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SIMULATION OF KINETIC DATA ON THE INFLUX AND EFFLUX OF CHLOROQUINE BY ERYTHROCYTES INFECTED WITH *PLASMODIUM FALCIPARUM*

EVIDENCE FOR A DRUG-IMPORTER IN CHLOROQUINE-SENSITIVE STRAINS

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Abstract—Literature data on influx and efflux kinetics of chloroquine (CQ) with erythrocytes infected with the malaria parasite *Plasmodium falciparum* were simulated using a four-compartment model with first-order exchange between the compartments. The four compartments represent (1) the buffer surrounding the infected erythrocyte; (2) the cytosol of the host erythrocyte; (3) the parasite cytosol; and (4) the food vacuole. Simulations showed that basal membrane transport of CQ, estimated from data on influx of CQ into uninfected red cells, largely accounts for uptake and release of CQ by erythrocytes infected with two different CQ-resistant (CQ-R) parasite strains. In contrast, the rate of uptake of CQ by erythrocytes infected with a CQ-sensitive (CQ-S) strain is substantially higher than predicted by uptake with membrane transfer by basal diffusion of CQ. Simulations also indicate that the difference in kinetics of CQ uptake by erythrocytes infected with the CQ-S and CQ-R strains can be explained by a net increase in the inward permeability coefficient at the host erythrocyte membrane, the composite membrane surrounding the parasite or the food vacuole membrane. The results are consistent with the presence of a drug-importer for CQ in erythrocytes infected with sensitive strains, which is absent in those infected with resistant strains. They are not consistent with the hypothesis that CQ resistance is attributable to a drug-exporter in resistant cells which is lacking in sensitive cells.

Chloroquine (CQ‡) has been shown to accumulate within the food vacuole of the intraerythrocytic malaria parasite [1] where it is generally thought to exert its antimalarial action [2–5]. A widely accepted view is that the accumulation of CQ within the food vacuole is largely [6–9], or in part [10], the result of the weak base properties of the drug and the pH gradient between the extracellular medium and the food vacuole. Based on this view, it has been proposed that differences in the accumulation of CQ between erythrocytes infected with CQ-sensitive (CQ-S) and CQ-resistant (CQ-R) parasites can be explained, for the most part, by a difference in the food vacuole pH of these parasites [8, 9]. Accumulation of CQ according to the weak base hypothesis assumes that biological membranes are predominantly permeable to the unionised drug species, and that membrane exchange of this species is symmetrical under equilibrium conditions; this is consistent with evidence from studies which suggest that the translocation of CQ across the membrane of the uninfected human erythrocyte is by a process of passive diffusion of the unionised drug species [11, 12]. However, several recent hypotheses claim that transport mechanisms of CQ other than simple passive diffusion operate in the infected erythrocyte. These alternative mechanisms are thought to be important determinants of the antimalarial actions of CQ and involved in the development of drug resistance by the malaria parasite.

Warhurst [13, 14] has suggested that a drug carrier or permease exists within the membrane of the intraerythrocytic parasite that transports CQ from the host erythrocyte cytosol to the parasite cytosol, thus facilitating drug uptake into the parasite. Drug resistance could then arise from either a decrease in the number or efficiency of the permease units. Another suggestion proposes the existence of a verapamil-sensitive drug-exporter which is thought to be responsible for an apparent increase in the rate of efflux of CQ from CQ-R parasites compared with CQ-S parasites, resulting in a greater reduction in the accumulation of drug within the infected erythrocyte in CQ-R parasites than in CQ-S parasites [15, 16]. It has also been suggested that asymmetrical CQ transporters may exist in the membranes of the parasitised erythrocyte which contribute to the accumulation of CQ within the cell [10]. Recently, it has been proposed that protonated CQ is exchanged with protons across the membrane of the food vacuole by means of a membrane carrier [17].

This study presents a kinetic model of the uptake of CQ by infected erythrocytes. The model is based on first-order exchange of CQ between the four main compartments of an infected erythrocyte (Fig. 1). Kinetic data generated using the model are compared with experimental kinetic data reported

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‡ Abbreviations: CQ, chloroquine; CQ-S, chloroquine-sensitive; and CQ-R, chloroquine-resistant.

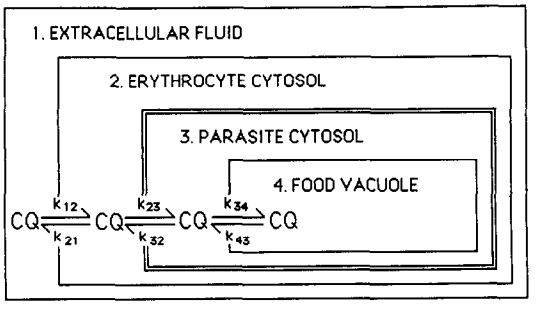

Fig. 1. Model of the malaria-infected erythrocyte showing the four compartments used to model the kinetics of CQ uptake, and the transport constants, k_{ij}, for exchange of CQ between compartments i and j; extracellular buffer i = 1, host erythrocyte cytosol i = 2, parasite cytosol i = 3 and food vacuole i = 4. The parasite cytosol is surrounded by a double boundary to represent the composite membrane comprising the membrane of the parasitophorous vacuole and the plasma membrane of the parasite.

by Geary *et al.* [8] (Study 1) which describe the uptake of CQ by erythrocytes infected with CQ-R and CQ-S strains of *Plasmodium falciparum*. Simulations are also compared with data describing the efflux of CQ from erythrocytes infected with different CQ-R and CQ-S strains of *P. falciparum*, reported by Krogstad *et al.* [15] (Study 2). The aim of the present study is to examine whether differences in the membrane permeability characteristics of CQ can explain observed differences in CQ transport between erythrocytes infected with CQ-S and CQ-R strains of *P. falciparum*.

THEORY

Four-compartment first-order model

The parasitised erythrocyte, suspended in buffer, is treated as a closed four-compartment system with first-order exchange between the compartments as illustrated in Fig. 1. The four compartments are: (1) the buffer surrounding the infected erythrocyte; (2) the cytosol of the host erythrocyte; (3) the parasite cytosol; and (4) the food vacuole. Assuming that the rate-determining process is movement of drug across the membranes separating these compartments, the following first-order equations describe the time dependence of the change in concentration of unbound CQ in solution in the various compartments of the infected erythrocyte suspended in buffer:

$$V_1 \frac{dC_1}{dt} = k_{21}C_2 - k_{12}C_1 \quad (1)$$

$$V_2 \frac{dC_2}{dt} = k_{12}C_1 - (k_{21} + k_{23})C_2 + k_{32}C_3 \quad (2)$$

$$V_3 \frac{dC_3}{dt} = k_{23}C_2 - (k_{32} + k_{34})C_3 + k_{43}C_4 \quad (3)$$

$$V_4 \frac{dC_4}{dt} = k_{34}C_3 - k_{43}C_4 \quad (4)$$

where C_i represents the unbound CQ concentration in the extracellular buffer (i = 1), host erythrocyte cytosol (i = 2), parasite cytosol (i = 3) and the food vacuole (i = 4); V_i represents the apparent volumes of distribution of the respective compartments; and k_{ij} represents the transport constant for transfer of CQ from compartment i to j. The transport constants are defined as

$$k_{ij} = P_{ij}S_{ij} \quad (5)$$

where P_{ij} is the CQ permeability coefficient for unidirectional transfer from compartment i to j and S_{ij} is the surface area of the membrane separating these compartments. P_{ij} is defined with respect to the total CQ concentration; interpretation in terms of transport of individual CQ species is dealt with later.

In the absence of binding of CQ to cell components, the volumes of distribution in Equations 1–4 equal the physiological volumes of cell water within the compartments. When binding is present, the apparent volume is greater than the true compartmental volume. The initial assumption in the present study is that binding of CQ to cell components is insignificant; provisions for cell surface binding are included in some simulations. Although the parasitemia is low in the experimental studies (see later), total uptake into infected erythrocytes is much greater than uptake into uninfected erythrocytes, and therefore uninfected erythrocytes do not contribute significantly to the observed uptake. If the role of uninfected erythrocytes is significant in other uptake studies, the model is easily adapted by adding an extra differential equation, but this is not necessary here.

Equations 1–4 were solved using standard techniques to yield an expression for C_1, the CQ concentration in the buffer. For comparison with the experimental data, the amounts of CQ in the infected erythrocyte fraction were calculated at each time point as the difference between the total amount of CQ present in suspension and the amount in the buffer. The analytical solution to the differential equations is discussed in Appendix 1. The simulations obtained from this solution were verified using simulations based on numerical integration of Equations 1–4 (see also Appendix 1).

Estimation of parameters for simulations

Dimensions of compartments. The volumes of intraerythrocytic *P. falciparum* and its food vacuole as fractions of the total volume of the host erythrocyte have been estimated to be 0.36 ± 0.09 and 0.032 ± 0.02, respectively, during the trophozite-early schizont stages [6]; parasites used in Study 1 were also synchronised to the trophozoite-schizont stage. (The growth stage of parasites used in Study 2 was not reported; for the purposes of the simulations, the compartmental dimensions of these parasites were assumed to be the same as those reported for the trophozoite-early schizonts.) Taking the total volume of the host erythrocyte to be the same as that of a normal erythrocyte (8.6×10^{-11} cm^3 [18]), since the size of the host erythrocyte is reported not to be altered significantly when infected with *P. falciparum* [19], and the cell water fraction

Table 1. Values of compartment volumes and surface areas used in simulations

Parameter*	Study 1	Study 2
V_1 (cm^3)	0.991	1.0†
V_2 (cm^3)	2.7×10^{-4}	7.4×10^{-5}
V_3 (cm^3)	1.6×10^{-4}	4.4×10^{-5}
V_4 (cm^3)	1.4×10^{-5}	3.8×10^{-6}
S_{12} (cm^2)	11.9	3.26
S_{23} (cm^2)	3.48	0.95
S_{34} (cm^2)	0.69	0.19

* Calculated for a 1-mL suspension volume.
† No haematocrit was reported in Study 2.

to be 0.7 [20] for all compartments, the volume of cell water within each compartment was calculated from the compartmental volume fractions. The surface area of the host erythrocyte, again based on the dimensions of the uninfected cell, was taken to be 1.63×10^{-6} cm^2 [21]. The surface areas of the intracellular parasite and parasite food vacuole were estimated from their volumes by assuming that these structures are spherical. To determine the total volume and surface areas of the host cell, parasite and food vacuole, the volume and surface area of a single compartment were multiplied by the number of infected erythrocytes in 1 mL of suspension. A 0.86% hematocrit and 7.3% parasitemia were reported in the data taken from Study 1. This represents 7.3×10^6 infected cells per mL of suspension (taking the volume of each cell to be equal to 8.6×10^{-11} mL). In Study 2, the reported number of infected cells per mL was 2×10^6. The values of parameters describing cell dimensions in terms of the compartment volumes and surface areas used in the simulations are shown in Table 1.

Permeability coefficients. Evidence from previous studies indicates that the unionised CQ species (CQ°) is the predominant membrane permeant in the uninfected erythrocyte membrane and that transport appears to be by a process of passive diffusion [11, 12]. A linear Arrhenius plot of the permeability coefficients of CQ° was reported over the temperature range 0 to 25° [12]. The permeability coefficient of the unionised species at the temperature of the experimental studies, 37°, was estimated to be 7.5 cm/sec, by assuming that this relationship remains linear to 37°. This value is of the same order of magnitude as the permeability coefficient of CQ° reported for transport across the membrane of the rat liver hepatocyte at 37° [22]. As no basal permeability values are available in the literature for the individual membranes of the infected erythrocytes, the preliminary assumption made for the purposes of the simulations was that the permeability coefficients for basal diffusion of CQ° across all membranes of the infected erythrocyte approximate the value estimated for the uninfected human erythrocyte membrane (7.5 cm/sec). As discussed later, this assumption was modified in some instances in the light of the results of the simulations.

One complication when modelling transport across the membranes of the infected erythrocyte is that the parasite is surrounded by two membranes, the parasite plasma membrane and the membrane of the parasitophorous vacuole. The latter is derived from the host erythrocyte plasma membrane. Since the resistance to transport across each membrane, which is inversely proportional to the permeability coefficient, is approximately additive [23], the permeability coefficient of CQ° across the composite membrane is expected to approximate 3.75 cm/sec, based on the value assumed for a single membrane (7.5 cm/sec).

pH of the compartments. In simulations where it is assumed that the membrane is permeable to one drug species (e.g. the unionised species), the transport rate constants, defined in Equation 5, can be written as

$$k_{ij} = f_i^* \, p_{ij}^* \, S_{ij} \tag{6}$$

where f_i^* is the concentration of the permeable species as a fraction of the total drug concentration in compartment i and p_{ij}^* is the permeability coefficient for that species. Values for f_i^* for the proposed transported species were estimated from the pH of the relevant compartment and the pK_a values of CQ at 37° (8.12 and 10.42 [24]).

The pH of the extracellular buffer used in all simulations was 7.4, as reported in Studies 1 and 2. Values of 7.1 and 7.4 were chosen for the pH of the host erythrocyte and parasite cytosols, respectively, as suggested by Warhurst [14]. Other pH values, which differ by as much as 0.5 units from these values, have been reported for the host and parasite cytosols (see, for example, Ref. 13); however, simulations (not shown) of CQ uptake using the model indicated that the rate and extent of uptake are insensitive to variations of this order in the pH of these compartments.

The reported kinetic data in Study 1 describe CQ uptake by erythrocytes infected with a CQ-S (FCN) and a CQ-R (VNS) strain of *P. falciparum*. As part of Study 1, the pH of the food vacuole of various parasite strains was estimated from the equilibrium distribution ratio of CQ between the food vacuole and the extracellular buffer (C_4/C_1) using the relationship

$$C_4/C_1 = [H^+]_4^2/[H^+]_1^2 \tag{7}$$

where $[H^+]_4$ and $[H^+]_1$ are the proton concentrations in the food vacuole and buffer, respectively. Equation 7 assumes that CQ distributes according to its weak base properties, with symmetrical exchange of CQ° across all membranes involved. The intravacuolar pH values, estimated from data reported in Study 1 at low CQ concentrations, are in the ranges 4.3 to 4.4 and 4.7 to 4.8 for the CQ-S (FCN) and CQ-R (VNS) parasites, respectively. Due to the reported effects of high CQ concentrations on the pH [4, 6] and osmotic balance [25] of the food vacuole, only data reported in Study 1 at low total CQ concentrations (10 nM) were examined.

The CQ-resistant strain used in Study 2 (Indochina I/CDC) was reported in another study [4] to have an intravacuolar pH range of 5.2 to 5.4. This range was determined independently of CQ distribution

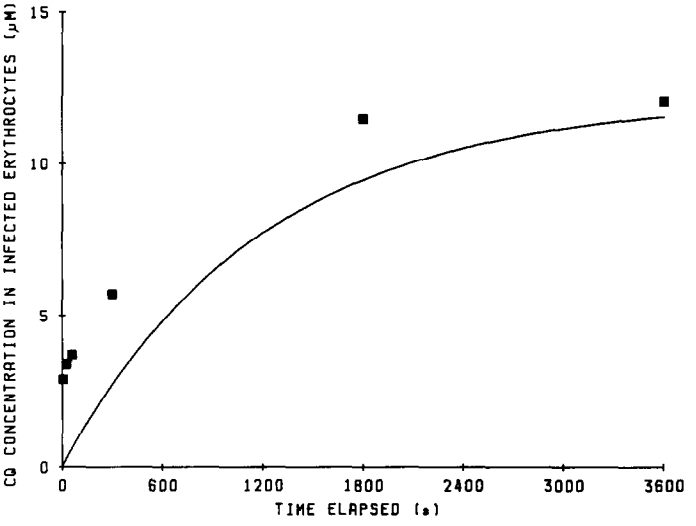

Fig. 2. Kinetics of CQ uptake by erythrocytes infected with the CQ-R (VNS) strain of *P. falciparum* reported in Study 1; simulation with basal permeability coefficients. The curve was generated assuming membrane transfer of only CQ°, with permeability coefficients of CQ° equal to 7.5 cm/sec across all membranes, and a food vacuole pH value of 4.68.

by spectrofluorimetry using fluorescein-dextran as a label. The CQ concentration used in Study 2 (1 nM) has been shown previously not to influence food vacuole pH [4]. Data on the kinetics of CQ uptake by infected erythrocytes were also reported in Study 2, but were unsuitable for the purposes of the present study.

RESULTS AND DISCUSSION

Interpretation of rapid initial uptake apparent in data reported in Study 1

Figure 2 shows data on the kinetics of uptake of CQ by erythrocytes infected with the CQ-R (VNS) strain reported in Study 1. The curve in Fig. 2 shows simulated uptake kinetics with membrane transfer by basal diffusion, generated by assuming membrane transfer solely of CQ°, with permeability coefficients equal to 7.5 cm/sec (the value estimated for the uninfected erythrocyte at 37°) across all membranes, and a food vacuole pH of 4.68. This pH value was chosen so that the predicted equilibrium concentration of CQ in the infected erythrocyte fraction equals the reported value (12.2 μM). It is clear from inspection of Fig. 2 that, although the simulated uptake profile is of the same order of magnitude as the data, the observed initial uptake of CQ by the infected erythrocytes is much too rapid to be explained by the initial rate of uptake predicted by the model. A similar initial rapid phase is also apparent in data reported in Study 1 describing uptake by erythrocytes infected with the CQ-S (FNC) strain, but was not observed in Study 2 or with uninfected erythrocytes [11, 12].

The most likely explanation for the initial rapid phase of uptake is surface binding to the plasma membrane of the infected erythrocytes. Simulations were conducted to investigate this possibility by incorporating a binding term into the model. The apparent amounts of CQ taken up in the initial rapid phase by erythrocytes infected with the FCN and VNS strains at 10^{-7} and 10^{-8} M CQ are approximately proportional to the CQ concentration which suggests that the proposed surface binding can be considered linear over this concentration range. A term describing equilibrium linear surface binding was incorporated into the model by using an apparent volume of distribution of the extracellular buffer, rather than the true volume (see Appendix 2). It was estimated that the extent of CQ uptake by the infected erythrocytes in the initial rapid uptake phase observed in Study 1 corresponds to apparent volumes of distribution for the extracellular buffer that are 1.21 and 1.22 times the true volumes of the buffer for infection with the CQ-R and CQ-S strains, respectively.

Uptake of chloroquine with equilibrium surface binding and membrane transport by basal diffusion

The data from Study 1 showing the uptake of CQ by erythrocytes infected with the CQ-R (VNS) strain are plotted again in Fig. 3. The unbroken line in Fig. 3 simulates uptake of CQ with membrane transfer by basal diffusion and rapid initial uptake by surface binding. This curve was generated assuming membrane transfer solely of CQ°, with permeability coefficients of 7.5 cm/sec across all membranes, the equilibrium binding term described above, and a food vacuole pH of 4.74. This pH value, which is within the range reported in Study 1 for the VNS strain (4.7 to 4.8), was again chosen so that the predicted equilibrium concentration equals the reported value (12.2 μM). (This value is slightly higher than the pH value of 4.68 used in the simulation described in Fig. 2 due to the residual effect at equilibrium of the binding term included in

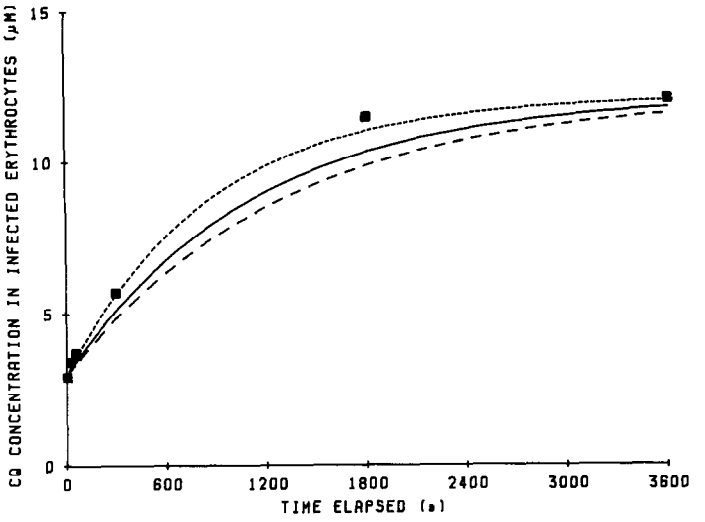

Fig. 3. Kinetics of CQ uptake by erythrocytes infected with the CQ-R (VNS) strain of *P. falciparum* reported in Study 1; simulations with basal permeability coefficients and equilibrium binding term. The unbroken line was generated using the equilibrium binding term (see text) and a food vacuole pH of 4.74, assuming membrane transfer of only CQ° with permeability coefficients of CQ° equal to 7.5 cm/sec across all membranes. The broken line was generated using the same parameters, except that the permeability coefficients of CQ° for exchange between the erythrocyte cytosol and the parasite cytosol were assigned a value of 3.75 cm/sec to account for the composite membrane surrounding the parasite. The dotted line was generated assuming the parameters used to generate the unbroken line, except that all permeability coefficients were increased by a factor of 1.5.

this simulation.) The same parameters were used to generate the broken line shown in Fig. 3, except that the inward and outward permeability coefficients of CQ° at the composite membrane separating the host cell and parasite cytosol were assigned a value of 3.75 cm/sec to simulate the additional resistance of the double membrane comprising the membrane of the parasitophorous vacuole and the parasite plasma membrane. The additional resistance results only in a marginally lower uptake rate, indicating that the composite membrane surrounding the parasite is unlikely to be a major rate-limiting barrier to overall transport.

The rates of CQ uptake obtained in both simulations illustrated in Fig. 3 are marginally lower than the observed rates but can be brought into line by increasing the inward and outward permeability coefficients at each membrane by a factor of 1.5 (illustrated by the dotted line in Fig. 3). This appears to be a small adjustment in basal permeability, given that the lipid composition of the membranes of the parasitised erythrocyte is substantially different from that of the uninfected erythrocyte [26–28], and this difference is expected to have some influence on the passive transport behaviour of CQ. For example, it has been suggested that an alteration in erythrocyte membrane lipid induced by *P. falciparum* results in a more fluid and permeable membrane [28]. This suggests that the assigned permeability coefficient of 7.5 cm/sec could underestimate slightly the true value and explain the small discrepancy noted in Fig. 3 between the simulations and the experimentally derived results.

Cellular efflux of chloroquine with membrane transport by basal diffusion

Figure 4 shows a plot of the data from Study 2 which describes the efflux of CQ from erythrocytes infected with a different CQ-R strain (Indochina I/CDC) from that used in Study 1. The data fall between the two lines which are generated using the minimum (pH 5.2; upper curve) and maximum (pH 5.4; lower curve) intravacuolar pH values reported for the Indochina I/CDC strain [4] and the conditions assigned earlier for basal diffusion (i.e. that each membrane is permeable only to the unionised drug species and a permeability coefficient of 7.5 cm/sec across the host erythrocyte and food vacuole membranes and 3.75 cm/sec across the composite membrane surrounding the parasite; no equilibrium binding term is used in these simulations). This result provides further evidence that membrane transport of CQ by a process of basal diffusion of the unionised drug species appears largely to explain the observed kinetic behaviour of CQ when erythrocytes are infected with CQ-R parasites. A higher rate of CQ efflux from erythrocytes infected with a CQ-R strain than from those infected with the CQ-S strain was reported in Study 2. To explain this observation, it was proposed that a membrane carrier operates in CQ-R parasites to facilitate the removal of CQ from the infected erythrocyte. This proposal conflicts with evidence presented here, that membrane transfer by basal diffusion can account largely for the observed kinetics of uptake and release of CQ by erythrocytes infected with a CQ-

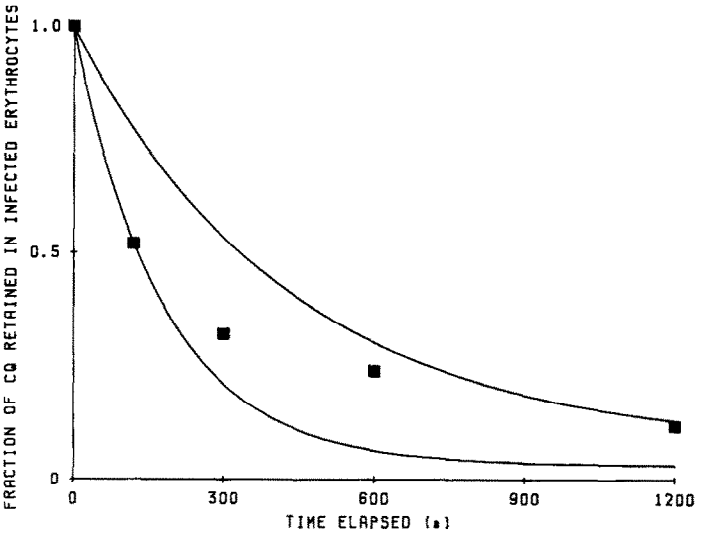

Fig. 4. Kinetics of CQ efflux from erythrocytes infected with the CQ-R (Indochina I/CDC) strain of *P. falciparum* reported in Study 2; simulations with basal permeability coefficients. Both curves were generated assuming efflux with membrane transfer by basal diffusion (i.e. assuming membrane transfer of only CQ°, with permeability coefficients of CQ° equal to 7.5 cm/sec for transfer across the membranes of the host erythrocyte and food vacuole, and 3.75 cm/sec for transfer across the composite membrane surrounding the parasite). The minimum reported food vacuole pH for this strain (5.2) was used to generate the upper curve, and the maximum reported pH (5.4) was used to generate the lower curve.

R strain. Simulations of kinetic data for CQ-S strains will be discussed in the following section.

The authors of Study 1 presented a fundamentally different interpretation of their uptake data than that advanced here. It was suggested that equilibrium was rapidly achieved between CQ in the extracellular fluid and the food vacuole by passive exchange of the drug. This process was thought to be accompanied by an increase in the food vacuole pH, due to the weak base properties of CQ. The gradual increase in intracellular CQ was interpreted as being the consequence of a slow return of the food vacuole pH to its normal resting value. A recent article [29] has presented a mathematical model of uptake based on this interpretation. However, the simulations in the present study do not support this interpretation, since they suggest that CQ transport by basal diffusion is much too slow to allow for the equilibrium approximation.

Uptake of chloroquine by erythrocytes infected with a chloroquine-sensitive parasite strain

The data reported in Study 1 describing CQ uptake by erythrocytes infected with the CQ-S (FCN) strain is plotted in Fig. 5. The curve shown in Fig. 5 was generated with all permeability coefficients at basal values, using the equilibrium binding term and assigning a value for the pH of the food vacuole of 4.30 (which is within the reported range for this CQ-S strain). The simulated rate of CQ uptake (after the rapid initial phase) is markedly lower than the observed uptake rate despite a comparable equilibrium cellular concentration (15.2 μM). The results of these simulations suggest that while basal permeability conditions adequately simulate kinetic data for the CQ-R strain, this is not the case for the CQ-S strain. This comparison also suggests that the difference in kinetics of CQ uptake observed between the two strains of Study 1 cannot be explained simply by the difference in food vacuole pH if basal membrane transport conditions exist in erythrocytes infected with either the CQ-R or CQ-S strain.

The following sections report simulations which explore possible explanations of the kinetic differences between erythrocytes infected with CQ-S and CQ-R strains.

Simulations of symmetrical changes in membrane permeability to chloroquine

The high rate of CQ uptake by erythrocytes infected with the CQ-S strain observed in Study 1 may possibly be explained by a symmetrical increase, relative to basal values, in the permeability to CQ of the membranes of the infected erythrocyte, induced by the CQ-S parasite. This could be related to the changes in erythrocyte membrane permeability characteristics reported for glucose [30], amino acids [31, 32], polyols [33, 34], anions [35] and nucleosides [36, 37] during the erythrocytic stages of malaria. It has been proposed that the membrane transport of amino acids, anions, glucose and sorbitol, modified during the trophozoite stage of malaria, appears to exhibit pore-like behaviour [33–35, 38] which facilitates the transport of small substrates and larger neutral molecules. This increase in membrane leakiness also could act to increase CQ permeation, for example by allowing passage of CQ cations (although cationic molecules appear to be excluded by the newly formed pores [33, 34]).

In simulations presented here, the presence of highly permeable or leaky membranes is mimicked by increasing the permeability of selected membranes

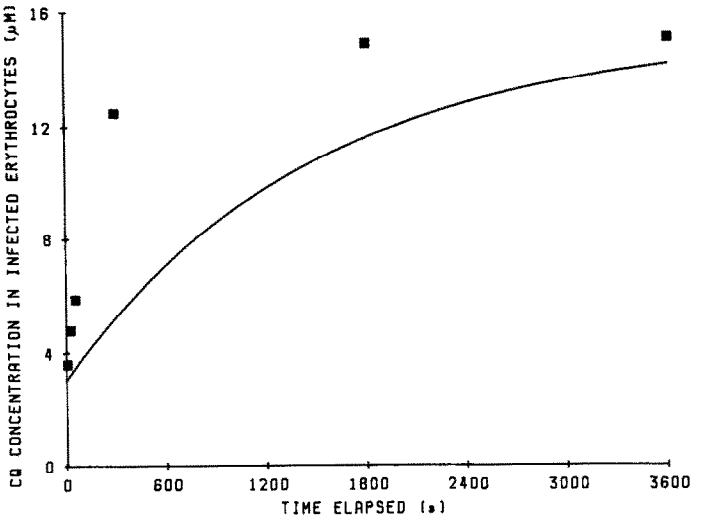

Fig. 5. Kinetics of CQ uptake by erythrocytes infected with the CQ-S (FCN) strain of *P. falciparum* reported in Study 1; simulation with basal permeability coefficients. The unbroken line simulates uptake of CQ with membrane transfer by basal diffusion (see legend to Fig. 4). The equilibrium binding term (see text) and a food vacuole pH value of 4.30 were used in this simulation.

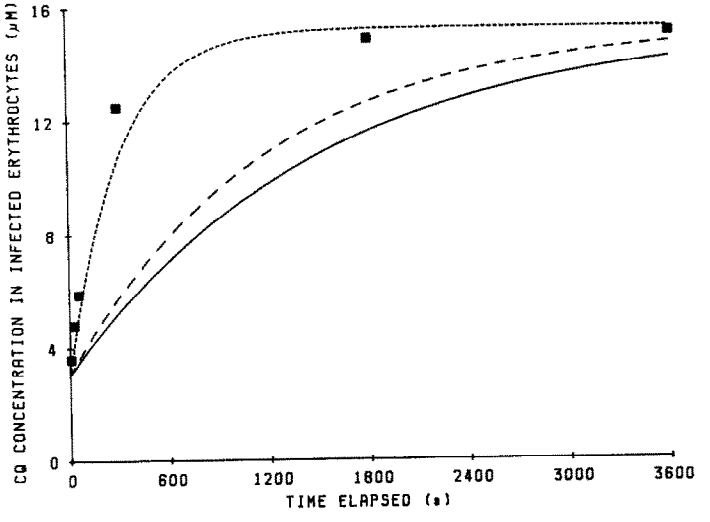

Fig. 6. Kinetics of CQ uptake by erythrocytes infected with the CQ-S (FCN) strain of *P. falciparum* reported in Study 1; simulations with basal and symmetrical nonbasal permeability coefficients. The unbroken line simulates uptake of CQ with membrane transfer by basal diffusion (see legend to Fig. 4). The equilibrium binding term (see text) and a food vacuole pH value of 4.30 were used in this simulation. The broken line was generated using the same parameters, except that the permeability coefficients for transfer of CQ across the host erythrocyte membrane (P_{12} and P_{21}) and the composite membrane surrounding the parasite (P_{23} and P_{32}) were increased by a factor of 10^4 over basal levels. The dotted line was generated assuming the parameter values used to generate the unbroken line, except that the permeability coefficients of CQ° for transfer across the membrane of the food vacuole were assigned a value of 10^5 cm/sec.

of the infected erythrocyte to very large values, which effectively removes the membrane as a barrier to CQ transport.

Preliminary studies showed that increasing the inward and outward permeability coefficients by a factor of 10^4 over basal levels effectively removed the host erythrocyte membrane and the composite membrane surrounding the parasite as barriers to transport of CQ (i.e. kinetics of uptake were independent of permeability coefficients when permeability coefficients were increased beyond this value). The data reported in Study 1 describing CQ

uptake by erythrocytes infected with the CQ-S (FCN) strain are shown again in Fig. 6, along with the curve (unbroken line) simulating uptake with equilibrium binding and membrane transfer by basal diffusion (and using a food vacuole pH of 4.30). The effect of raising the permeability across the erythrocyte membrane and the composite membrane surrounding the parasite by a factor of 10^4 over basal levels is shown by the broken line in Fig. 6. It is apparent that the effective removal of these membranes as barriers to the transport of CQ is insufficient to increase the predicted rate to that observed in Study 1 for erythrocytes infected with the CQ-S strain. This result also indicates that, although the host erythrocyte membrane and the composite membrane surrounding the parasite have some influence on uptake kinetics, they are unlikely to provide the major rate-determining barrier to overall transport of CQ; the major transport barrier appears to be the food vacuole membrane.

Modelling possible permeability increases at the food vacuole membrane requires some restrictions. To comply with the proposal that unbound CQ accumulates within the food vacuole by a process of ion trapping, permeation across the membrane of the food vacuole must remain selective to CQ° and maintain symmetry of transfer of CQ° at equilibrium. The dotted line in Fig. 6 shows that a close fit to the data describing the uptake of CQ by erythrocytes infected with the CQ-S strain of Study 1 can be obtained by introducing a sufficiently large symmetrical increase in permeability of the food vacuole membrane to CQ° (while maintaining basal transport conditions at the erythrocyte and parasite membranes). In this simulation, agreement with the experimental data required that the inward and outward permeability coefficients at the food vacuole membrane be assigned a value of 10^5 cm/sec (1 km/sec) for the unionised species, which is clearly unreasonably large for permeation under physiological conditions. This result suggests that the food vacuole membrane cannot be reasonably expected to allow unionised CQ to permeate with the high permeability coefficient required to explain the observed kinetic data. It is possible, however, that the membrane of the food vacuole also allows symmetrical exchange of CQ cations, consistent with a leaky food vacuole membrane, but it is difficult to see how CQ could accumulate to the high levels observed within the food vacuole in such a situation.

Simulations of asymmetrical changes in membrane permeability to chloroquine

The lower half-time of CQ uptake by erythrocytes infected with the CQ-S strain, compared with that for erythrocytes infected with the CQ-R strain, could be explained by a larger inward permeability coefficient at any membrane (P_{12}, P_{23} or P_{34}; see Fig. 1) for CQ-S strains relative to CQ-R strains while the outward permeability coefficients (P_{21}, P_{32} and P_{43}) are unchanged. This would simulate the presence of an asymmetrical membrane carrier which favours inward permeation, such as the permease proposed by Warhurst [13, 14].

One complication for modelling an asymmetrical change in permeability is that it is associated with a change in the equilibrium distribution ratio of the permeant across the relevant membrane. The presence of asymmetrical transport across any of the three membrane systems in the infected erythrocyte would invalidate the calculations of the pH of the food vacuole by the method used in Study 1 since these calculations, based on CQ distribution between the buffer and infected cell, assume symmetrical transfer of the unionised drug species across all membranes at equilibrium. A net increase in the inward permeability coefficient at any membrane requires an increase in the food vacuole pH to maintain the observed distribution ratio.

The data reported in Study 1 describing the uptake of CQ by erythrocytes infected with the CQ-S (FCN) strain are plotted again in Fig. 7. The curve shown in Fig. 7 was generated by increasing P_{23} (host erythrocyte cytosol to parasite cytosol; see Fig. 1) by a factor of 10 compared with the basal value (with basal values for the permeability coefficients at all other membranes, and using the equilibrium binding term). The pH of the food vacuole was chosen to be 4.82 in this simulation to maintain the equilibrium CQ concentration in erythrocytes infected with the CQ-S strain reported in Study 1 (15.2 μM). The generated line gives an excellent fit to the observed data. An almost identical curve (not shown) can be generated by increasing P_{12} 7-fold over basal levels (while maintaining basal transport conditions at all other membranes) and adjusting the food vacuole pH to 4.74. These results indicate that an asymmetrical carrier system which favours the inward transport of CQ, operating at the host erythrocyte membrane or the composite membrane surrounding the parasite, can explain the observed kinetics of uptake exhibited by erythrocytes infected with a CQ-S strain.

The adjustment in the food vacuole pH of the CQ-S (FCN) strain in simulations of the asymmetrical permeability changes (i.e. from pH 4.30 to pH 4.82 and 4.74 when increasing P_{23} and P_{12}, respectively) brings the food vacuole pH values into line with the values reported for the CQ-R (VNS) strain (4.7 to 4.8), for which no asymmetrical permeability change was required to simulate uptake. This suggests the possibility that the food vacuole pH may not differ between the two parasite strains, and that differences in the apparent food vacuole pH, calculated in Study 1 from the CQ distribution in different parasite strains, could arise not from a true pH difference, but from an alteration in membrane permeability to CQ.

A reasonable fit to data from Study 1 describing the uptake of CQ by the CQ-S strain can also be obtained by altering the inward permeability coefficient of the food vacuole membrane (P_{34}). However, the requirement in this case is that P_{34} be increased by a factor of at least 100 over the basal value, and the food vacuole pH adjusted to compensate; for a 100-fold increase in P_{34}, a food vacuole pH of 5.3 was required to achieve the correct equilibrium cellular concentration of CQ. (Note that increasing P_{34} by a factor of 100 over the basal value is reasonable if CQ cations are transported from the parasite cytosol to the food vacuole since the cation

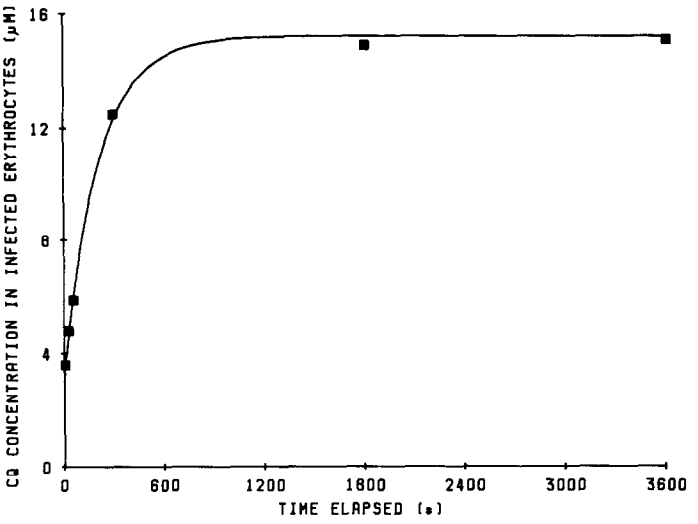

Fig. 7. Kinetics of CQ uptake by erythrocytes infected with the CQ-S (FCN) strain of *P. falciparum* reported in Study 1; simulation with basal and asymmetrical nonbasal permeability coefficients. The curve was generated by increasing P_{23} by a factor of 10 compared with the basal value (and maintaining all other permeability coefficients at basal levels; see legend to Fig. 4). The equilibrium binding term (see text) and a food vacuole pH of 4.82 were used in this simulation.

concentration exceeds that of CQ° by about four orders of magnitude at pH 7.4.)

The concept of an asymmetrical transport system which resides within the membranes surrounding the parasite is broadly consistent with the permease hypothesis raised by Warhurst [13, 14], who has proposed that a carrier molecule or permease is responsible for transporting diprotonated CQ from the host erythrocyte cytosol to the parasite cytosol. However, the present simulations provide no information to allow distinctions to be drawn between different possible mechanisms of increased permeability.

Effects of changes in asymmetrical permeability and food vacuole pH on the cellular efflux of chloroquine

A comparison of data reported in Study 2 describing efflux of CQ from erythrocytes infected with a CQ-R (Indochina I/CDC) strain and a CQ-S (Haiti 135) strain provides a further opportunity to test the findings of the present studies. These data are illustrated in Fig. 8, and show that the rate and extent of CQ release from infected erythrocytes appear to be reduced markedly in erythrocytes infected with the CQ-S strain. Krogstad *et al.* [15, 16] have raised the possibility that a drug-exporter operating in CQ-R parasites can explain the higher rate of CQ release from erythrocytes infected with resistant parasites. As discussed previously, this proposal is inconsistent with evidence that membrane transfer by basal diffusion accounts largely for the kinetics of CQ uptake and release by erythrocytes infected with CQ-R strains. However, it is possible that a transport system operating preferentially in the inward direction reduces the rate and extent of CQ release from the infected erythrocyte since the kinetics of release from the infected erythrocytes depend not only on the rate of transfer out of each compartment of the infected erythrocyte, but also on the rate of transfer in the reverse direction (see Equations 1–4 and Fig. 1). For example, if P_{34} were much greater than P_{32}, due to the presence of a drug-importer in the food vacuole membrane, drug entering the parasite cytosol during efflux from the food vacuole would be preferentially returned to the food vacuole, rather than transferred to the erythrocyte cytosol; this would result in a lower net rate of efflux. The aim of this section is to investigate by simulation whether a transport system operating in erythrocytes infected with a CQ-S strain that imports CQ in the direction of the food vacuole may be an alternative explanation of the observations of Study 2 to that proposed by its authors.

The curves illustrated in Fig. 8 show the effect of increasing the inward permeability coefficient of CQ on the kinetics of CQ efflux reported in Study 2. The food vacuole pH was assumed to be 5.3 in these simulations, which is within the range reported for the CQ-R (Indochina CDC) strain of Study 2 [4]. (The influence of intravacuolar pH on efflux will be discussed later.) The unbroken line in Fig. 8 was generated assuming membrane transfer solely by basal diffusion, and the broken line was generated by increasing P_{23} by a factor of 10 over the basal value (with other permeability coefficients maintained at the basal value), consistent with the increase in this value required to reasonably fit the uptake of CQ by the erythrocytes infected with the CQ-S (FCN) strain of Study 1 (see Fig. 7). While this curve confirms that a net increase in the inward membrane permeability signifiantly reduces the rate and extent of CQ release, the 10-fold increase in P_{23} is clearly insufficient to account adequately for data describing the efflux of CQ from erythrocytes infected with the CQ-S strain. To achieve a reasonable fit of these data (dotted line in Fig. 8),

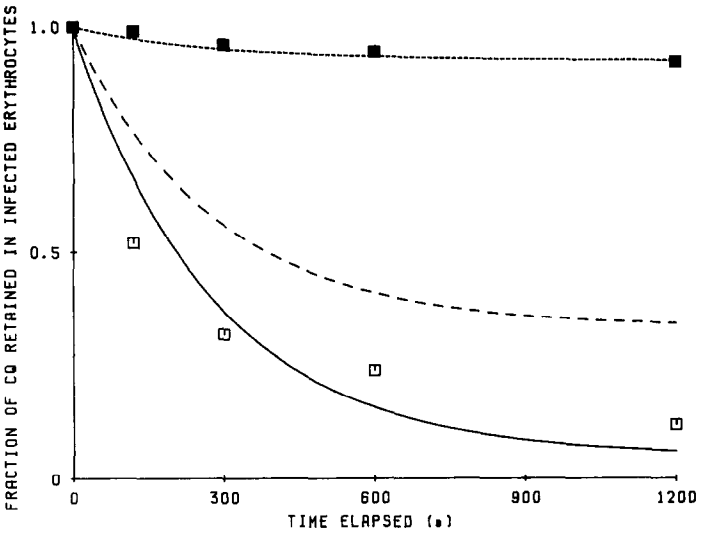

Fig. 8. Kinetics of CQ efflux from erythrocytes infected with the CQ-R (Indochina I/CDC; □) and CQ-S (Haiti 135; ■) strains of *P. falciparum* reported in Study 2; simulations with basal and nonbasal permeability coefficients. The unbroken line simulates efflux of CQ with membrane transfer by basal diffusion (see legend to Fig. 4). The broken line was generated by increasing P_{23} by a factor of 10 over the basal value (and maintaining all other permeability coefficients at basal levels). The dotted line was generated by increasing P_{23} by a factor of 250 over the basal value (and maintaining all other permeability coefficients at basal levels). A food vacuole pH of 5.3 was used in all three simulations.

P_{23} must be increased by a factor of 250 over the basal value (while maintaining the other permeability coefficients at basal levels). A similar fit of these efflux data was also achieved by increasing P_{34} (parasite cytosol to food vacuole) by a factor of about 150. (Simulating an increase in P_{12} by a factor of 250 gave essentially the same equilibrium position as that obtained when increasing P_{23} by this factor, but in this case equilibrium is attained in less than 120 sec which is too rapid to account for the observed data.) These increases in permeability coefficients are much greater than those required for the CQ-S strain of Study 1. This may indicate a drug-importer operating in erythrocytes infected with the CQ-S strain of Study 2 which is much more active than in erythrocytes infected with the CQ-S strain of Study 1. Alternatively, as discussed below, the discrepancy may be a consequence of the estimate of intravacuolar pH in the CQ-R strain of Study 2.

Ginsburg [9] has suggested that the reduced rate of efflux from erythrocytes infected with a CQ-S strain is also consistent with a reduction in the concentration of membrane permeable unionised CQ within the food vacuole, arising from a lower intravacuolar pH in CQ-S parasites. This proposal is tested in simulations shown in Fig. 9. The unbroken and broken lines shown in this figure were generated by assuming efflux with membrane exchange by basal diffusion. The unbroken line was obtained by choosing a food vacuole pH of 5.3 (as in Fig. 8), whereas the broken line, which reasonably simulates the observed efflux of CQ from erythrocytes infected by the CQ-S strain, was generated using a food vacuole pH of 4.5. The difference in the food vacuole pH values chosen in these simulations agrees with the difference stipulated by Ginsburg [9] to account for the difference in CQ efflux from erythrocytes infected with CQ-R and CQ-S strains. It is important to note that a reasonable fit to the data shown in Fig. 9 also can be obtained by simulating a decrease in intravacuolar pH in combination with an increase in the inward membrane permeability to CQ. To illustrate this proposal, a reasonable fit to the efflux of CQ from erythrocytes infected with the CQ-S strain observed in Study 2 (shown by the dotted line in Fig. 9) was achieved when increasing P_{23} by a factor of 10 over the basal value (and maintaining the other permeability coefficients at basal levels) and assigning a food vacuole pH of 4.7; this combination is consistent with the P_{23} value and food vacuole pH used to model the uptake of CQ by erythrocytes infected with the CQ-S (FCN) strain reported in Study 1 (see Fig. 7).

A reduction in efflux rate of CQ on addition of the calcium channel blocker verapamil was interpreted in Study 2 as competition between CQ and verapamil for sites on the proposed drug-exporter. Ginsburg [9] offered the alternative explanation that verapamil may act by reducing the food vacuole pH in CQ-R parasites, rather than as an inhibitor of a drug-exporter. The overall effect would be a reduction in the rate of CQ efflux out of the infected erythrocyte and an increase in the amount of CQ retained by the infected erythrocyte. Experiments in Study 2 have shown that 10 μM verapamil increases the equilibrium concentration of CQ in erythrocytes infected with the CQ-R strain by a factor of 2–3. Simulations (not shown) have demonstrated that, under basal transport conditions, a reduction in food vacuole pH of about 0.2 to 0.3

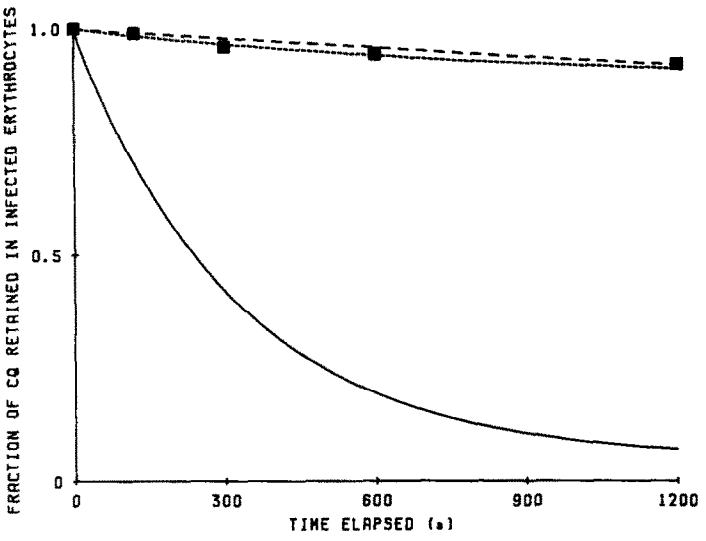

Fig. 9. Kinetics of CQ efflux from erythrocytes infected with the CQ-S (Haiti 135) strains of *P. falciparum* reported in Study 2; simulations with basal and nonbasal permeability coefficients and altered food vacuole pH values. The unbroken line was generated assuming efflux with membrane transfer by basal diffusion (see legend to Fig. 4) and using a food vacuole pH of 5.3. The broken line was generated using the same parameters, except that a food vacuole pH of 4.5 was used. The dotted line was generated by increasing P_{23} by a factor of 10 over the basal value (and maintaining all other permeability coefficients at basal levels) and using a food vacuole pH of 4.7.

units is sufficient to mimic this observed effect of verapamil.

Conclusions

Krogstad *et al.* [15, 16] have proposed that the increased cellular efflux of CQ from erythrocytes infected with CQ-R strains, compared with efflux from erythrocytes infected with CQ-S strains, is due to an increase in membrane permeability which favours the outward transport of CQ, consistent with the presence of a drug-exporter. This drug-exporter is thought to be absent or deficient in erythrocytes infected with CQ-S parasites. In contrast to this proposal, results from the present study show that the kinetics of uptake and release of CQ by erythrocytes infected with two different CQ-R strains can largely be explained by membrane transfer of CQ by basal diffusion without a need to postulate a drug-exporter. On the other hand, simulation of cellular uptake of CQ by erythrocytes infected with the CQ-S strain of Study 1 requires an additional increase in membrane permeability that favours inward transport of CQ, consistent with a drug-importer system. A drug-importer that operates preferentially in erythrocytes infected with a CQ-S strain can also explain the low rate and extent of CQ efflux from erythrocytes infected with a CQ-S strain. On the basis of the limited data available, the simulations of the present study suggest that CQ resistance is not the consequence of a specialised drug-exporter in erythrocytes infected with CQ-R parasites, which is absent or deficient in CQ-S strains, but rather the consequence of the presence of a drug-importer in erythrocytes infected with CQ-S parasites, which is deficient in CQ-R parasites.

APPENDICES

Appendix 1. Analytical and numerical solutions to the differential equations

The analytical solution to Equations 1–4 with respect to C_1, the unbound CQ concentration in buffer, is

$$C_1 = \left\{\frac{k_{21}k_{32}k_{43}}{\alpha\beta\gamma}\left[\frac{C_{1,0}}{V_2V_3V_4} + \frac{C_{2,0}}{V_1V_3V_4} + \frac{C_{3,0}}{V_1V_2V_4} + \frac{C_{4,0}}{V_1V_2V_3}\right]\right\}$$

$$- \left\{\frac{k_{21}k_{32}k_{43}}{V_1V_2V_3}C_{4,0} + \frac{k_{21}k_{32}}{V_1V_2}\left[\frac{k_{43}}{V_4} - \alpha\right]C_{3,0}\right.$$

$$+ \frac{k_{21}}{V_1}\left[\alpha^2 - \alpha\left(\frac{k_{32}}{V_3} + \frac{k_{34}}{V_3} + \frac{k_{43}}{V_4}\right) + \frac{k_{32}k_{43}}{V_3V_4}\right]C_{2,0}$$

$$\left. - [\alpha^3 - \alpha^2X + \alpha Y - Z]C_{1,0}\right\}\frac{e^{-\alpha t}}{\alpha(\beta-\alpha)(\gamma-\alpha)}$$

$$- \left\{\frac{k_{21}k_{32}k_{43}}{V_1V_2V_3}C_{4,0} + \frac{k_{21}k_{32}}{V_1V_2}\left[\frac{k_{43}}{V_4} - \beta\right]C_{3,0}\right.$$

$$+ \frac{k_{21}}{V_1}\left[\beta^2 - \beta\left(\frac{k_{32}}{V_3} + \frac{k_{34}}{V_3} + \frac{k_{43}}{V_4}\right) + \frac{k_{32}k_{43}}{V_3V_4}\right]C_{2,0}$$

$$\left. - [\beta^3 - \beta^2X + \beta Y - Z]C_{1,0}\right\}\frac{e^{-\beta t}}{\beta(\alpha-\beta)(\gamma-\beta)}$$

$$- \left\{\frac{k_{21}k_{32}k_{43}}{V_1V_2V_3}C_{4,0} + \frac{k_{21}k_{32}}{V_1V_2}\left[\frac{k_{43}}{V_4} - \gamma\right]C_{3,0}\right.$$

$$+ \frac{k_{21}}{V_1}\left[\gamma^2 - \gamma\left(\frac{k_{32}}{V_3} + \frac{k_{34}}{V_3} + \frac{k_{43}}{V_4}\right) + \frac{k_{32}k_{43}}{V_3V_4}\right]C_{2,0}$$

$$\left. - [\gamma^3 - \gamma^2X + \gamma Y - Z]C_{1,0}\right\}\frac{e^{-\gamma t}}{\gamma(\beta-\gamma)(\alpha-\gamma)}$$

where $C_{1,0}$, $C_{2,0}$, $C_{3,0}$ and $C_{4,0}$ are unbound CQ concentrations in the extracellular buffer, erythrocyte cytosol, parasite cytosol and food vacuole, respectively, at time zero,

$$X = \frac{k_{21}}{V_2} + \frac{k_{23}}{V_2} + \frac{k_{32}}{V_3} + \frac{k_{34}}{V_3} + \frac{k_{43}}{V_4}$$

$$Y = \frac{k_{21}k_{32}}{V_2V_3} + \frac{k_{21}k_{34}}{V_2V_3} + \frac{k_{21}k_{43}}{V_2V_4} + \frac{k_{23}k_{34}}{V_2V_3} + \frac{k_{23}k_{43}}{V_2V_4} + \frac{k_{32}k_{43}}{V_3V_4}$$

$$Z = \frac{k_{21}k_{32}k_{43}}{V_2V_3V_4}$$

and α, β abd γ are the roots of the cubic equation

$$s^3 + \left[\frac{k_{12}}{V_1} + \frac{k_{21}}{V_2} + \frac{k_{23}}{V_2} + \frac{k_{32}}{V_3} + \frac{k_{34}}{V_3} + \frac{k_{43}}{V_4}\right]s^2 + \left[\frac{k_{12}k_{23}}{V_1V_2} + \frac{k_{12}k_{32}}{V_1V_3} + \frac{k_{12}k_{34}}{V_1V_3} + \frac{k_{12}k_{43}}{V_1V_4} + \frac{k_{21}k_{32}}{V_2V_3} + \frac{k_{21}k_{34}}{V_2V_3} + \frac{k_{21}k_{43}}{V_2V_4} + \frac{k_{23}k_{34}}{V_2V_3} + \frac{k_{23}k_{43}}{V_2V_4} + \frac{k_{32}k_{43}}{V_3V_4}\right]s + \frac{k_{12}k_{23}k_{34}}{V_1V_2V_3} + \frac{k_{12}k_{23}k_{43}}{V_1V_2V_4} + \frac{k_{12}k_{32}k_{43}}{V_1V_3V_4} + \frac{k_{21}k_{32}k_{43}}{V_2V_3V_4} = 0$$

α, β and γ were obtained numerically [39]. The above equations were written as part of computer programs designed to generate predicted cellular CQ concentrations at given time values.

The analytical solution to Equations 1–4 was validated by numerical integration of these equations using the NAG (Numerical Algorithm Group, Oxford) Fortran library routine D02EBF, which integrates systems of ordinary first-order equations by the Gear method [40]. The assumptions and codings used in the numerical solutions are different from those used in the analytical solution. Cellular uptake and efflux data generated using the analytical and numerical solutions to Equations 1–4 did not differ significantly.

Appendix 2. Addition of the equilibrium surface binding term using the apparent volume of the buffer

The rapid initial uptake phase observed in the cellular uptake data reported in Study 1 was interpreted as the equilibrium binding of CQ to the surface of the host erythrocyte. This surface binding term was included in the kinetic model by using an apparent volume, rather than the true volume, of the extracellular buffer. The apparent volume was determined as follows. The concentration of unbound CQ in the buffer (C_1) immediately after initial rapid uptake is given by the expression

$$C_1 = (A_t - A_m)/V_b \qquad \text{(A1)}$$

where A_t is the total amount of CQ present in the erythrocyte suspension, A_m is the amount of CQ taken up by infected erythrocytes in the rapid uptake phase and V_b is the true volume of the buffer. Values for A_m were estimated by curve fitting the data from Study 1 using nonlinear regression with a single exponential function containing a separate binding term. Equation A1 can also be written as

$$C_1 = A_t/V_1 \qquad \text{(A2)}$$

where V_1 is the apparent volume of the buffer. Combining Equations A1 and A2 and rearranging gives

$$V_1 = V_bA_t/(A_t - A_m) \qquad \text{(A3)}$$

V_1 was shown to be 1.21 and 1.22 times V_b for uptake of CQ by erythrocytes infected with the CQ-R (VNS) and CQ-S (FCN) strains of Study 1, respectively.

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