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UPTAKE OF CHLOROQUINE BY HUMAN ERYTHROCYTES

VICTOR FERRARI and DAVID J. CUTLER*
Department of Pharmacy, University of Sydney, NSW 2006, Australia

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Abstract—Analysis of studies of the pH dependence of the kinetics of chloroquine (CQ) uptake by human erythrocytes indicates that the unionised CQ species is the major membrane permeant at physiological pH even though the concentration of this species as a fraction of the total CQ concentration in solution is extremely small (0.01% at pH 7.4). CQ concentration-dependence studies and studies performed in the presence of various substrates and inhibitors of erythrocyte membrane transport failed to provide evidence of saturation or inhibition of CQ transport, which suggests that the likely mechanism of CQ transport across human erythrocyte membranes is by passive diffusion. Results of equilibrium binding studies of CQ to intact and lysed human erythrocytes indicated that the mechanism of CQ accumulation in intact human erythrocytes appears to be by a combination of ion trapping (a consequence of the basic nature of the drug and the pH gradient across the human erythrocyte membrane) and binding of CQ to cell components.

The antimalarial activity of chloroquine (CQ) is generally regarded to be a consequence of its ability to accumulate within the food vacuole of the intraerythrocytic parasite [1, 2]. Several mechanisms of accumulation within the food vacuole have been proposed. One mechanism involves high-affinity binding within the food vacuole of CQ to ferriprotoporphyrin IX, a haemoglobin breakdown product [3]. Another proposal is that CQ, a basic drug with two sites of ionisation (the reported pK_a values at 37° are 8.11 and 10.41 [4]), accumulates within the acidic food vacuole by trapping of the protonated forms of the drug within the compartment of lower pH [5, 6]. This mechanism assumes that only the unionised CQ species is freely permeable across biological membranes. One of the difficulties with this assumption is that CQ is extensively ionised, with less than 0.01% of CQ being present in the unionised form at physiological pH. This has led to the suggestion that the relatively rapid uptake of CQ into parasitised cells must involve transport of ionised species [7, 8]. Yayon and Ginsburg [8] have suggested that the uptake of CQ by uninfected human erythrocytes is mediated by a simple symmetrical carrier that is normally responsible for the transport of basic amino acids such as lysine and arginine. These amino acids are thought to be transported as a diprotonated species [9] which suggests that CQ may also be transported in the diprotonated form. Recently, Warhurst [10, 11] has proposed a model to describe the uptake of CQ by infected erythrocytes which involves a carrier or permease to transport the diprotonated CQ species from the host cytoplasm to the parasite cytoplasm.

The mechanism by which CQ crosses biological membranes is of fundamental importance in the antimalarial activity of the drug. To reach its proposed site of action within the parasite food vacuole, CQ must cross at least four different biological membranes: the host erythrocyte plasma membrane; the parasitophorous vacuole membrane (which envelopes the parasite and is derived from the host erythrocyte plasma membrane); the plasma membrane of the parasite; and the food vacuole membrane.

The aim of the present study is to investigate the mechanism of transport of CQ across the plasma membrane of uninfected human erythrocytes. It appears from the results of the present study that the unionised species is transported across this membrane by a mechanism of passive diffusion. The kinetic data presented here provide the basis for a kinetic analysis of previously reported [12, 13] transport rates in *Plasmodium falciparum* infected erythrocytes. This work will be presented in a subsequent report.

MATERIALS AND METHODS

Chloroquine (CQ), as the diphosphate salt, was obtained from the Sigma Chemical Co. (St Louis, MO). Hydroxychloroquine (HCQ), as the sulphate salt, was donated by Winthrop Laboratories (Ermington, NSW, Australia). To prevent CQ and HCQ binding, all glassware coming into contact with CQ solutions was treated with Aquasil silanising agent (Pierce, Rockford, IL, U.S.A.); plastic-ware was also tested for CQ binding prior to use.

Erythrocyte suspensions were prepared by first removing the plasma and buffy coat from freshly donated blood. The cells were then washed four times with buffer. The principal buffer system used in the present study contained 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES; Boehringer Mannheim GmbH, F.R.G.) and 147 mM NaCl adjusted to the appropriate pH (6.5 to 8.5). The cells were then suspended in buffer to achieve the desired haematocrit which was accurately measured using a haematocrit centrifuge. In one

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* Corresponding author.

study, NaCl in the HEPES buffer system was replaced with 294 mOsM KCl. In another study, recently expired whole blood was obtained from the Red Cross Blood Bank, Sydney, and erythrocytes were prepared as previously described using phosphate-buffered saline instead of the HEPES-based buffer. The pH of the erythrocyte suspension was measured after incubating the suspension for about 45 min at the desired temperature using a Ross pH electrode (Orion Research Inc., model 8104, Cambridge, MA, U.S.A.) attached to a digital pH meter. Once stabilised, the pH of the erythrocyte suspension was shown not to vary significantly over the duration of the study.

Kinetic studies

The technique used to obtain kinetic data first involved melting a 1-mm diameter hole in the caps of 1.5-mL micro-centrifuge tubes (Eppendorf, Microtube type 3810, Hamburg, F.R.G.) and then adding 0.5 mL of erythrocyte suspension to each tube—the haematocrit being about 30%. The tubes containing the erythrocytes were then equilibrated at the desired temperature (0 to 25 ± 1°) in a shaking water bath. A buffered CQ solution was also equilibrated in the water bath and adjusted to the same pH as the erythrocyte suspension.

To commence the uptake reaction, 0.49 mL of the CQ solution was injected rapidly into a tube containing erythrocytes using a syringe and needle via the hole in the cap. At the desired time, the tube containing the reaction mixture was taken from the water bath and centrifuged (Eppendorf Centrifuge, model 5414s) to stop the uptake reaction. We estimate, based on performance figures supplied by Eppendorf, that it takes less than 2–3 sec after commencing centrifugation to sediment the cells completely. By placing a tube containing erythrocytes in the centrifuge just prior to adding the CQ solution, the uptake reaction can be stopped within 3–4 sec of introducing the CQ solution.

At lower temperatures and erythrocyte suspension pH values (for which much slower uptake was seen), an alternate kinetic technique was used to validate the previously described technique. It involved the addition of a small volume of CQ solution to a well-stirred erythrocyte suspension, taking 1-mL samples of the suspension at desired times and terminating the uptake reaction by centrifugation. Results using this technique were not significantly different from those obtained using the previously described technique.

For both techniques, an aliquot (50 μL) of HCQ solution was added to 100 or 200 μL supernatant fraction and the cell pellet as an internal standard. The CQ and HCQ in the samples were extracted by adding 100 μL of 1 M NaOH, or 200 μL of ammonia solution in the case of the cell pellet, and 1 mL of freshly distilled diethyl ether and then vigorously shaking the mixture for 2 min. The ether phase was collected after freezing the aqueous phase using a mixture of acetone and dry ice. After evaporating the ether, the samples were reconstituted with a solution containing an equal volume of acetonitrile and 0.1 M H_2SO_4 and then assayed using the HPLC/fluorimetric detection technique described by Tett and co-workers [14]. A minor alteration to the mobile phase involving an increase in the methanol component from 70 to 85% resulted in shorter retention times.

Initially, both the supernatant fraction and the cell pellet were assayed to confirm total recoveries. In subsequent studies, only the supernatant fraction was assayed and the CQ concentration in the cell pellet was calculated by difference.

Inhibition and competition studies

Kinetic studies, conducted at 15 ± 1°, were also performed in the presence of substrates and transport inhibitors. The erythrocyte suspensions and CQ solutions used in the kinetic runs were prepared in the presence of substrates and inhibitors as follows:

Amino acids. An aliquot (100 μL) of 150 mM L-lysine (Sigma Chemical Co.) was added to 15 mL of erythrocyte suspension.

Erythrocytes and CQ solution were prepared as previously described with buffer containing either 10 mM L-histidine or L-tryptophan (Sigma Chemical Co.). The total osmolarity of the amino acid containing buffer was adjusted to 304 mOsM by reducing the NaCl concentration.

Erythrocytes and CQ solution were prepared with a solution containing 10 mM DL-4-azaleucine (as the dihydrochloride salt; Calbiochem, La Jolla, CA), 10 mM HEPES and 132 mM KCl.

Choline. Erythrocytes and CQ solution were prepared with buffer containing 2 mM choline chloride (Hopkins & Williams, Chadwell Heath, Essex, U.K.).

Glucose. Erythrocytes and CQ solution were prepared with a solution containing 120 mM D-glucose (Ajax Chemicals, Sydney, Australia), 10 mM HEPES and 87 mM NaCl.

Inhibitors. An aliquot (50 μL) of 1 M fluorodinitrobenzene (FDNB; Fluka AG, Buchs, Switzerland) in ethanol (freshly prepared) was added to 10 mL buffer followed by the addition of 5 mL concentrated erythrocyte suspension (haematocrit equal to about 90%). The erythrocyte suspension was then gently stirred at 20° for 90 min to allow the pH to stabilise since preliminary studies showed that FDNB causes a slow reduction in erythrocyte suspension pH over this time period.

An aliquot (100 μL) of 6 mg/mL 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (disodium salt; DIDS; Sigma Chemical Co.) solution (freshly prepared) was added to 15 mL of erythrocyte suspension and allowed to equilibrate at 37° for 45 min.

Erythrocytes and CQ solution were prepared with buffer containing either 20 μM *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI; Sigma Chemical Co.) or 1 mM verapamil hydrochloride (Sigma Chemical Co.)

Binding studies

The binding of CQ to lysed erythrocytes at various pH values and temperatures was investigated using the following technique: erythrocytes were washed as described for the kinetic technique and reconstituted with buffer previously adjusted to a desired pH. The haematocrit (about 50%) was then measured to determine total cell volume prior to lysis. The cells were then subjected to three cycles of freeze–thaw lysis using a mixture of dry ice in acetone. The pH of the lysed cell suspension was then measured at the appropriate temperature. A small volume of CQ solution was added to a known volume of lysed cell suspension and buffer (adjusted to the same pH as the lysed cell suspension) to obtain a final CQ concentration of 10 μM. CQ binding to the lysed erythrocyte components was determined by equilibrium dialysis using 1 mL semi-micro teflon dialysis cells (Spectrin Medical Industries Inc., Los Angeles, CA) and a cellophane semi-permeable membrane (Type 20, Union Carbide Corp., Chicago, IL). Dialysis was conducted in a thermostated water bath with a run time of 2 hr. Samples (200 μL) from both the lysed cell suspension and buffer compartments were then collected in triplicate, internal standard was added, and the CQ and HCQ were extracted and assayed as previously described.

RESULTS AND DISCUSSION

Figure 1 illustrates typical profiles of the time course of CQ uptake by human erythrocytes at 15° at different extracellular pH values. The lines shown are nonlinear regression fits (FUNFIT [15]) to the data using the first-order model

$$CQ_c = CQ_{c,\infty}(1 - e^{-k_{app}t}). \qquad (1)$$

CQ_c is the CQ concentration in cells (total amount in cell fraction divided by the total cell volume) at time t; the unknown parameters to be determined by the nonlinear regression are the CQ concentration in the cell at equilibrium ($CQ_{c,\infty}$) and the apparent transport rate constant (k_{app}).

The coefficients of variation, determined as part of the curve-fitting procedure, are about 1–3% for $CQ_{c,\infty}$ and 5–10% for k_{app}. No systematic deviation of the data about the fitted curves was evident, and the coefficients of determination (r^2) varied from 0.90 to 1.00. These values indicate that fitting the data to a first-order model is appropriate.

One complication in analysing uptake kinetics is that the pH of the human erythrocyte interior varies with the external pH [16]. Another complication arises from the possibility that drug is bound to intracellular components. Analysis is simplified by considering first initial uptake rates, since these are independent of intracellular pH and binding. Initial uptake rates (denoted by $J_{in,o}$) were calculated using the fitted curve represented by Equation 1;

$$\begin{aligned} J_{in,o} &= V_c[dCQ_c/dt]_{t\to o} \\ &= V_c k_{app} CQ_{c,\infty} \qquad (2) \end{aligned}$$

where V_c is the cell volume. Alternatively, in terms of the permeability coefficient, P_{in}, for transport from buffer to cell,

$$J_{in,o} = P_{in}\cdot S\cdot CQ_{b,o} \qquad (3)$$

where $CQ_{b,o}$ is the drug concentration in buffer at time zero and S is the surface area of the cell. Combining Equations 2 and 3, and rearranging,

$$P_{in} = V_c k_{app} CQ_{c,\infty}/S\cdot CQ_{b,\infty} \qquad (4)$$

Thus the permeability coefficient in the inward direction can be determined from the initial buffer concentration, the parameters from fitting Equation 1 to the uptake data, and an estimate of the volume to surface ratio for the cell. The mean cell volume of erythrocytes used in the present study was

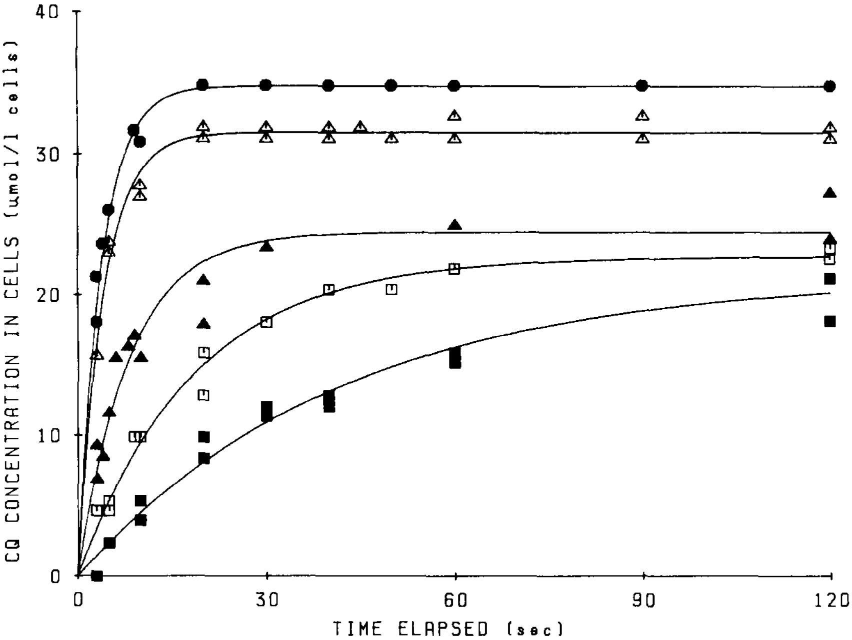

Fig. 1. Time course of CQ uptake by human erythrocytes determined at 15° at various extracellular pH values. Key: pH 7.20 (■), pH 7.35 (□), pH 7.55 (▲), pH 7.70 (△), and pH 7.80 (●). The lines shown are nonlinear regression fits to the data points.

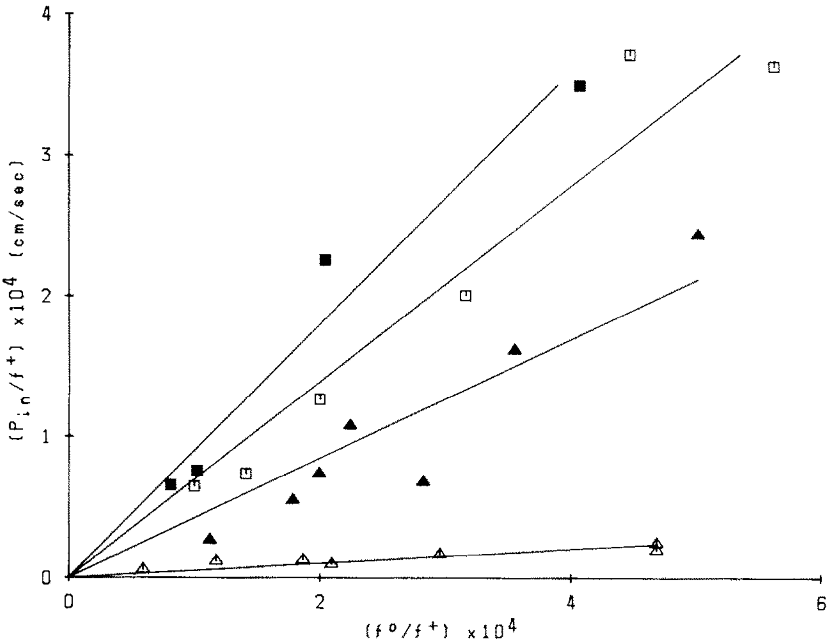

Fig. 2. Plots of P_{in}/f^+ versus f^o/f^+ determined at various temperatures. Key: 0° (△), 10° (▲), 15° (□) and 20° (■). P_{in} values were estimated by nonlinear regression from individual kinetic runs. The lines shown are linear regression fits to the data points.

8.5×10^{-11} cm^3 and the erythrocyte surface area taken to be 1.63×10^{-6} cm^2 [17].

The permeability coefficient P_{in} in Equation 4 is defined in terms of the total drug, rather than for particular permeant species. Recognising the neutral (concentration CQ°), monoprotonated (concentration CQ^+) and diprotonated species (concentration CQ^{2+}) as possible permeant species and assuming uptake by separate parallel processes allows P_{in} to be written as

$$P_{in} = p_{in}^{o} f^{o} + p_{in}^{+} f^{+} + p_{in}^{2+} f^{2+} \tag{5}$$

where p_{in}^o, p_{in}^+ and p_{in}^{2+} are the permeability coefficients for neutral, monoprotonated and diprotonated species and f^o, f^+ and f^{2+} the concentrations of these species as a fraction of the total buffer concentration of CQ. The f^o, f^+ and f^{2+} values can be calculated given the extracellular pH and the pk_a values of CQ at the appropriate temperature [4]. With increasing pH, P_{in} increases sharply. CQ^{2+} decreases with increasing pH, while both CQ° and CQ^+ increase with pH over the range studied. This suggests that CQ^{2+} is not a major permeant. Taking p_{in}^{2+} to be negligibly small, Equation 5 can be rearranged to give

$$\frac{P_{in}}{f^+} = p_{in}^{+} + \frac{f^o}{f^+} p_{in}^{o} \tag{6}$$

Table 1. Permeability coefficients for the transport of the unionised CQ species into human erythrocytes (P_{in}^{o})

Temperature (°C)	P_{in}^{o}* (cm/sec)	r_2*
0	0.052	0.96
10	0.42	0.96
15	0.70	0.98
20	0.90	0.99

* Determined by linear regression of the lines shown in Fig. 2.

If the above assumptions are correct, and the only influence of pH is on the fractions of the species present as the neutral species and the monocation (specifically, if the permeability coefficients, p_{in}^o and p_{in}^+, are independent of pH), Equation 6 predicts that a plot of P_{in}/f^+ versus f^o/f^- should be linear. Figure 2 shows these plots at various temperatures, indicating reasonable agreement with the prediction of linearity. The calculated intercepts (equal to p_{in}^+, according to Equation 6) were either extremely small or not statistically different from zero, indicating that the permeability coefficient of the monoprotonated CQ species for transport into the cells is not significant relative to the permeability of the unionised species. Since the calculated intercepts were not significant, the regression lines shown in Fig. 2 have been constrained to pass through the origin. Values of p_{in}^o, calculated as the slope of the lines in Fig. 2, are shown in Table 1. These results support the proposal that the human erythrocyte membrane is significantly permeable only to the unionised CQ species.

Since an increase in extracellular pH is expected to lower the erythrocyte membrane potential [16], it is possible that the observed pH dependence of P_{in} is, in fact, due to an effect of altered membrane potential on transport across the membrane of a cationic species, rather than (as supposed above) due to shifting proportions of the unionised species. This possibility can be investigated further by comparing the observed effects of pH on the rate of CQ uptake by erythrocytes with those calculated from the Nernst–Planck equation. The flux (J) of a permeable ion in the presence of a membrane potential gradient ($\delta\Psi/\delta x$) is given by the Nernst–Planck equation,

$$J = -D\left[\frac{\delta C}{\delta x} + ZC\frac{F}{RT}\frac{\delta\Psi}{\delta x}\right] \quad (7)$$

D is the diffusion coefficient of the ion in the membrane; $\delta C/\delta x$ is the concentration gradient of the transported species across the membrane; Z is the charge of the transported ion; C is the concentration of the transported species in the membrane as a function of position; and F, R and T are the Faraday constant, gas constant and absolute temperature respectively. Assuming that the concentration and electrical gradients are linear across the membrane, Equation 7 can be used to derive the following equation which relates the initial uptake rate ($J_{\mathrm{in,o}}$) to the membrane potential.

$$J_{\mathrm{in,o}} = P_m\left[1 + Z\frac{F}{RT}\Delta\Psi\right]C_{b,\mathrm{o}} \quad (8)$$

where P_m is a constant, $\Delta\Psi$ is the potential difference across the membrane and $C_{b,\mathrm{o}}$ is the initial buffer concentration of the permeable species. The relationship between membrane potential and extracellular pH, reported by Funder and Wieth [16] for human erythrocytes at 20°, indicates that the membrane potential is zero at pH 6.5; hence

$$J_{\mathrm{in,o}}\,(\mathrm{pH}\ 6.5) = P_m C_{b,\mathrm{o}}$$

Using the same reported relationship, the membrane potential at pH 7.5 is about −15 mV. When this value is inserted into Equation 8,

$$J_{\mathrm{in,o}}(\mathrm{pH}\ 7.5) = 1.6 P_m C_{b,\mathrm{o}}$$

(assuming $Z = 1$ and taking $ZF/RT = 0.04\ \mathrm{mV}^{-1}$) which indicates that the predicted $J_{\mathrm{in,o}}$ value at pH 7.5 is 1.6 times greater than the value at pH 6.5 for a monocation (or 2.2 times greater for dications, taking $Z = 2$). From our kinetic results at 20°, a 25-fold difference in $J_{\mathrm{in,o}}$ values was observed over the smaller pH range 6.80 to 7.50. The predicted pH dependence of initial flux of CQ cations into human erythrocytes due to a transmembrane potential is at least one order of magnitude too low to account for the observed pH dependence.

Yayon and Ginsburg [8] reported that CQ is transported across the human erythrocyte membrane by means of a symmetrical carrier. From preliminary results, they reported that the transport of CQ was inhibited by the amino acids lysine, arginine, histidine and tryptophan. It has also been claimed that the transport of tryptophan is weakly inhibited by CQ [18]. These findings suggest that CQ may be transported by an amino acid carrier. Amino acids are thought to be predominately transported as ions under physiological conditions [9]. The proposal that CQ is transported by an amino acid carrier is contrary to our conclusion that the neutral CQ species is the major permeant. To further investigate whether the transport of CQ across the erythrocyte membrane is mediated by facilitated diffusion, kinetic studies were performed in the presence of arginine, tryptophan and histidine, other substrates reported to be transported by facilitated diffusion, and known inhibitors of carrier-mediated pathways in human erythrocytes.

Table 2 shows a comparison of the kinetic parameters and permeability coefficients P_{in} (calculated from the kinetic parameters as described above) determined in the presence and absence of various substrates and inhibitors of membrane transport. To correct for variations in erythrocyte suspension

Table 2. Comparison of CQ kinetic parameters (k_{app} and $CQ_{c,\infty}$) and permeability coefficients (P_{in} and p°_{in}) for transport into human erythrocytes determined in the absence and presence of various substrates and membrane transport inhibitors

Substrate or Inhibitor	pH$_e$	k_{app} (per sec)	$CQ_{c,\infty}$ (μmol/L cells)	P_{in} ($\times 10^{-6}$ cm/sec)	p°_{in} ($\times 10^{-1}$ cm/sec)
Control 1	7.35	0.054	22.8	5.5 ± 0.4*	6.4 ± 1.7†
Control 2	7.55	0.121	24.5	13.3 ± 1.6	6.4 ± 2.1
Control 3	7.70	0.241	31.6	33.8 ± 1.5	5.2 ± 1.3
Lysine	7.48	0.066	27.6	8.2 ± 0.7	5.3 ± 1.5
Tryptophan	7.47	0.079	24.9	8.8 ± 0.7	6.0 ± 1.7
Histidine	7.43	0.064	28.0	8.1 ± 0.7	6.6 ± 1.9
KCl‡	7.42	0.071	31.4	10.1 ± 0.6	8.7 ± 2.3
Azaleucine‡	7.42	0.081	29.0	10.6 ± 0.7	9.1 ± 2.4
Choline	7.50	0.059	29.9	7.9 ± 1.0	4.8 ± 1.6
Glucose	7.42	0.102	24.3	10.1 ± 1.1	9.5 ± 2.9
FDNB	7.39	0.050	30.2	6.7 ± 0.3	6.6 ± 1.6
DIDS	7.44	0.071	20.4	6.5 ± 0.4	5.1 ± 1.3
NBTI	7.49	0.065	27.7	8.0 ± 0.8	5.0 ± 1.5
Verapamil	7.60	0.121	30.7	17.0 ± 1.2	6.5 ± 1.8

Results were obtained at 15° at various extracellular pH values (pH$_e$) using a total CQ concentration of 10 μM.

* Confidence intervals calculated from the sum of the coefficients of variation determined for k_{app} and $CQ_{c,\infty}$ as part of the nonlinear regression procedure (N = 9–20 for individual kinetic runs).

† Confidence intervals calculated from the percentage errors in P_{in} and f°. An experimental error of 0.05 pH units for pH$_e$ was chosen which translates to a 20% error in f° over the pH range studied.

‡ NaCl in buffer system was replaced with KCl.

pH (pH_e), p_{in}° values are also listed in Table 2. The p_{in}° values were estimated by dividing the P_{in} values by f° calculated at the suspension pH (which is equivalent to assuming that the membrane is permeable only to the unionised species). The results show that the P_{in} values calculated in the presence of the substrates and inhibitors, over the pH range 7.39 to 7.50 (except in the case of verapamil), were within the P_{in} ranges calculated in the absence of substrates and inhibitors at pH 7.35 and 7.55 (controls 1 and 2). The P_{in} value determined for verapamil at pH 7.60 fell within the range of values determined for controls 2 and 3 (pH 7.55 to 7.70). The p_{in}° values determined in the presence of the substrates and inhibitors were shown not to be significantly different from the control p_{in}° values.

The L-lysine and L-tryptophan concentrations in solution used in the erythrocyte suspension (0.5 and 10 mM respectively) were chosen to be considerably higher than the reported K_m values for uptake by human erythrocytes (0.068 mM at 37° [19] for L-lysine and 3.5 mM at 25° [20] for L-tryptophan). The results (Table 2) suggest that the transport of CQ across erythrocyte membranes is not mediated by a lysine or tryptophan membrane carrier system. The transport of L-histidine across the human erythrocyte membrane is not well characterised. The lack of inhibition of L-histidine uptake by human erythrocytes at 10 mM histidine, under Na^+ free conditions, indicates the possibility of a high K_m for this system [21]. To further investigate the proposed Na^+-independent histidine carrier as a possible carrier for CQ, studies were carried out in the presence of 10 mM azaleucine, a reported inhibitor of Na^+-independent histidine transport [21]. Shown in Table 2 is the result of a preliminary study where extracellular Na^+ was replaced with K^+. No significant effect on CQ uptake was observed. The addition of azaleucine (with extracellular K^+ in place of Na^+ to obtain Na^+-free conditions) also showed no significant effect. This result and the lack of inhibition of CQ transport in the presence of 10 mM L-histidine suggest that CQ transport does not involve a histidine carrier system.

Both choline and D-glucose (used at concentrations of 2 and 120 mM respectively) have been shown to be transported by complex carrier-mediated systems with reported K_m values ranging from 6.3 to 33 μM for choline [22, 23] and 3 to 25 mM for D-glucose [24]. It has been reported that FDNB acts as an irreversible inhibitor of glucose transport in human erythrocytes [25]. The transport of purine and pyrimidine nucleosides across erythrocyte membranes has also been shown to occur by a process of facilitated diffusion which is inhibited by NBTI [26]. The results for these substrates and inhibitors (Table 2) suggest that CQ is not transported across human erythrocyte membranes by either a choline, glucose or nucleoside membrane carrier system.

It has been reported that verapamil inhibits the release of chloroquine from erythrocytes infected by CQ-resistant strains of the malarial parasite, *P. falciparum*. A proposed mechanism for this inhibition is that verapamil competes with CQ for binding to a carrier that transports CQ out of the parasite [13]. One of the membranes that surrounds the parasite, the parasitophorous vacuole membrane, is derived from the host cell plasma membrane. If the proposed verapamil-sensitive CQ carrier is present within the parasitophorous vacuole membrane, then this raises the possibility that the carrier is native to the host erythrocyte plasma membrane. The lack of a significant effect of verapamil (Table 2) on the kinetics of uptake of CQ indicates that the proposed verapamil-sensitive CQ carrier is not present in the uninfected human erythrocyte membrane.

Kinetic studies were also performed in the presence of the anion channel blocker DIDS [27], to further investigate the possibility of transport of CQ cations under the influence of a transmembrane potential. DIDS could be expected to inhibit CQ transport in the case in which CQ cations are transported, with electroneutrality maintained by rapid anion exchange. The lack of a significant effect of DIDS on CQ transport rate into the erythrocytes supports our earlier conclusion that the charged CQ species are not transported to a significant extent.

Studies were also carried out to investigate possible concentration dependence of membrane transport. The kinetic parameters and permeability coefficients determined over a wide CQ concentration range are shown in Table 3. The results show that the P_{in} values calculated at the high CQ concentrations (up to 1000 μM at pH_e 7.45) fell within the range of values determined using 10 μM CQ (at pH_e 7.35 and 7.55). No significant difference in the calculated p_{in}° values was observed over the CQ concentration range studied. The results of the CQ concentration dependence studies indicate that saturation of a transport system over the CQ concentration range studied is not evident.

Shown in Table 4 are parameter values determined at 10 and 100 μm CQ using erythrocytes from

Table 3. Comparison of kinetic parameters (k_{app} and $CQ_{c,\infty}$) and calculated CQ permeability coefficients into human erythrocytes at different total CQ concentrations ($[CQ]_t$)

$[CQ]_t$ (μM)	pH_e	k_{app}* (per sec)	$CQ_{c,\infty}$* (μmol/L cells)	P_{in} ($\times 10^{-6}$ cm/sec)	p_{in}° ($\times 10^{-1}$ cm/sec)
10	7.35	0.054	22.8	5.5 ± 0.4†	6.4 ± 1.7†
10	7.55	0.121	24.5	13.3 ± 1.6	6.4 ± 2.1
100	7.45	0.104	228	10.6 ± 1.3	7.9 ± 2.5
500	7.45	0.110	1156	11.4 ± 1.0	8.5 ± 2.5
1000	7.45	0.115	1749	9.0 ± 0.9	6.7 ± 2.0

* Determined at various extracellular pH values (pH_e) at 15°.

† Confidence intervals as defined in Table 2.

Table 4. Kinetic parameters (k_{app} and $CQ_{c,\infty}$) and calculated CQ permeability coefficients into human erythrocytes using erythrocytes from recently expired blood bank blood and phosphate-buffered saline as the cell medium (10 and 100 μM CQ) and erythrocytes from freshly donated blood and the HEPES-based buffer as the cell medium (10 μM CQ)

$[CQ]_t$ (μM)	pH_e	k_{app} (per sec)	$CQ_{c,\infty}$ (μmol/L cells)	P_{in} (× 10^{-6} cm/sec)	p°_{in} (× 10^{-1} cm/sec)
Recently expired blood bank blood					
10	7.10	0.18	21.5	17.8 ± 3.0*	17.4 ± 6.4*
100	7.10	0.21	201	19.0 ± 1.7	18.6 ± 5.4
Freshly donated blood					
10	7.40	0.52	27.7	64.4 ± 5.1	16.5 ± 4.6

All results were obtained at 25°.

* Confidence intervals as defined in Table 2.

recently expired blood bank blood and phosphate-buffered saline (pH 7.4) at 25°. The results show that the kinetic parameters and permeability coefficients calculated at 10 μM CQ were almost identical to the values determined at 100 μM CQ. The absence of a saturable transport system demonstrated by these results, is consistent with results obtained using freshly donated blood (Table 3). Results at 10 μM CQ using erythrocytes from recently donated blood suspended in the HEPES-based buffer at 25° are also shown in Table 4. The p°_{in} value determined under these experimental conditions was not significantly different from values obtained using recently expired blood bank blood (note that the p°_{in} values are higher than those of Table 3 because data in Table 3 were obtained at a lower temperature).

Stein [28] has published a list of reported K_m and V_{max} values for numerous facilitated diffusion systems. These values can be used to calculate equivalent influx permeability coefficients. At subsaturation substrate concentrations, the initial flux into a cell, $J_{in,o}$, can be written as

$$J_{in,o} = (V_{max}/K_m)V_{c,w}C_{b,o} \qquad (9)$$

where $C_{b,o}$ is the initial substrate concentration in the buffer and $V_{c,w}$ is the volume of cell water. Since the K_m and V_{max} values are expressed in mmol and mmol/L cell water/sec, respectively, $V_{c,w}$ is included in Equation 9 so that $J_{in,o}$ can be expressed in the same units as in the present study (μmol/sec). Combining Equations 3 (with $CQ_{b,o}$ replaced by $C_{b,o}$) and 9, and rearranging, we obtain an expression describing the inward substrate permeability coefficient (P_{in}) in terms of V_{max} and K_m,

$$P_{in} = (V_{max}/K_m)(V_{c,w}/S). \qquad (10)$$

For human red cells $V_{c,w}/S$ is about 3.7 × 10^{-5} cm. The P_{in} value calculated for CQ at pH 7.4, using data from the present study, is 1.9 × 10^{-5} cm/sec at 20°. This value is considerably higher than permeability coefficients calculated from V_{max} and K_m values reported for erythrocyte facilitated diffusion systems. For example, the permeability coefficients calculated for the amino acids published by Stein [28] are at least two orders of magnitude lower than the value calculated for CQ. The only erythrocyte carrier system described by Stein [28] that has similarly high permeability coefficients is that of glucose transport (with typical calculated values of about 1 × 10^{-5} cm/sec at 20°). If, as suggested in the present study, the unionised CQ species is the major permeant, it is appropriate to use p°_{in} in the comparison rather than P_{in}. The p°_{in} value calculated at pH 7.4 at 20° was 0.90 cm/sec (listed in Table 1). This value is five orders of magnitude greater than the highest permeability coefficients calculated from the list of parameters for human red cells published by Stein [28].

The results reported in the present study strongly suggest that CQ is transported across the erythrocyte membrane as the unionised species by passive diffusion, contrary to an earlier report by Yayon and Ginsburg [8]. It seems that these authors may have encountered methodological problems in their study since they observed CQ accumulation ratios equal to one in human erythrocytes, which is not only inconsistent with our findings but also with those reported in a number of other studies [12, 29, 30]. Using Equation 10, the P_{in} value calculated from the K_m and V_{max} values reported by Yayon and Ginsburg [8] for CQ transport in the buffer to cell direction is 3.2 × 10^{-6} cm/sec. The P_{in} value determined in the present study at the same pH and temperature (pH 7.4 and 25°; Table 4) was considerably higher at 6.44 × 10^{-5} cm/sec. This discrepancy may be due to the fact that Yayon and Ginsburg [8] used erythrocytes from recently expired blood bank blood. When we repeated the kinetic study using recently expired blood bank blood as the source of erythrocytes and phosphate-buffered saline as the cell medium (Table 4), although pH 7.4 buffer was used to wash and suspend the red cells, the pH of the erythrocyte suspension, measured after 1 hr of incubation at 25°, was only 7.1. This lower pH is presumably due to either an increase in cellular lactic acid concentration (reported to occur during blood storage [31]) or the residual effect of the citrate–phosphate–dextrose–adenine solution used to store the blood bank blood. A reduced extracellular pH can explain an apparently low reported transport rate and accumulation ratio for CQ uptake by human erythrocytes. Yayon and Ginsburg [8] did not report measuring the pH of the erythrocyte suspension and may have encountered a similar problem. These authors reported the presence of a saturable process for CQ uptake by human erythrocytes at 10–100 μM CQ. No saturation of CQ transport was evident in our studies using either erythrocytes from freshly donated blood or recently expired blood bank blood (Table 3 and 4).

The kinetic data reported in the present study can also be used to estimate permeability coefficients for efflux, with the aid of further data on intracellular binding. This can be done using the apparent rate constant for uptake, k_{app}, estimated from from kinetic runs, or (mathematically simpler) using the equilibrium accumulation ratios obtained from the same runs. Fitting the entire uptake curve by nonlinear regression ensures the same results are obtained by either method; here we describe only the simpler method based on equilibrium accumulation ratios.

At equilibrium

$$\frac{CQ_{c,\text{free}}}{CQ_b} = \frac{P_{\text{in}}}{P_{\text{out}}} \tag{11}$$

where $CQ_{c,\text{free}}$ and CQ_b are the unbound CQ concentrations inside the cell and in the buffer at equilibrium, and P_{out} is the CQ permeability coefficient for transport from the cell to the buffer. The cell concentrations measured in the present study are derived from a measure of the total amount of drug in the cell (divided by the cell volume), which differs from the free concentration $CQ_{c,\text{free}}$ in Equation 11 for two reasons. First, the volume of the cell does not coincide with the volume of cell water. Second, drug may be bound to intracellular components, in which case the free concentration will be less than the concentration estimated from the total amount in the cell. The first factor is easily dealt with, since the fraction of the cell volume which is free water is known (taken to be 0.71 [32]). The second factor presents more difficulties (the same difficulties arise when the analysis is based on k_{app}). Studies on intact cells do not provide the required information, since uptake depends on both permeability and binding. Consequently, studies were carried out using lysed cells, which can be expected to retain the binding component but not the permeability component.

The use of lysed cell preparations introduces a problem, concerning the appropriate pH for binding studies. Most of the components within an intact erythrocyte are presumably in contact with the intracellular fluid. However, the pH of the intracellular fluid does not in general equal that of the surrounding buffer. Funder and Wieth [16] published the following empirical relationship that predictes an intracellular pH (pH_i) given an extracellular pH (pH_e) for intact human erythrocytes over the pH range 6.5 to 8.5:

$$pH_i = pH_e + \log(3.094 \pm 0.014 - 0.335 \pm 0.005\, pH_e) \tag{12}$$

For a study of uptake into intact cells at a buffer pH equal to pH_e, the corresponding intracellular pH (which influences intracellular binding) is pH_i as determined by Equation 12. This is the appropriate pH for a binding study using lysed cells, for comparison with an uptake study using intact cells with external pH equal to pH_e.

Corrected pH values (not the actual experimental values) were used when plotting the lysed cell binding data shown in Fig. 3. Lysed cell binding, K(lysed), is defined as the amount of CQ bound (μmol) per

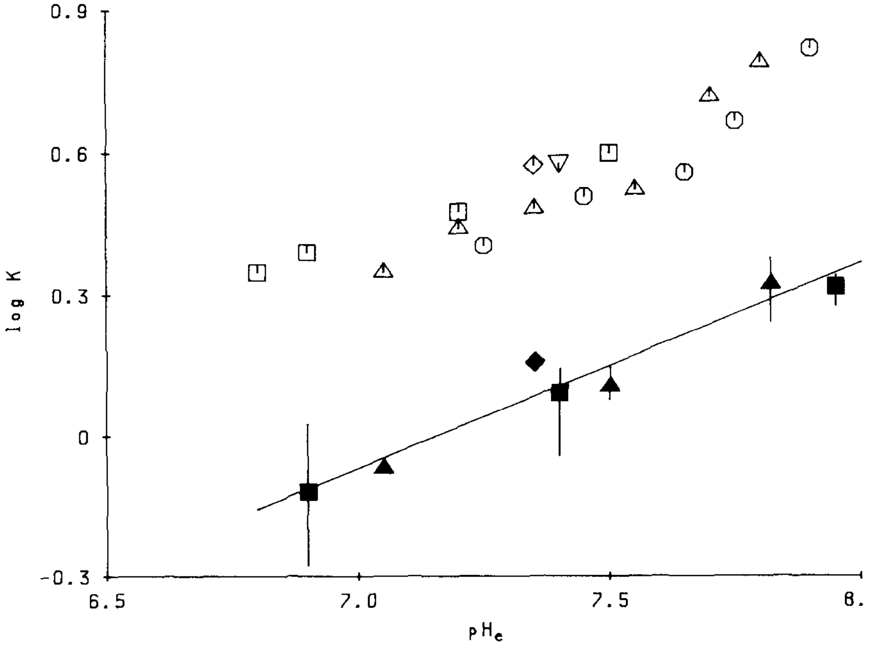

Fig. 3. CQ accumulation ratios in intact (log K(intact)) and lysed (log K(lysed)) erythrocytes at various temperatures plotted as a function of extracellular pH (pH_e). Intact erythrocyte data: 10° (○), 15° (△), 20° (□), 25° (▽) and 37° (◇). Lysed cell binding data: 15° (▲), 20° (■) and 37° (◆). Intact erythrocyte data were estimated by nonlinear regression from individual kinetic runs. Error bars for lysed cell data indicate range of triplicate determinations. The line shown is a linear regression fit to the lysed cell data points.

litre of original cell suspension (prior to lysis) divided by the unbound CQ concentration. Also shown in Fig. 3 are the CQ equilibrium accumulation ratios in intact cells, K(intact), calculated from the $CQ_{c,\infty}$ values determined in the kinetic study. The K(intact) values are defined as the amount of CQ bound (μmol) per litre of cell suspension divided by the unbound CQ concentration. It is evident from Fig. 3 that binding to lysed cells is considerable and therefore binding to intracellular components cannot be neglected (it is estimated that about 35% of CQ in intact cells is bound). A reasonable log-linear relationship is evident between the lysed cell binding data and pH in Fig. 2 ($r^2 = 0.95$). For each pH at which equilibrium accumulation data were available for intact cells, the corresponding lysed cell value was interpolated from Fig. 3 using the log-linear relationship. For each data point the free intracellular concentration in intact cells was calculated by subtracting the amount bound estimated from the lysed cell study from the total amount of CQ in the intact cell (this assumes that lysed cell binding reflects intracellular binding) and dividing by the estimated cell water volume. The resulting value, with the corresponding buffer concentration of CQ, was used, at each pH, to calculate P_{in}/P_{out} according to Equation 11. The results are shown in Table 5.

It can be seen from Table 5 that the P_{in}/P_{out} ratio was substantially greater than 1, and increased with extracellular pH. This pH dependence can be largely explained on the assumption that it is the unionised CQ species which is transported in both directions. Writing $P_{in} = p^\circ_{in} f^\circ_b$ and $P_{out} = p^\circ_{out} f^\circ_c$ (where f°_b and f°_c are the fractions of CQ in the unionised form in the buffer and cell water respectively) we have

$$\frac{P_{in}}{P_{out}} = \frac{p^\circ_{in} f^\circ_b}{p^\circ_{out} f^\circ_c}$$

Using this equation (and Equation 12 for intracellular pH) the values of $p^\circ_{in}/p^\circ_{out}$ reported in Table 5 were calculated. It can be seen that at 10 and 15° the calculated ratio was close to the expected value, 1, if a symmetrical transport system (such as passive diffusion) were operating. The ratios for temperatures above 20° were slightly higher than 1. However, these values were consistent with a ratio $p^\circ_{in}/p^\circ_{out} = 1$ and an underestimate of the intracellular binding of only about 10%. In view of the possible changes in binding which could occur on lysis, it is unlikely that the calculated value indicates a genuine departure from symmetry in the transport system. Furthermore, even if the calculated ratio is accepted as indicating a genuine asymmetry in transport, it is unlikely that this indicates a carrier-mediated efflux process. If the process of transfer from buffer to cell is, as argued previously, one of passive diffusion, a carrier-mediated efflux should result in P_{out} (passive diffusion and carrier-mediated diffusion) exceeding P_{in} (passive diffusion only). In fact, calculated values of P_{out} were smaller than P_{in}.

Table 5. Accumulation ratios in human erythrocytes for unbound CQ (P_{in}/P_{out}), calculated at various temperatures and extracellular pH values (pH$_e$) from the observed accumulation ratio in intact erythrocytes (K_{obs}), and accumulation ratios for the unionised CQ species ($p^\circ_{in}/p^\circ_{out}$), calculated at various temperatures and extracellular pH values (pH$_e$) from K_{obs} and the predicted accumulation ratios due to ion trapping (f°_b/f°_c)

Temp (°C)	pH$_e$	f°_b/f°_c	K_{obs}	P_{in}/P_{out}	$P^\circ_{in}/P^\circ_{out}$
10	7.25	2.00–2.63*	2.54	2.03	0.8–1.0
10	7.45	2.40–3.31	3.22	2.64	0.8–1.1
10	7.65	2.88–4.37	3.62	2.79	0.6–1.0
10	7.75	3.31–5.01	4.64	3.98	0.8–1.2
10	7.90	3.98–6.31	6.59	6.31	1.0–1.6
15	7.05	1.66–2.19	2.23	1.87	0.9–1.1
15	7.20	1.91–2.51	2.76	2.42	1.0–1.3
15	7.35	2.19–3.02	3.04	2.57	0.9–1.2
15	7.55	2.63–3.80	3.33	2.60	0.7–1.0
15	7.70	3.16–4.79	5.22	4.92	1.0–1.6
15	7.80	3.47–5.50	6.16	5.99	1.1–1.7
20	6.80	1.32–1.66	2.23	2.16	1.3–1.6
20	6.90	1.45–1.82	2.46	2.38	1.3–1.6
20	7.20	1.91–2.51	2.99	2.74	1.1–1.4
20	7.50	2.51–3.63	3.98	3.62	1.0–1.4
25	7.40	2.29–3.16	3.78	3.57	1.1–1.6
37	7.35	2.19–3.02	3.75†	3.59	1.2–1.6

* Range of values presented for f°_b/f°_c was determined using the maximum and minimum estimates of the intracellular pH using Equation 12.

† K_{obs} at 37° was determined from an equilibrium binding study.

Values for P_{in}/P_{out} can also be calculated for CQ uptake in the presence of substrates and inhibitors (listed in Table 2). These values (not shown) generally agreed with the controls although they appeared to be more variable. This variability was presumably due to an influence on intracellular pH by some of the substrates and inhibitors (e.g. changes in cellular lactic acid production or as a result of an alteration in anion transport). The general agreement between these P_{in}/P_{out} values and controls supports the proposal that efflux of CQ in human erythrocytes is not mediated by facilitated diffusion.

Evidence provided in this study strongly supports the proposal that CQ transport across the human erythrocyte membrane is predominatly by passive diffusion of the unionised CQ species. The likely mechanism for accumulation of CQ in uninfected human erythrocytes is by a combination of ion trapping and binding to cell components.

REFERENCES

1. Aikawa M, High-resolution autoradiography of malarial parasites treated with [^{3}H]chloroquine. *Am J Pathol* **67**: 277–284, 1972.
2. Yayon A and Ginsburg H, Chloroquine inhibits the degradation of endocytic vesicles in human malaria parasites. *Cell Biol Int Rep* **7**: 895, 1983.
3. Fitch CD, Mode of action of antimalarial drugs. In: *Malaria and the Red Cell, Ciba Foundation Symposium 94* (Eds. Evered D and Whelan J), pp. 222–232. Pitman, London, 1983.
4. Ferrari V and Cutler DJ, Temperature dependence of the acid dissociation constants of chloroquine. *J Pharm Sci* **76**: 554–556, 1987.
5. Yayon A, Cabantchik ZI and Ginsburg H, Identification of the acidic compartment of *Plasmodium falci-*

parum-infected human erythrocytes as the target of the antimalarial drug chloroquine. *EMBO J* **3**: 2695–2700, 1984.

6. Yayon A, Cabantchik ZI and Ginsburg H, Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc Natl Acad Sci USA* **82**: 2784–2788, 1985.
7. Homewood CA, Warhurst DC, Peters W and Baggaley VC, Lysosomes, pH and the antimalarial action of chloroquine. *Nature* **235**: 50–52, 1972.
8. Yayon A and Ginsburg H, The transport of chloroquine across human erythrocyte membranes is mediated by a simple symmetrical carrier. *Biochim Biophys Acta* **686**: 197–203, 1982.
9. Christensen HN, Organic ion transport during seven decades. The amino acids. *Biochim Biophys Acta* **799**, 255–269, 1984.
10. Warhurst DC, Antimalarial schitzontocides: Why a permease is necessary. *Parasitol Today* **2**: 331–334, 1986.
11. Warhurst DC, Mechanism of chloroquine resistance in malaria. *Parasitol Today* **4**: 211–213, 1988.
12. Geary TG, Jensen JB and Ginsburg H, Uptake of [^{3}H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite *Plasmodium falciparum*. *Biochem Pharmacol* **35**: 3805–3812, 1986.
13. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AMJ, Martin SK, Milhous WK and Schlesinger PH, Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science* **238**: 1283–1285, 1987.
14. Tett SE, Cutler DJ and Brown KF, High-performance liquid chromatographic assay for hydroxychloroquine and metabolites in blood and plasma, using a stationary phase of poly(styrene divinylbenzene) and a mobile phase at pH 11, with fluorimetric detection. *J Chromatogr* **344**: 241–248, 1985.
15. Pedersen PV, Curve fitting and modelling in pharmacokinetics and some practical experiences with NONLIN and a new program FUNFIT. *J Pharmacokinet Biopharm* **5**: 513–531, 1977.
16. Funder J and Wieth JO, Chloride and hydrogen ion distribution between human red cells and plasma. *Acta Physiol Scand* **68**: 234–245, 1966.
17. Ponder E, *Hemolysis and Related Phenomena*. Grune & Stratton, New York, 1971.
18. Ginsburg H and Krugliak M, Uptake of L-tryptophan by erythrocytes infected with malaria parasites (*Plasmodium falciparum*). *Biochim Biophys Acta* **729**: 97–103, 1983.
19. Young JD, Jones SEM and Ellory JC, Amino Acid transport in human and in sheep erythrocytes. *Proc R Soc Lond [Biol]* **209**: 355–375, 1980.
20. Rosenberg R, Young JD and Ellory JC, L-Tryptophan transport in human red blood cells. *Biochim Biophys Acta* **598**: 375–384, 1980.
21. Vadgama JV and Christensen HN, Discrimination of Na$^+$-independent transport systems L, T and ASC in erythrocytes. *J Biol Chem* **260**: 2912–2921, 1985.
22. Devés R and Krupka RM, The binding and translocation steps in transport as related to substrate structure. A study of the choline carrier of erythrocytes. *Biochim Biophys Acta* **557**: 469–485, 1979.
23. Krupka RM and Devés R, the choline transport system of erythrocytes. Distribution of the free carrier in the membrane. *Biochim Biophys Acta* **600**: 228–232, 1980.
24. Baker GF and Naftalin RJ, Evidence of multiple operational affinities for *d*-glucose inside the human erythrocyte membrane. *Biochim Biophys Acta* **550**: 474–484, 1979.
25. Bowyer F and Widdas WF, The action of inhibitors on the facilitated hexose transfer system in erythrocytes. *J Physiol (Lond)* **141**: 219–232, 1958.
26. Cass CE and Paterson ARP, Mediated transport of nucleosides by human erythrocytes. Specificity towards purine nucleosides as permeants. *Biochim Biophys Acta* **291**: 734–746, 1973.
27. Cabantchik ZI and Rothstein A, Membrane proteins related to anion permeability of human red blood cells. I. Localisation of disulfonic stilbene binding sites in proteins involved in permeation. *J Membr Biol* **15**: 207–226, 1974.
28. Stein WD, *Transport and Diffusion Across Cell Membranes*. Academic Press, Orlando, FL, 1986.
29. Fitch CD, Chevli R, Kanjananggulpan P, Dutta P, Chevli K and Chou AC, Intracellular ferriprotoporphyrin IX is a lytic agent. *Blood* **62**: 1165–1168, 1983.
30. Orjih AU, Chevli R and Fitch CD, Toxic heme in sickle cells: an explanation for death of malaria parasites. *Am J Trop Med Hyg* **34**: 223–227, 1985.
31. Beutler E, Experimental blood preservatives for liquid storage. In: *The Human Red Cell* in Vitro, *Proceedings of the American National Red Cross Fifth Annual Scientific Symposium, Washington, DC, 7–8 May 1973* (Eds. Greenwalt TJ and Jamieson GA), pp. 189–216. Grune & Stratton, New York, 1974.
32. Gary-Bobo CM and Solomon AK, Properties of hemoglobin solutions in red cells. *J Gen Physiol* **52**: 825–853, 1968.