Biochemical Pharmacology, Vol. 41, No. 1, pp. 23–30, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

KINETICS AND THERMODYNAMICS OF CHLOROQUINE AND HYDROXYCHLOROQUINE TRANSPORT ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

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(*Received* 1 *November* 1989; *accepted* 15 *June* 1990)

Abstract—Chloroquine (CQ) and hydroxychloroquine (HCQ) have almost identical molecular volumes but showed very different permeability characteristics. The permeability coefficient for the unionised species of CQ (2.0 cm/sec at 25°) was about fifty times that of HCQ (0.039 cm/sec at 25°), but the apparent activation energy for transport (85 kJ/mol for CQ, 81 kJ/mol for HCQ) was almost identical for the two drugs. The partition coefficient of CQ into various organic solvents was much higher than for HCQ, but the different permeability behaviour cannot be quantitatively explained by partitioning behaviour into hexane or octanol, two solvents commonly used to mimic the membrane interior. A comparison of permeability and partitioning characteristics suggests that the barrier phase for these drugs within the membrane can be modelled by a mixed solvent of 5% octanol in hexane. The results suggest that interactions with hydrogen bonding groups within the membrane are important in the membrane transport of these drugs, and that the membrane does not behave functionally as a simple hydrocarbon barrier.

In a previous article [1], chloroquine (CQ) was shown to cross the human erythrocyte membrane as the unionised species by a process of passive diffusion, and the rate of transport of the unionised species was found to be substantially more rapid than expected. The present study attempts to obtain further insight into the transport mechanism by a comparative study of the kinetics and thermodynamics of the membrane transport of CQ and its congener hydroxychloroquine (HCQ).

According to the conventional view of passive membrane transport, the permeability coefficient, P, can be expressed as

$$P = K_{mem} D_{mem} / \lambda \qquad (1)$$

where K_{mem} is the partition coefficient of the permeant species between the membrane interior and water, D_{mem} is the diffusion coefficient of the permeant species within the membrane interior, and λ is the thickness of the membrane. It is generally accepted that D_{mem} is primarily a function of the molecular size of the permeant, that K_{mem} is a function of the physicochemical properties of the permeant and the membrane, and that λ is independent of the nature of the permeant.

The traditional approach to investigating mechanisms of passive membrane transport has been the study of homologous series of permeants (e.g. Naccache and Sha'afi [2]; Brahm [3]). One of the difficulties with this approach is that within a homologous series (particularly for the small nonelectrolytes that have been studied most widely) changes in both chemical character and size and shape occur with the transition from one member to another of the series. In the present study, the substitution of an *N*-ethyl group in CQ with an *N*-hydroxyethyl group in HCQ (Fig. 1) had a minor effect on the overall size and shape of the molecule, which is dominated by the aminoquinoline nucleus and side chain. However, this apparently minor structural change had a major influence on the partition coefficient (see Results) between organic solvents and water. A comparative study of membrane transport of CQ and HCQ allowed an investigation of the influence of chemical character without the complication of a simultaneous change in molecular size and shape.

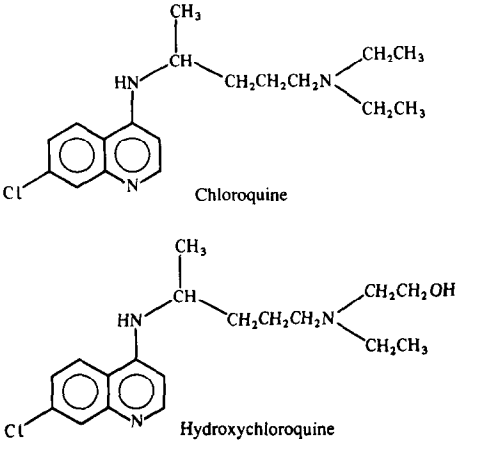

Fig. 1. Chemical structures of chloroquine and hydroxychloroquine.

Based on a review of a wide range of data, Lieb and Stein [4] have provided evidence that passive diffusion of small nonelectrolytes can be accounted for adequately by Equation 1, with a hydrocarbon

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solvent (hexadecane) as a model solvent for partitioning into the membrane interior. In another study [5], these authors suggested that a solvent with slightly higher polarity than hexadecane may provide an even better partitioning model. We conclude, on kinetic and thermodynamic grounds, that the first of these proposals cannot be extended to CQ and HCQ. Evidence from the present study suggests that the transport barrier within the membrane has a significant hydrogen bonding capacity, most likely associated with the polar region of the membrane bilayer or possibly the polar groups on intrinsic membrane proteins.

MATERIALS AND METHODS

Hydroxychloroquine (HCQ), as the sulphate salt, was donated by Winthrop Laboratories (Ermington, NSW, Australia). Chloroquine (CQ), as the diphosphate salt, was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). To prevent HCQ and CQ binding, all glassware coming into contact with solutions of CQ and HCQ was treated with Aquasil silanising agent (Pierce, Rockford, IL, U.S.A.), and plasticware was tested for CQ binding prior to use. Analytical grade solvents were used in the partitioning studies.

Kinetic studies. The method employed to determine the time and temperature dependence of CQ and HCQ uptake has been described previously [1]. Briefly, the method involved washing and reconstituting erythrocytes (obtained from freshly donated blood) in buffer of varying pH [10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and 147 mM NaCl], and then placing 0.5 mL of the erythrocyte suspension into 1.5-mL centrifuge tubes (Eppendorf, Microtube type 3810, Hamburg, Germany). The tubes were then equilibrated in a thermostated shaking water bath. A buffered HCQ or CQ solution (adjusted to the same pH as the erythrocyte suspension) was also equilibrated in the water bath. To commence uptake, 0.49 mL of HCQ or CQ solution (20 μM) was injected rapidly into the erythrocyte suspension via a 1-mm diameter hole which was melted previously in the cap of the centrifuge tube. At the desired time after introduction of the drug, the tube was removed from the water bath and centrifuged (Eppendorf Centrifuge, model 5414s) to stop uptake. For longer time samples (i.e. >5 min), tubes containing the erythrocyte suspensions were vortexed briefly during the uptake reaction to ensure that the erythrocytes remained adequately suspended. In the HCQ uptake studies, CQ was added to 200 μL of supernatant as an internal standard and, after extraction, the drugs were assayed by the HPLC technique described by Tett and co-workers [6]. In the CQ uptake studies, HCQ was used as the internal standard. The amount of drug in the cell fraction was calculated by difference.

Partitioning studies. A rapid equilibration method was developed to determine the effect of temperature on the CQ and HCQ partition coefficients in *n*-hexane, octanol and solvent mixtures containing various proportions of hexane and octanol. A watertight perspex cell was constructed with water inlet and outlet nozzles to allow thermostated water to circulate through the cell. Capped tubes containing the drug and aqueous and organic phases were placed in the thermostated cell and the phases vigorously mixed by shaking the cell containing the tubes on a planetary shaker at 1400 rpm (Janke & Kunkel, model IKA-Vibrax VXR, Staufen, Germany). Preliminary time and drug concentration dependence studies were used to determine the time to equilibrium and confirm the absence of drug saturation within the phases.

The following protocols were used to determine the CQ and HCQ partition coefficient from water to various organic solvents:

(a) hexane: 200 μL *n*-hexane was added to a tube containing 4 mL of 100 μM CQ in 0.1 M NaOH and the phases were mixed for 2 hr. Two milliliters of *n*-hexane was added to 2 mL of 100 μM HCQ in 0.1 M NaOH and the phases were mixed for 1 hr.

(b) Octanol: 100 μL of octan-1-ol solvent was added to a tube containing 4 mL of 100 μM CQ or HCQ in 0.1 M NaOH and the phases were mixed for 15 min.

(c) Mixed solvent: 100 μL of a mixture containing 5% (v/v) octanol in hexane was added to a tube containing 4 mL of 100 μM CQ or HCQ in 0.1 M NaOH (presaturated with octanol at the experimental temperature) and the phases were mixed for 1 hr.

The aqueous phase was assayed directly using the HPLC method used in the kinetic studies. Hexane samples (normally 50 μL) were evaporated and reconstituted in an appropriate volume of 0.1 M HCl prior to assaying. CQ and HCQ were extracted from the octanol samples (50 μL) into 2 mL of 0.1 M HCl and then assayed.

RESULTS

Partitioning studies. Partition coefficients of the unionised drug species (the proposed transported species) for the solvent systems studied are shown in Table 1. The partition coefficients were determined using 0.1 M NaOH as the aqueous phase. Under these conditions, the unionised drug species was the only species present to a significant extent; the partition coefficients are therefore those of the unionised species.

Enthalpies and entropies of partitioning (Table 1) were estimated from the temperature dependence of the partition coefficients using the van't Hoff equation,

$$\ln K = \Delta S/R - \Delta H/RT \qquad (2)$$

where K is the partition coefficient, ΔS and ΔH are the entropy and enthalpy changes of the partitioning process (from water to the organic solvent), R is the gas constant, and T is the absolute temperature. Partitioning data were also obtained with ethyl acetate as the organic solvent; the results (not shown) were very similar to those for octanol. Also included in Table 1 are hypothetical CQ and HCQ partition coefficients, enthalpies and entropies for transfer between organic solvents ("hypothetical" because the solvents are miscible). These are calculated from the corresponding parameters for transfer between water and the individual solvents:

Table 1. Partition coefficients between various solvents of the unionised CQ and HCQ species and calculated enthalpies (ΔH) and entropies (ΔS) of the partitioning process

Solvent combination	Partition coefficient* (at 25°)	ΔH (kJ/mol)	ΔS (kJ/(mol·K)
Chloroquine			
Hexane/water	124	37 ± 2†	0.16 ± 0.01
Octanol/water	21000	4 ± 5	0.10 ± 0.02
Mixed solvent/water‡	3800	33 ± 10	0.18 ± 0.03
Hexane/octanol§	0.0059	33 ± 7	0.06 ± 0.03
Hydroxychloroquine			
Hexane/water	0.53	43 ± 2	0.14 ± 0.01
Octanol/water	3500	−17 ± 2	0.012 ± 0.007
Mixed solvent/water‡	74	23 ± 2	0.114 ± 0.006
Hexane/octanol§	0.00015	59 ± 4	0.13 ± 0.02

* Expressed as a ratio of the molar concentrations in the two phases.
† Standard deviation was calculated from residuals of least squares fit; N = 8–22.
‡ Mixed solvent comprises 5% (v/v) octanol in hexane.
§ Hypothetical partition coefficients, ΔH and ΔS values determined for these solvent combinations were calculated from the experimentally derived partition coefficients, ΔH and ΔS values.

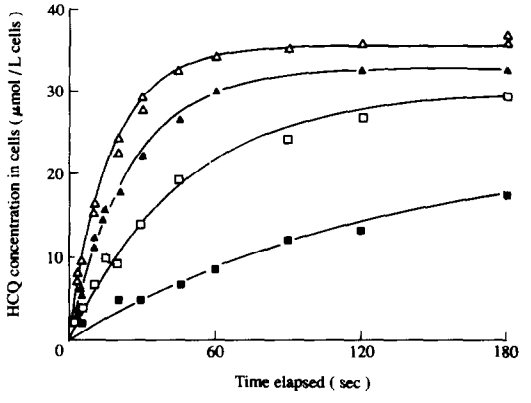

Fig. 2. Time course of HCQ uptake by human erythrocytes determined at 20° at various extracellular pH values: pH 7.17 (■), pH 7.41 (□), pH 7.63 (▲) and pH 7.70 (△). The lines shown are nonlinear regression fits to the data points. Symbols refer to individual measurements.

$$\Delta H_{\text{hexane/octanol}} = \Delta H_{\text{hexane}} - \Delta H_{\text{octanol}} \quad (3)$$

and

$$K_{\text{hexane/octanol}} = K_{\text{hexane/water}}/K_{\text{octanol/water}}. \quad (4)$$

It is evident from Table 1 that substitution of the *N*-ethyl of CQ with the *N*-hydroxyethyl of HCQ substantially influenced the partitioning behaviour.

Kinetic studies. Figure 2 illustrates the time course of HCQ uptake by human erythrocytes at 20° at different extracellular pH values. As previously described for CQ uptake [1], a first-order model was fitted to the HCQ concentrations at various times by nonlinear regression (FUNFIT [7]), and the permeability coefficient for transport into the cell (P_{in}) was calculated from the drug concentration in the cell at equilibrium and the apparent transport rate constant.

HCQ, like CQ, is a basic drug with two sites of ionisation. The pH dependence of uptake (Fig. 2) showed the same trends as observed previously with CQ [1]. With increasing pH, corresponding to a decrease in the fraction of HCQ as the diprotonated species, P_{in} increased sharply. Taking this to indicate a negligible role for the diprotonated species (as previously indicated for CQ [1]), the following equation relates P_{in} to the inward permeability coefficients of the unionised (p°_{in}) and monoprotonated (p^{+}_{in}) HCQ species and the fraction of these species (f° and f^{+} respectively) present in solution at a given extracellular pH [1].

$$P_{\text{in}}/f^{+} = p^{+}_{\text{in}} + p^{\circ}_{\text{in}} f^{\circ}/f^{+}. \quad (5)$$

Equation 5 predicts that a plot of P_{in}/f^{+} versus f°/f^{+} should be linear with slope and intercept equal to p°_{in} and p^{+}_{in} respectively. The f° and f^{+} values for HCQ were calculated at the experimental extracellular pH using the two HCQ pK_a values estimated at the appropriate temperature from the pK_a values determined at 37° and 20° [8.13 ± 0.03 (SD) and 9.62 ± 0.07; and 8.45 ± 0.03 and 10.29 ± 0.014 respectively*), by the method described by Ferrari and Cutler [8] for CQ. Figure 3 shows that plots of P_{in}/f^{+} versus f°/f^{+} for HCQ at different temperatures, as in the case with CQ uptake by human erythrocytes [1], were consistent with the major permeant being the unionised species (nonzero positive slope) with negligible transport of the monocation (zero intercept). Linear regression (using the general straight line) yield intercepts (p^{+}_{in}) which are insignificant compared with the p°_{in} values (equal to the slope of the lines). The regression

* McLachlan AJ, personal communication, cited with permission.

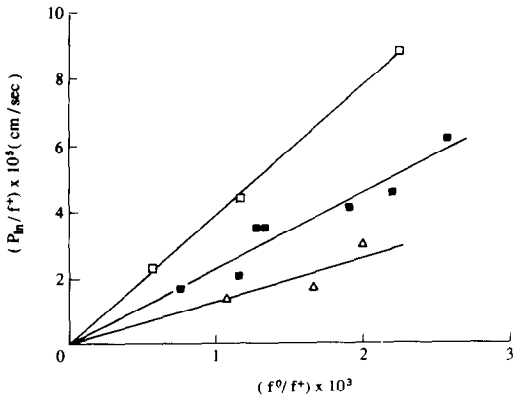

Fig. 3. Plots of P_{in}/f^+ versus f^0/f^+ for HCQ determined at 15° (△), 20° (■) and 25° (□). P_{in} values were estimated by nonlinear regression from individual kinetic runs. The lines shown are linear regression fits to the data points. Symbols refer to individual measurements.

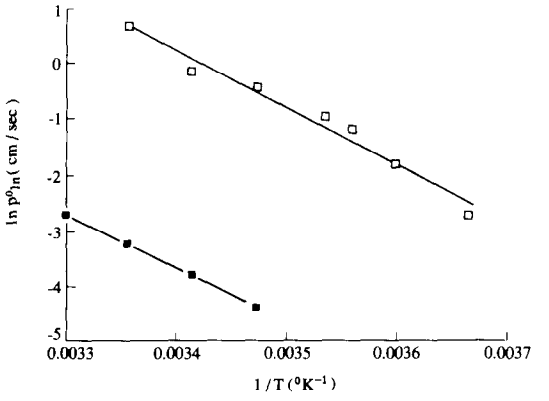

Fig. 4. Arrhenius plots of the temperature dependence of the permeability coefficient across the human erythrocyte membrane of the unionised CQ (□) and HCQ (■) species. These permeability coefficients were estimated by nonlinear regression from individual kinetic runs. The lines shown are linear regression fits to the data points. Symbols refer to individual measurements.

Table 2. Permeability coefficients of CQ and HCQ (P_{in}) and the unionised CQ and HCQ species (p°_{in}) for transport into erythrocytes and the calculated activation energies (E_a) for the transport process

Permeant	P^*_{in}† (cm/sec)	$p^{\circ *}_{in}$ (cm/sec)	E_a (kJ/mol)	r^2
CQ	7.5×10^{-5}	1.9	85 ± 6‡	0.97
HCQ	8.2×10^{-6}	0.039	81 ± 1	1.00

* Determined at 25°.
† Determined at pH 7.40.
‡ Standard deviation was calculated from residuals of least squares fit; N = 7, 4.

lines shown in Fig. 3 have been subsequently constrained to pass through the origin (all r^2 values exceeded 0.98). The p°_{in} values calculated for CQ and HCQ at 25° are listed in Table 2. As a comparison, the apparent P_{in} values obtained at pH 7.40 are also listed. It is clear from this comparison that the p°_{in} values are as much as 5 orders of magnitude higher than the corresponding P_{in} values due to the very low fractions of CQ and HCQ present in the unionised form at physiological pH.

Figure 4 shows plots of $\ln p^\circ_{in}$ values versus $1/T$ for HCQ and CQ. The p°_{in} values for CQ at four temperatures were reported previously [1]; results for CQ for a further three temperatures were obtained in the present study. Using linear regression, reasonable straight line fits of both sets of data were obtained (r^2 greater than 0.97). This temperature dependence of p°_{in} was interpreted in terms of the Arrhenius equation,

$$p^\circ_{in} = Ae^{-E_a/RT} \quad (6)$$

where E_a is the activation energy for permeation of the unionised drug species and A is the frequency factor. According to the absolute rate theory [9], A is linearly dependent on T. In preliminary calculations, where estimates of A and E_a were determined by nonlinear regression (using Equation 6 as the model), equivalent results were obtained when either p°_{in} or p°_{in}/T were assumed to have the exponential form as on the righthand side of Equation 6; in the following, Equation 6 was used in the analysis with A taken to be temperature independent.

The E_a values determined from the slope of the regression lines were similar (Table 2). However, it can be seen from Fig. 4 that the p°_{in} values for CQ were about fifty times higher than the values for HCQ at a given temperature.

DISCUSSION

Interpretation of partitioning data. The ΔH value describing CQ partitioning from water to hexane (37 kJ/mol) does not favour partitioning; the large partition coefficient arises as a result of a large entropy increase (the entropy component, $T\Delta S$, equals 48 kJ/mol at 25°). This is a typical hydrophobic effect and presumably is a consequence of the ordering effect on water structure by the compound. The partitioning behaviour of CQ represents a fine balance between large opposing tendencies; in the case of CQ, the entropic effects dominate. This fine balance is important in understanding the differing partitioning behaviour of CQ and HCQ. The ΔH value for HCQ partitioning from water to hexane (43 kJ/mol) was slightly larger than for CQ (37 kJ/mol) and is in agreement with the greater hydrogen bonding capacity of HCQ in water. The ΔS value for HCQ describing the same process [0.14 kJ/(mol·K)] was slightly lower than the value obtained for CQ [0.16 kJ/(mol·K)] and is consistent with expectations if we attribute a reduction in water structuring to the N-hydroxyethyl group on HCQ. Since the partitioning behaviour is the result of a

fine balance between enthalpic and entropic factors, the net effect of these two relatively small changes is a substantial reduction in the partition coefficient of HCQ (0.53 at 25°) compared with CQ (124 at 25°).

Information on the type of interaction that might be experienced by CQ and HCQ in the hydrophobic environment of a biological membrane may be provided by the partition coefficient into octanol. This solvent provides an essentially hydrophobic environment with a hydrogen bonding capacity and may mimic the polar region of the membrane bilayer which comprises phospholipid ester groups and hydroxyl groups from cholesterol molecules or possibly the polar groups on intrinsic membrane proteins. The ΔH value for CQ partitioning from water to octanol was positive and again indicates that partitioning is not enthalpically driven. However, the enthalpy for transfer for this organic solvent was much less than for hexane. This suggests a large contribution due to hydrogen bonding interactions in octanol. The lower ΔS values observed for drug partitioning from water into this solvent (especially in the case of HCQ; see Table 1) also suggest that the hydrogen bonding difference between water and octanol for these drugs is not as pronounced as for water and hexane. The ΔH values for the partitioning of HCQ from water to octanol were negative, indicating that stronger hydrogen bonding interactions involving the *N*-hydroxyethyl group are to be found in the organic phase than in water. The ΔS values for the same process were very small [0.01 kJ/(mol · *K*)], indicating only a small net change in solvent structuring due to HCQ exchange.

Another means of analysing the thermodynamic parameters is to calculate hypothetical values for drug exchange from octanol to hexane. The ΔH value for CQ determined for this hypothetical process was 33 kJ/mol. Assuming that the hydrocarbon environment in octanol is essentially the same as in hexane, and that the two solvents differ only in the availability of hydrogen bonding sites in octanol, the 33 kJ/mol appears to be attributable to the hydrogen bonding in the hydrocarbon environment in octanol. This suggests that hydrogen bonding of CQ to membrane components is likely to be a significant factor in its transport across membranes. The estimate of the hypothetical ΔH values for HCQ exchange from octanol to hexane (59 kJ/mol) was substantially higher than for CQ exchange between the same solvents; the additional hydrogen bonding in HCQ is equivalent to about 25 kJ/mol in the octanol. This value is within the range of values normally cited for the enthalpy of hydrogen bonds (e.g. 8–42 kJ/mol [10]). A similar interpretation can be made from the high hypothetical ΔS values for these processes; these values correspond to an increase in disorder as both drugs pass from octanol to hexane. This favourable entropy change was considerably higher for HCQ, consistent with a greater ordering effect in octanol arising from additional hydrogen bonding.

Possible effects of unstirred layers. In a previous article [1], the possible role of unstirred layers in determining the uptake rate was investigated by comparing the rate measured by the present technique, with relatively little agitation (except for longer incubations which were vortexed occasionally; see Materials and Methods), with the rate obtained in studies with a well-stirred erythrocyte suspension. No significant differences were observed. The results of the present study support the view that unstirred layers represent at most a minor contribution to the calculated permeability coefficients. First, if the measured permeability coefficients were largely due to unstirred layers, similar values would be expected for CQ and HCQ, in view of their similar size and the fact that the permeability coefficients would in that case be determined by the aqueous diffusion coefficients of the two drugs. The observed values of the permeability coefficients were around fifty times higher for CQ than for HCQ. Second, the activation energies, at around 80 kJ/mol, were much higher than expected for a process controlled by aqueous diffusion (for which typical values of the order of 20 kJ/mol are expected; [4]). Additional evidence favouring an insignificant role for unstirred layers comes from the pH dependence of the permeability coefficients, indicating transport of the unionised species. If unstirred aqueous layers were the major transport barrier, no significant pH dependence would be expected, since aqueous diffusion coefficients are not significantly different for ionised and unionised species.

The conclusion that unstirred layers are not significant in the present study is in agreement with the theoretical considerations of Barry and Diamond [11], which indicate that with an unstirred layer as large as 100 μm (compared with the diameter of the red cell of about 8 μm) an apparent permeability coefficient of approximately 7.5×10^{-5} cm/sec is not significantly different from the true permeability coefficient. The value of 7.5×10^{-5} cm/sec was the highest value observed in the present study (for CQ, the more rapidly transported drug) for the permeability coefficient with respect to total CQ (Table 2). This is the relevant value for comparison purposes, since an aqueous diffusion layer would not discriminate between ionised and unionised molecules, and the reaction rates involved in proton exchange are orders of magnitude greater than observed here. Reasonable values for the thickness of unstirred layers are not obtained easily (in part because they cannot be defined precisely), but the value of 100 μm cited above can be put into perspective by noting that a red cell, with a typical sedimentation rate of 10 mm/hr [12], would travel 200 μm in 1 min, a typical uptake half-time. Even in the absence of any convection in the bulk solution, extensive surface renewal would occur, and it does not appear likely that an unstirred layer of the magnitude assumed above could be maintained for any significant period during a kinetic run.

Interpretation of membrane transport data. The thermodynamic analysis of the CQ and HCQ partitioning behaviour indicates that a substantial difference exists in the hydrogen bonding capacity of the two drugs in water and octanol. Such a difference in hydrogen bonding capacity would be expected to result in a significant difference in the activation energies for overall membrane permeation (E_a in Equation 6) if drug transfer across the

Table 3. Molecular volumes and diffusion coefficients (D_{mem}) for diffusion within the erythrocyte membrane of the unionised CQ (CQ°) and HCQ (HCQ°) species and a selection of small nonelectrolytes

		$D_{mem} \times 10^9$ (cm²/sec)			
		n-Alkane†	Octanol†		5% Octanol† in hexane
Diffusant	Volume* (cm³/mol)	(λ = 4 nm)	(λ = 4 nm)	(λ = 1 nm)	(λ = 1 nm)
CQ°	197	6.5	0.038	0.0095	0.053
HCQ°	201	29	0.0044	0.0011	0.053
n-Hexanol‡	72.9	2.7	0.032		
Glycerol‡	51.4	32	0.023		
Methanol‡	21.7	390	8.2		

* Molecular volume was calculated using data provided by Bondi [13].

† Solvent used to estimate K_{mem}, with corresponding value of λ, used to calculate D_{mem} (Equation 7). Hexane was used as the *n*-alkane in the present study; hexadecane was used in the small nonelectrolyte study.

‡ From Lieb and Stein [4, 5].

membrane/water interface is the predominant barrier to membrane transport. However, the activation energies for CQ and HCQ membrane permeation were almost identical (Table 2). These results are consistent with the conventional view that overall membrane transport of these compounds is rate-limited by transport within the membrane rather than by the rate of permeant transfer across the membrane/water interface.

If the rate-determining barrier in membrane transport is diffusion of the permeant within the hydrocarbon membrane interior, it is expected that K_{mem} in Equation 1 could be estimated adequately by the partition coefficient of the permeant into a hydrocarbon solvent (hexadecane) [4, 5]. In our studies, hexane was used as the hydrocarbon solvent instead of hexadecane since the relatively low melting point of hexadecane (18°) limits the temperature range over which a thermodynamic study can be conducted. From Equation 1, with p°_{in} (the permeability coefficient for the unionised species) in place of P,

$$D_{mem} = p^\circ_{in}\lambda/K_{mem}. \quad (7)$$

If K_{mem} is approximated adequately by the partition coefficient into a hydrocarbon solvent, this equation allows calculation of the diffusion coefficient within the membrane interior, D_{mem}. Following Lieb and Stein [4, 5], we take $\lambda = 4$ nm for the thickness of the hydrocarbon interior of the membrane. The calculated values for D_{mem}, using drug partition coefficients into hexane to estimate K_{mem}, are shown in Table 3, with a selection of values calculated by Lieb and Stein [4, 5]. Clearly, there is a major discrepancy between the values for CQ and HCQ and the values for the smaller nonelectrolytes when size considerations are taken into account; for example, the calculated value of D_{mem} for HCQ is more than ten times the value of the much smaller molecule, *n*-hexanol. This result directly contradicts the steep size dependence of D_{mem} established for the series of small nonelectrolytes [4, 5]. Furthermore, since HCQ and CQ have very similar molecular volumes (Table 3), the calculated values for D_{mem} were expected to be similar. However, the data in Table 3 indicate that D_{mem} for HCQ was four times the value for CQ. If the dependence of membrane transport on size is so extreme that even the small difference in size between CQ and HCQ is significant, it would be expected that HCQ, slightly larger than CQ, would have the smaller D_{mem}, not the larger value as observed.

Many workers have used solvents with some polar character, such as octanol, ether or ethyl acetate, to mimic the membrane interior (e.g. Naccache and Sha'afi [2]). Repeating the calculation of D_{mem}, according to Equation 1, but with octanol/water partition coefficients in place of hexane/water partition coefficients, the values of D_{mem} reported in Table 3 were obtained. When octanol was used as the model solvent for all permeants, major discrepancies were again observed when size considerations are taken into account. D_{mem} for CQ was slightly larger than for the much smaller molecule *n*-hexanol; the value of D_{mem} for CQ was nine times the value for HCQ, in spite of the close similarity in size.

These calculations suggest that a single model solvent may not be adequate to mimic the transport behaviour of all permeants. This is to be expected if the barrier to transport is different for different permeants. The detailed analysis by Lieb and Stein [4, 5] provides a convincing case for the use of a hydrocarbon solvent to model the membrane interior for permeants which are small nonelectrolytes, suggesting that the rate-limiting barrier for these compounds is the hydrocarbon interior of the membrane. A likely alternative barrier for transport is the polar region of the membrane bilayer. The observation that strong hydrogen bonding interactions occur for CQ and HCQ in octanol suggests that hydrogen bonding interactions in the polar regions of the membrane may provide a substantial barrier to transport.

The polar region, comprising glycerol ester groups from phospholipids and hydroxyl groups from cholesterol [14], is not located precisely within the membrane, but, from X-ray and neutron diffraction data [14] on phospholipid bilayers, a reasonable value for total thickness of the polar region appears to be about 1 nm. Assuming this value for λ, the calculated values of D_{mem} (when using partition coefficients into octanol to estimate K_{mem}) are also shown in Table 3. It is now seen that the value of D_{mem} for CQ and HCQ, with octanol as the model solvent and an appropriate value for λ, were somewhat smaller than the values for the small nonelectrolytes, with hexane as the model solvent and λ equal to the thickness of the hydrocarbon interior of the membrane. However, a discrepancy remains in that D_{mem} for CQ and HCQ differed by a factor of nine (note that this is independent of uncertainty about the exact value for λ), whereas a closer agreement was expected on the basis of the close similarity in size.

If it is supposed that D_{mem} is strictly a function of size, so that D_{mem} is approximately the same for CQ and HCQ, it follows from Equation 1 that

$$p^{\circ}_{in,CQ}/p^{\circ}_{in,HCQ} = K_{mem,CQ}/K_{mem,HCQ}. \quad (8)$$

The ratio on the left, in our studies, is essentially independent of temperature, equal to about 50; Equation 8 indicates that a suitable solvent to estimate K_{mem} for the barrier region should have a partition coefficient for CQ about fifty times that for HCQ. Hexane and octanol both fail on this criterion; however, after preliminary studies with a range of solvents with various proportions of octanol and hexane, it was found that a solvent mixture comprising 5% (v/v) octanol in hexane as the organic phase had the required ratio of partition coefficients of 50 (see Table 1). D_{mem} values calculated for CQ and HCQ using 5% octanol in hexane as a model solvent and $\lambda = 1$ nm (Table 3) were roughly two orders of magnitude smaller than D_{mem} calculated for *n*-hexanol (hexane as the model solvent, $\lambda =$ 4 nm). The observation that the CQ and HCQ partition coefficients between water and 5% octanol in hexane adequately approximate K_{mem} is in contrast to evidence provided by Lieb and Stein [4, 5] that the barrier phase for small nonelectrolytes within the erythrocyte membrane can be modelled by the hydrocarbon hexadecane, which mimics the hydrocarbon region of the membrane bilayer (or possibly a solvent that is only slightly more polar than hexadecane that may account for the presence of unsaturated hydrocarbon chains within the hydrocarbon region [5]).

If as seems likely, CQ and HCQ advance through the membrane with their minimum cross-sectional areas presented to the direction of transport, the length of both molecules is such that they could not be fully accommodated within the polar region. The transport "barrier," if associated with the polar region, must then be interpreted as a barrier for only a part of the molecule (presumably the hydrogen bonding regions). These observations also present a potential complication to the interpretation of K_{mem}, since this would need to be understood as a "partition coefficient" for only part of the molecule, again presumably the hydrogen bonding groups. However, the partition coefficients in hydrogen bonding solvents are so much larger than those in hexane (Table 1) that the complications arising from hydrophobic portions of the molecule extending partly into the hydrophobic interior of the membrane are likely to be minor.

Further insight into the transport mechanism follows on analysis of the enthalpies of partitioning and the apparent activation energies of membrane transport. The close agreement between experimentally determined activation energies for CQ and HCQ permeation (Table 2) appears at first sight to support the conventional view. The activation energy appears to be independent of chemical nature, and the agreement in the values for the two compounds can be understood to be due to their similar molecular sizes. However, a number of factors complicate this interpretation. It is important to note that the acid dissociation constants of weak bases are significantly temperature dependent (ΔH values for the ionisation of CQ are 46 and 49 kJ/mol [8]). The values of p°_{in} reported in this study have been corrected for the temperature dependence of ionisation (by using the pK_a values appropriate to each temperature). It is evident from Equation 1 that the temperature dependence of p°_{in} (which the experimental activation energy reflects) may be due to the temperature dependence of K_{mem}, D_{mem} or λ. According to Lieb and Stein [4], membrane thickness varies only slightly with temperature. However, the data of Table 1 indicate that the same cannot be said of K_{mem}. We assume that the temperature dependence of K_{mem} and D_{mem} can be expressed by the equations

$$K_{mem} = e^{-\Delta H_{mem}/RT} e^{\Delta S_{mem}/R} \quad (9)$$

and

$$D_{mem} = D_{\infty} e^{-E_d/RT} \quad (10)$$

where ΔH_{mem} and ΔS_{mem} are the enthalpy and entropy of partitioning into the membrane phase, D_{∞} is a constant and E_d is the activation energy of diffusion within the rate-limiting barrier in the membrane. Combining these equations with Equation 1 gives an equation of the form of Equation 6, with

$$E_a = \Delta H_{mem} + E_d. \quad (11)$$

If the conventional view is correct, the temperature dependence of K_{mem} can be approximated by the temperature dependence of partitioning into organic solvents. Table 1 shows that the enthalpies of partitioning of CQ and HCQ into the hexane-based solvents are large and of the same order of magnitude as the observed activation energies and consequently cannot be neglected when interpreting the thermodynamic data. The E_d values, shown in Table 4, were estimated from Equation 11 (i.e. $E_d = E_a - \Delta H_{mem}$), where ΔH_{mem} is approximated by the enthalpy of partitioning into hexane, octanol and a mixture of 5% octanol in hexane. Although the differences in E_d values between CQ and HCQ were not large for any solvent, the values were closest for

Table 4. Activation energies for the diffusion of CQ and HCQ through the human erythrocyte membrane (E_d), calculated using Equation 11 with estimates of ΔH_{mem} from different organic solvents to mimic the barrier phase

Diffusant	E_a (kJ/mol)	E_d (kJ/mol) Hexane*	Octanol*	5% Octanol in hexane*
CQ	85 ± 6†	48 ± 8	81 ± 11	52 ± 16
HCQ	81 ± 1	38 ± 3	98 ± 3	58 ± 3

* Solvent used to mimic partitioning into membrane.
† Standard deviation was calculated from residuals of least squares fit; N = 7, 4.

the 5% octanol in hexane mixture. If it is accepted that D_{mem} is a function of size, not chemical nature, this result provides further support for the proposal that the 5% octanol in hexane mixture adequately mimics the barrier phase for CQ and HCQ.

Lieb and Stein [4, 5] have provided evidence that basal diffusion of small nonelectrolytes across the human erythrocyte membrane is rate-limited by movement across the hydrocarbon region of the membrane bilayer. The findings of the present study suggest that the transport barrier for the larger molecules CQ and HCQ within the human erythrocyte membrane is best modelled by a solvent with significant polar character (5% octanol in hexane), presumably indicating involvement of the polar region of the membrane bilayer.

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