^+}$ remains constant. In both cases, we observed an increase in the time required to reach half-maximal alkalinizing effect (given in seconds at 0.1 μM CQ): from 114 in the absence of valinomycin to 183 in its present and from 92 in the absence of KCl to 442 in the presence of 90 mM KCl in the second case, both resulting from the expected decrease in $\Delta\psi$. The same effects were observed with Q and MQ (results not shown). As shown in Fig. 4, substitution of phosphate with chloride in the medium resulted in an increased $\Delta pH$ (upward shift in the 'apparent pH' scale) with increasing [KCl]. The fact that steady-state alkalinization by CQ was obtained in the same concentration range and that the dose–response profile remained unaltered irrespective of [KCl] suggests that the larger drug accumulation in KCl medium (due to increased $\Delta pH$) must have been compensated for by a smaller efflux of $CQH^+$, as expected from its decreased pH-dependent relative concentration.

Most importantly, the alkalinizing effects of all drugs were observed at concentrations which are substantially higher than their therapeutic levels. One cannot exclude,

however, an alkalinizing effect in malarial parasites, since partitioning of basic amphiphiles into membranes depends on their lipid constitution [22].

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