*Biochimica et Biophysica Acta*, 511 (1978) 152—162
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BBA 78079

# ELECTROPHORETIC DETECTION OF REVERSIBLE CHLORPROMAZINE · HCl BINDING AT THE HUMAN ERYTHROCYTE SURFACE

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(Received January 2nd, 1978)

## Summary

The binding of chlorpromazine · HCl at the human erythrocyte surface has been detected through its effect on cellular electrophoretic mobility. Incubation of erythrocytes (approx. $5 \cdot 10^6$/ml) in 23 $\mu$M chlorpromazine · HCl resulted in a reduction of negative electrophoretic mobility from the control value of $-1.11 \pm 0.01$ $(\mu m \cdot s^{-1})/(V \cdot cm^{-1})$ to $-1.00 \pm 0.02$ $(\mu m \cdot s^{-1})/(V \cdot cm^{-1})$ (pH 7.2, ionic strength 0.155). This mobility change was completely reversed when chlorpromazine · HCl was removed by centrifugal washing. Increasing the drug concentration to 70 $\mu$M did not affect the mobility change, indicating saturation of the electrophoretically detectable drug binding sites over the chlorpromazine · HCl concentration range studied here. The effect of the 23 $\mu$M chlorpromazine · HCl on electrophoretic mobility was also measured in isotonic media of reduced ionic strength. The drug-induced reduction in negative surface charge density was found to be independent of ionic strength over the range 0.155 (Debye length, 0.8 nm) to 0.00310 (Debye length, 5.7 nm).

Fixation of erythrocytes with glutaraldehyde affected neither the normal electrophoretic mobility of discocytes nor the reduced electrophoretic mobility of chlorpromazine · HCl-induced stomatocytes. When these stomatocytes were first fixed with glutaraldehyde, then washed free of chlorpromazine · HCl, they retained the stomatocyte form while regaining a normal control electrophoretic mobility. Conversely, when discocytes fixed in that form were treated with chlorpromazine · HCl, they showed the same mobility change as did fixed or unfixed stomatocytes. The drug-induced mobility change is therefore independent of the shape change, but reflects a contribution to cellular surface charge density from the membrane-bound chlorpromazine · HCl molecules. From the charge reduction, it is estimated that about $10^6$ chlorpromazine · HCl molecules are bound at the electrokinetic cell surface and occupy approximately 0.4% of the total surface area.

## Introduction

The tranquilizer chlorpromazine · HCl is known to induce a change in erythrocyte morphology from the normal biconcave disk form (discocyte) to a cup-shaped form (stomatocyte) [1,2]. The degree of morphological transformation is correlated with the concentration of chlorpromazine · HCl in the suspending medium and the net cellular uptake of drug [2]. Stomatocytes formed at relatively low chlorpromazine · HCl concentrations have been demonstrated to have less sensitivity to osmotic hemolysis than normal discocytes [3]. At high chlorpromazine · HCl concentrations, stomatocytes attain a spherical shape and display an increased sensitivity to osmotic lysis, as compared with normal discocytes [2,3]. Five transitional stages between the discocyte and the most advanced sphero-stomatocyte form have been described [2]. As long as the transformation has not proceeded to a final spherical stage, the morphological transition from discocyte to stomatocyte can be completely reversed by removing chlorpromazine · HCl from the medium [2].

The mechanism by which chlorpromazine · HCl leads to changes in erythrocyte shape and osmotic fragility is not yet well established. From studies on volume changes of ghosts exposed to chlorpromazine · HCl, Seeman and associates [4,5] concluded that this drug led to a 1.5—3% membrane expansion. However, using an isotopic technique to quantitate drug binding, they found that the bulk volume occupied by membrane-bound chlorpromazine · HCl could not account for the total calculated increase in membrane area [6]. These workers also demonstrated that the binding of chlorpromazine · HCl to ghost membranes is an exothermic process, and they proposed that this is consistent with the site of drug binding being "at the immediate interface of the membrane-aqueous region" [6]. More recently, Sheetz and Singer [7] have hypothesized that chlorpromazine · HCl binding occurs preferentially at the inner (cytoplasmic) membrane surface. The basis for this hypothesis was the finding that the chemical derivative chlorpromazine methiodide, in which the amine functional group of chlorpromazine is quaternized and thus bears a permanent positive charge, produces crenated erythrocytes forms (echinocytes) rather than stomatocytes. They proposed that this cationic chlorpromazine derivative cannot diffuse across the membrane and gain access to the cytoplasmic membrane-water interface, and thus preferentially localizes at the outer membrane surface. In contrast, chlorpromazine · HCl contains a tertiary amine which exists in a partially uncharged state at physiological pH, thereby facilitating molecular diffusion across the membrane and intercalation of the drug at the interior membrane-water interface. Sheetz and Singer [7] propose that expansion of the exterior half of the membrane bilayer resulting from chlorpromazine methiodide binding at the outer surface of the plasma membrane produces the echinocyte morphology. In their model, chlorpromazine · HCl binding at the inner surface expands the cytoplasmic half of the membrane bilayer, and thereby produces the stomatocytic morphology.

In the studies reported here, the microscope method of cell electrophoresis (microelectrophoresis) has been used to quantitate chlorpromazine · HCl binding at the erythrocyte outer membrane surface over a wide range of solution ionic strengths. Our results demonstrate that stomatocytes formed in the pres-

ence of chlorpromazine · HCl have a reduced net negative surface charge density. In addition, we have explored the interrelationships between the chlorpromazine · HCl-induced in erythrocyte morphology, and the effect of this drug on cellular surface charge. For this purpose, glutaraldehyde was used to fix erythrocytes in both non-deformable discocyte and in stomatocyte forms, thereby preventing the changes in morphology that accompany the addition or removal of chlorpromazine · HCl from unfixed, deformable cells. Our microelectrophoretic studies on cells treated by these procedures show that the reduction in surface charge resulting from exposure to chlorpromazine · HCl is independent of the drug-induced shape change, but results only from a contribution by membrane-bound chlorpromazine · HCl molecules to cellular surface charge density.

## Methods

*Preparation of erythrocytes.* Fresh human blood from the same normal donor was used in all experiments. Several ml of blood were drawn quickly by venipuncture into a glass syringe without added anticoagulant, and immediately dispensed into approximately 30 vols. of Dulbecco phosphate-buffered saline (Grand Island Biological Co., Grand Island, New York). The erythrocytes were pelleted by centrifugation at 300 $\times g$ for 10 min, and the supernatant and buffy coat removed by aspiration. The cells were resuspended in phosphate-buffered saline, washed once under the same centrifugal conditions and stored on ice as a concentrated stock suspension. They were then treated as described below and studied experimentally within a few hours following preparation of the stock cell suspension.

*Treatment with chlorpromazine · HCl.* Chlorpromazine · HCl was obtained from Smith, Kline and French (Philadelphia, Pa.). The purity of this drug preparation was examined by means of gas-liquid chromatography using a fluorescence detector for analysis of sulfoxide and sulfone oxidation products (measurements kindly performed by Mr. David Takahashi of the Federal Drug Administration, San Francisco, and Professor Neal Castagnoli of the Department of Pharmaceutical Chemistry, University of California Medical Center, San Francisco). This analysis indicated that the actual chlorpromazine · HCl concentration was within 2% of the nominal value stated by the manufacturer, and that the concentrations of sulfoxide and sulfone contaminants were less than 0.1% of the total.

The washed erythrocytes were suspended in room temperature phosphate-buffered saline containing either 23 or 70 $\mu$M chlorpromazine · HCl at a cell concentration of approx. $5 \cdot 10^6$/ml. As verified in the phase microscope for both of these drug concentrations, a 100% conversion of discocytes to stomatocyte form occured within approximately 5 min.

*Glutaraldehyde fixation.* Erythrocytes were fixed for 20 min at room temperature with 0.25% (w/v) glutaraldehyde prepared in phosphate-buffered saline from an 8% solution (Polyscience, Englewood, N.J.). Sufficient NaCl was added to increase the osmolality of the fixative solution to 355 mosM/kg, under which condition it has been shown that no change in erythrocyte volume occurs during the fixation process [8]. After the fixation was completed, glu-

taraldehyde was removed by centrifugal washing, and the cells were resuspended in phosphate-buffered saline. When cells were glutaraldehyde-fixed following incubation with chlorpromazine · HCl, the same concentration of chlorpromazine · HCl was added to both the fixative solution and to the phosphate-buffered saline used as a resuspending medium.

*Microelectrophoresis.* Cell electrophoresis was carried out using a Zeiss cytopherometer (Carl Zeiss, New York) with a laterally oriented rectangular chamber thermostated at 25 ± 0.1 °C, and a $Zn/ZnSO_4$ electrode assembly [9]. Electrophoretic velocities of individual erythrocytes in an applied electric field ($1.3—3.5\ V \cdot cm^{-1}$) were recorded at both the front and back stationary levels [10]. For each erythrocyte, mobility measurements were made with both polarities of the applied electric field in order to cancel out the influence of any mechanical fluid drift within the microelectrophoresis chamber.

In order to determine the influence of solution ionic strength, mobilities were measured for the 23 μM chlorpromazine · HCl preparation in phosphate-buffered saline suspending media having a wide range of ionic strengths: 0.155, 0.031, 0.0155, 0.00775, and 0.00310. Each of these suspending solutions was made isotonic (290 mosM/kg) by adding sucrose prior to the addition of erythrocytes. The pH of all cell suspensions was adjusted to 7.2 ± 0.1 by the dropwise addition of either NaOH or HCl at the same ionic strength as the phosphate-buffered saline suspending medium.

## Results

As a guide to following the various sequential erythrocyte treatments described below, these are summarized schematically in Fig. 1, and the results are presented in tabular form in Table I.

*Unfixed erythrocytes treated with chlorpromazine · HCl.* The majority of our microelectrophoretic studies were carried out with stomatocytes formed in the presence of 23 μM chlorpromazine · HCl in a phosphate-buffered saline solution of ionic strength 0.155 (pH 7.2). At the cell concentration used during microelectrophoresis, (approx. $5 \cdot 10^6$/ml), this drug concentration was found to produce a nearly immediate, and completely reversible, morphological transformation from the discocyte to the stomatocyte form. In addition, parallel studies on cellular size, shape, and deformability using the technique of "resistive pulse spectroscopy" [8] demonstrated that a chlorpromazine · HCl concentration of 23 μM led to a maximal protection against erythrocyte swelling and lysis in hypotonic NaCl solutions (Mel, H.C. and Yee, J.P., unpublished).

As summarized in Table I, the addition of 23 μM chlorpromazine · HCl to a suspension of human erythrocytes reduced the negative cellular electrophoretic mobility by 9.9%. In comparison with the mobility of control erythrocytes, this reduction was highly significant ($P < 0.001$). The chlorpromazine · HCl-induced decrease in mobility was completely reversed upon removal of the drug by centrifugal washing. Subsequent reintroduction of 23 μM chlorpromazine · HCl into the washed cell suspension once again led to a 9.0% decrease in mobility relative to untreated control erythrocytes.

A test was also made to determine any possible effect on erythrocyte surface charge of additives (antioxidant and preservative materials) contained in the

TABLE I

CHLORPROMAZINE · HCl-INDUCED CHANGES IN HUMAN ERYTHROCYTE ELECTROPHORETIC MOBILITY

Electrophoretic mobility values represent the mean ± 1 S.D. of measurements conducted on the number of individual cells stated in parentheses. Mobilities were measured in a phosphate-buffered saline suspending medium (ionic strength 0.155, pH 7.2). The percent alteration in negative mobility of treated erythrocytes is expressed relative to unfixed controls. Values of $P$ were determined by Student's $t$-test; $P > 0.01$ was considered not to be significant (n.s.).

| Erythrocyte treatment | Negative electrophoretic mobility $((\mu m \cdot s^{-1})/(V \cdot cm^{-1}))$ | Percent alteration | $P$ |
|---|---|---|---|
| Unfixed control | 1.11 ± 0.01 (135) | | |
| Unfixed control + 'additives' * | 1.13 ± 0.03 (40) | +1.8 | n.s. |
| Unfixed + 23 $\mu$M chlorpromazine · HCl | 1.00 ± 0.02 (100) | −9.9 | <0.001 |
| Unfixed + 70 $\mu$M chlorpromazine · HCl | 1.01 ± 0.02 (40) | −9.0 | <0.001 |
| Unfixed + 23 $\mu$M chlorpromazine · HCl + wash | 1.13 ± 0.01 (50) | +1.8 | n.s. |
| Unfixed + 23 $\mu$M chlorpromazine · HCl + wash + 23 $\mu$M chlorpromazine · HCl | 1.01 ± 0.01 (50) | −9.0 | <0.001 |
| Gltaraldehyde-fixed | 1.13 ± 0.01 (100) | +1.8 | n.s. |
| Glutaraldehyde + 23 $\mu$M chlorpromazine · HCl | 1.00 ± 0.01 (50) | −9.9 | <0.001 |
| Unfixed + 23 $\mu$M chlorpromazine · HCl + glutaraldehyde | 1.00 ± 0.01 (50) | −9.9 | <0.001 |
| Unfixed + 23 $\mu$M chlorpromazine · HCl + glutaraldehyde + wash | 1.15 ± 0.02 (110) | +3.6 | n.s. |
| Unfixed + 23 $\mu$M chlorpromazine · HCl + glutaraldehyde + wash + 23 $\mu$M chlorpromazine · HCl | 1.01 ± 0.02 (50) | −9.0 | <0.001 |

* 'Additives' refers to antioxidant and preservative materials added to the commercial chlorpromazine · HCl preparation. The nature and concentrations of these additives are described under Results.

commercial 25 mg/ml chlorpromazine · HCl preparation. These additives include 2 mg/ml ascorbic acid, 1 mg/ml sodium bisulfite, 1mg/ml sodium sulfite, 1 mg/ml NaCl, and 2% benzyl alcohol. A stock solution of these chemicals was prepared and diluted with 3058 vols. of phosphate-buffered saline solution, as required, to reduce the chlorpromazine · HCl concentration in the commercial preparation to 23 $\mu$M. As shown in Table I, incubation of erythrocytes in this additive-containing solution (without chlorpromazine · HCl) led to no measurable effect on erythrocyte electrophoretic mobility.

*Influence of chlorpromazine · HCl concentration.* Electrophoretic mobility measurements were also carried out with erythrocytes exposed to 70 $\mu$M chlorpromazine · HCl. This drug concentration level was three times higher than that used in the other experiments reported here, but it did not lead to a greater reduction in electrophoretic mobility (Table I). It therefore appears that incubation of erythrocytes with as little as 23 $\mu$M chlorpromazine · HCl results in saturation of the electrophoretically detectable drug binding sites at the outer membrane surface.

*Ionic strength variation.* Electrophoretic measurements made on unfixed erythrocytes exposed to 23 $\mu$M chlorpromazine · HCl solutions at various reduced ionic strengths are given in Table II. The mobilities measured at 25° C and pH 7.2, for control erythrocytes and for the erythrocytes exposed to chlorpromazine · HCl, are plotted in Fig. 2 as a function of the Debye length,

TABLE II

CHLORPROMAZINE · HCl INFLUENCE ON ELECTROPHORETIC MOBILITY AND SURFACE CHARGE DENSITY AT LOW IONIC STRENGTH

Negative electrophoretic mobilities ($\mu_E$) are the mean ± 1 S.E. determined for 20 individual unfixed erythrocytes at each of the indicated values of ionic strength (pH 7.2). The calculated Debye length ($1/\kappa$) is given for each value of ionic strength. Net negative surface charge densities ($\sigma_E$) are the mean ± 1 S.E. calculated from mobilities using the Gouy-Chapman relation [11,12]. Erythrocyte treatment: A, control; B, 23 $\mu$M chlorpromazine · HCl.

| Erythrocyte treatment | Ionic strength | $1/\kappa$ (nm) | $\mu_E$ ($(\mu m \cdot s^{-1})/(V \cdot cm^{-1})$) | $\sigma_E$ ($10^{-3}$ C · $m^{-2}$) | $\lvert\Delta\sigma_E\rvert$ ($10^{-4}$ C · $m^{-2}$) |
|---|---|---|---|---|---|
| A | 0.155 | 0.81 | 1.09 ± 0.03 | 12.00 ± 0.33 | 8.8 ± 4.0 |
| B | 0.155 | 0.81 | 1.01 ± 0.02 | 11.12 ± 0.22 | |
| A | 0.0310 | 1.82 | 1.72 ± 0.03 | 8.47 ± 0.15 | 5.4 ± 2.1 * |
| B | 0.0310 | 1.82 | 1.61 ± 0.03 | 7.93 ± 0.15 | |
| A | 0.0155 | 2.57 | 2.31 ± 0.06 | 8.04 ± 0.21 | 11.8 ± 2.5 * |
| B | 0.0155 | 2.57 | 1.97 ± 0.04 | 6.86 ± 0.14 | |
| A | 0.00775 | 3.63 | 2.46 ± 0.07 | 6.60 ± 0.17 | 7.8 ± 2.4 * |
| B | 0.00779 | 3.62 | 2.14 ± 0.07 | 5.28 ± 0.17 | |
| A | 0.00310 | 5.74 | 2.80 ± 0.10 ** | 4.36 ± 0.16 | 5.0 ± 1.8 * |
| B | 0.00314 | 5.70 | 2.46 ± 0.06 *** | 3.86 ± 0.09 | |

* Not significantly different from $\lvert\Delta\sigma_E\rvert$ measured at ionic strength 0.155 ($P > 0.4$).

** When resuspended in phosphate-buffered saline at ionic strength 0.155, the negative mobility of this sample was 1.12 ± 0.02; this value does not differ significantly from that for control erythrocytes which were not previously exposed to low ionic strength ($P < 0.001$).

*** When resuspended in a phosphate-buffered saline solution with ionic strength 0.155 and containing 23 $\mu$M chlorpromazine · HCl, the negative mobility of this sample was 1.01 ± 0.02; this value is identical to the mobility of drug-treated erythrocytes not previously exposed to low ionic strength ($P < 0.001$).

$1/\kappa$ (defined as the reciprocal of the Debye-Hückel constant, $\kappa$ [11]). The parameter $1/\kappa$ increases proportionately to the reciprocal square root of the ionic strength of the suspending medium, and is a measure of the thickness of the ionic double layer associated with fixed charges at the membrane surface [11]. As $1/\kappa$ increases (ionic strength decreases), mobility measurements reflect a contribution from membrane-bound charged groups lying at greater depths relative to the hydrodynamic surface of shear surrounding the cell. It is clear from Fig. 2 that the magnitude of the electrophoretic mobility progressively increases as the Debye length increases, although the positive slope of the curve is less for values of $1/\kappa \geq 2.5$ nm *. At all values of $1/\kappa$ in the range 0.8 to 5.7 nm, the erythrocytes exposed to 23 $\mu$M chlorpromazine · HCl were found to have a reduced mobility relative to control erythrocytes.

In order to test whether irreversible membrane damage was incurred upon exposure to low ionic strength media, erythrocytes incubated in a phosphate-buffered saline solution of ionic strength 0.00310 were resuspended in phosphate-buffered saline at ionic strength 0.155 and their mobilities measured.

* We have no explanation for the apparent biphasic nature of the slope of Fig. 2, but it may lie in the approximate relationship: $\mu_E \propto \epsilon \cdot \zeta/\eta$, where $\epsilon$ is the dielectric constant, $\zeta$ is the zeta potential, and $\eta$ is viscosity [12,13]. It is not unreasonable to assume that, in the transition region between bulk aqueous and near-membrane phases, a decrease in $\epsilon$ and an increase in $\eta$ occur at about the same position relative to the surface of shear. Alternatively, the slope discontinuity could be interpreted in terms of a zeta potential effect such as the unmasking of a layer of positive charge at low strength.

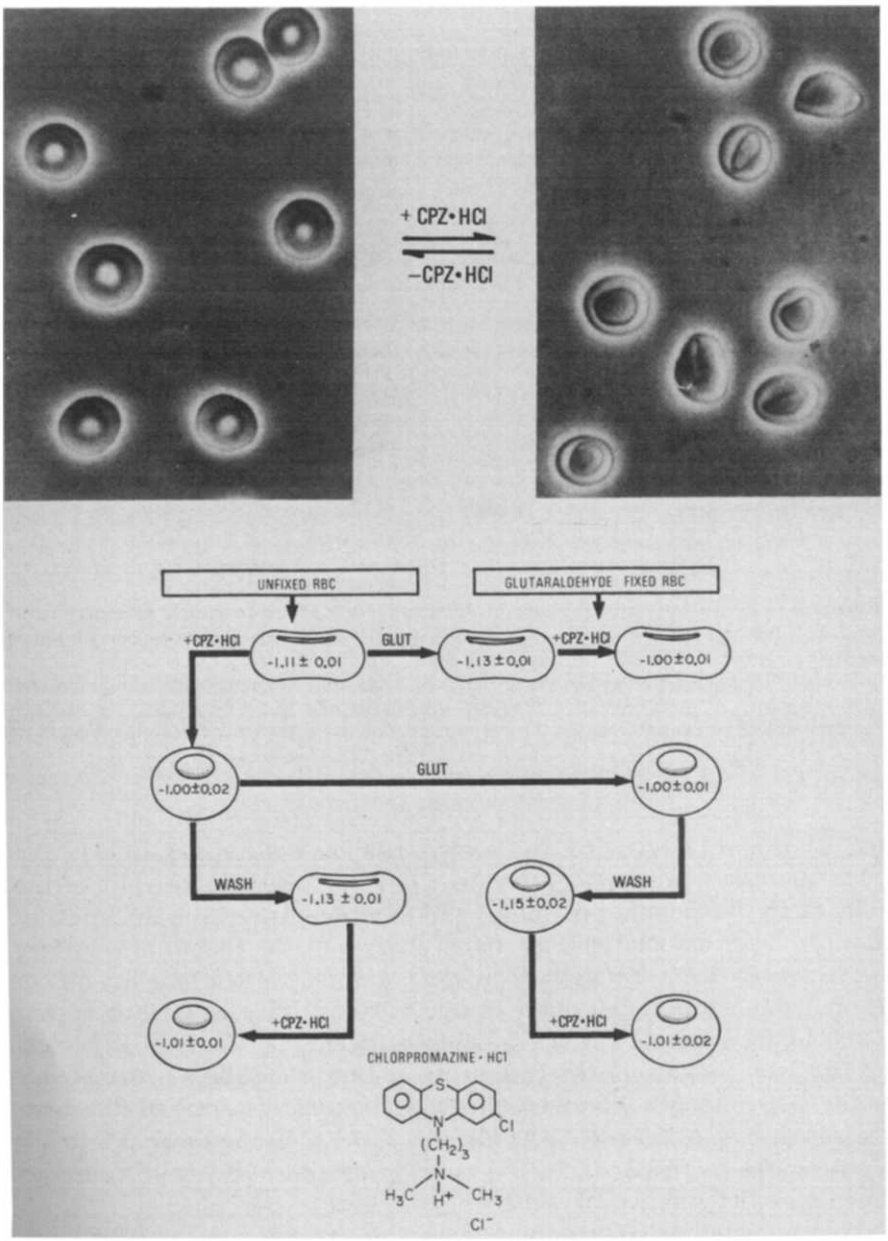


Fig. 1. Schematic diagram showing changes in erythrocyte (RBC) shape and electrophoretic mobility (± 1 S.E.) resulting from various sequential chlorpromazine · HCl (CPZ · HCl, 23 μM) and fixation treatments as indicated. The abbreviation GLUT refers to isovolume fixation with 0.25% glutaraldehyde as described in Methods.

Both in the presence and absence of chlorpromazine · HCl, the usual electrophoretic mobilities at ionic strength 0.155 were observed, thus demonstrating the complete reversibility of the cellular electrokinetic properties determined at low ionic strength (see Table II).

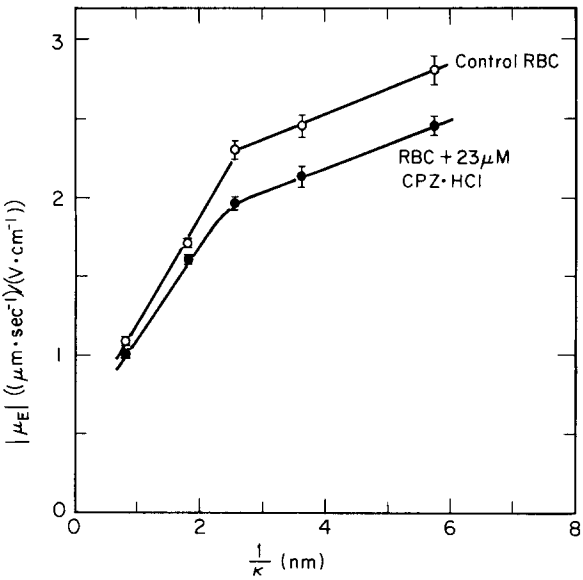


Fig. 2. Mobilities of native (open circles) and chlorpromazine · HCl-treated erythrocytes (closed circles) at different ionic strengths (pH 7.2). The magnitude of the negative electrophoretic mobility ($|\mu_E|$) measured at 25°C is plotted as a function of the Debye length $1/\kappa$. Error limits on the experimental data represent ± 1 S.E. of the mean.

Using the mobility data presented in Fig. 2, the contribution of 23 μM chlorpromazine · HCl to the cellular surface charge density ($\sigma_E$) was estimated from the Gouy-Chapman equation [11,12]. Lacking reliable data on the solution viscosity and dielectric constant in the close vicinity of the cell membrane (see footnote and ref. 11), values of these parameters appropriate to the bulk aqueous phase were used in calculating $\sigma_E$. It is reasonable to assume that the low concentrations of chlorpromazine · HCl used in our studies have little influence on the viscosity and dieletric constant. Therefore, this calculation should still provide reliable information on the relative magnitude of the drug-induced change in $\sigma_E$ occurring at each value of solution ionic strength. A precise calculation of the absolute magnitude of $\Delta\sigma_E$ as a function of $1/\kappa$ must await further knowledge of the physical chemistry of electrolyte solutions close to the membrane surface.

From the $\sigma_E$ values presented in Table II, the binding of chlorpromazine · HCl to the membrane surface is seen, within experimental error, to lead to the same reduction in surface charge density ($\Delta\sigma_E$) for values of the Debye length ranging from 0.8 nm (ionic strength, 0.155) to 5.7 nm (ionic strength, 0.00310). The drug-induced change in surface charge density is therefore predominantly attributable to chlorpromazine · HCl groups lying within 0.8 nm of the hydrodynamic surface of shear.

*Glutaraldehyde-fixed erythrocytes.* In order to determine whether the shape change induced by chlorpromazine · HCl influenced the erythrocyte electrophoretic mobility, several experiments were conducted with glutaraldehyde-fixed cells. As shown in Table I and Fig. 1, fixation with glutaraldehyde had no significant effect on the cellular electrophoretic mobility. When incubated with

23 μM chlorpromazine · HCl, glutaraldehyde-fixed erythrocytes retained a discocyte morphology but exhibited a reduced electrophoretic mobility comparable to that observed for unfixed erythrocytes in the presence of chlorpromazine · HCl. This result indicates that the drug-induced decrease in erythrocyte electrophoretic mobility is not dependent upon the morphological transition to a stomatocyte form.

To elucidate this point further, a second series of experiments was conducted with glutaraldehyde-fixed stomatocytes, the results of which are also summarized in Table I and Fig. 1. Erythrocytes were first converted to stomatocytes with 23 μM chlorpromazine · HCl, and then fixed with glutaraldehyde in the presence of chlorpromazine · HCl. These cells were found to have a decreased electrophoretic mobility identical to that of unfixed stomatocytes in 23 μM chlorpromazine · HCl. When the glutaraldehyde-fixed stomatocytes were washed free of chlorpromazine · HCl, the electrophoretic mobility increased to a value comparable to that of control erythrocytes. However, in contrast to unfixed erythrocytes, the stomatocyte morphology was retained by the glutaraldehyde-fixed cells following removal of chlorpromazine · HCl. This finding thus provided further evidence that the discocyte- to stomatocyte-shape change induced by chlorpromazine · HCl does not, per se, lead to an altered erythrocyte electrophoretic mobility.

The possibility that glutaraldehyde fixation might itself alter cell surface binding sites for chlorpromazine · HCl is ruled out by an additional experiment; the readdition of 23 μM chlorpromazine · HCl to washed, glutaraldehyde-fixed stomatocytes induced a 9.0% decrease in electrophoretic mobility (Table I, Fig. 1) comparable to the decrease seen for the unfixed cells. This conclusion is also supported by the chlorpromazine · HCl-induced reduction in electrophoretic mobility of glutaraldehyde-fixed discocytes, discussed above in regard to the possible interrelation between charge and morphology.

## Discussion

The microelectrophoretic data presented here indicate that chlorpromazine · HCl binding at the outer membrane surface can be detected as a statistically significant 9—10% decrease in electrophoretic mobility. The fact that this alteration in mobility can be achieved with erythrocytes maintained at constant volume in the discocyte form by glutaraldehyde fixation demonstrates that the drug effect on surface charge is independent of its effect on cell morphology. This conclusion is reinforced by the observation that electrophoretically detectable drug binding can be completely reversed under conditions where the morphological reversion from stomatocyte to discocyte form is blocked by glutaraldehyde fixation. Our results therefore indicate that chlorpromazine · HCl alteration of electrophoretic mobility reflects a reversible interfacial adsorption of drug with a resultant contribution to cellular surface charge density, undoubtedly from the protonated tertiary amine group.

At physiological ionic strength (0.155), the associated Debye length is 0.8 nm so that the chlorpromazine · HCl amine group which would influence the electrophoretic mobility must lie principally within this distance from the hydrodynamic surface of shear. No additional drug-induced change in surface

charge density was detected as the solution ionic strength was decreased from 0.155 to 0.00310, with a corresponding increase in the Debye length from 0.8 to 5.7 nm. This finding indicates that all of the electrophoretically detectable chlorpromazine · HCl tertiary amine groups were, in fact, revealed by mobility measurements at ionic strength 0.155.

From the electrophoresis data presented here, a calculation can be made of the number of chlorpromazine · HCl binding sites at the human erythrocyte periphery. In making this calculation, it will be assumed that the tertiary amine of chlorpromazine · HCl is fully charged. This assumption is reasonable since the $pK_a$ of this functional group appears to be in the range 9.2—9.3 [13,14], and, consequently, this group would be approx. 99% protonated at the pH 7.2 maintained during electrophoretic measurements. It will further be assumed that the electrophoretically detectable surface binding sites for chlorpromazine · HCl were fully occupied, consistent with our experimental observations comparing the effects of 23 and 70 $\mu$M chlorpromazine · HCl. From the measured electrophoretic mobilities and the Gouy-Chapman equation relating mobility and surface charge density [11,12], it can be calculated that the negative surface charge densities of native and chlorpromazine · HCl-treated erythrocytes are 0.0122 and 0.0110 $C \cdot m^{-2}$, respectively. The difference in surface charge densities of these cells thus corresponds to 7620 elementary electron charge units per $\mu m^2$ of cellular surface area. If the human erythrocyte surface area is taken to be 134 $\mu m^2$ [4], and if it is assumed that reduction of the negative surface charge density by one electron charge unit results from the binding of a single chlorpromazine · HCl molecule, then the number of electrophoretically detectable chlorpromazine · HCl binding sites at the human erythrocyte surface is $1 \cdot 10^6$ per cell. It is of interest to compare this value for the surface binding of chlorpromazine · HCl with the total number of chlorpromazine · HCl molecules present in the entire cell membrane when the drug binding has reached a saturation level (as in the present experiments). According to Kwant and Seeman [6], this saturation binding is $6.6 \cdot 10^8$ mol of chlorpromazine · HCl per mg of membrane. Assuming the membrane weight to be approximately $10^{-9}$ mg [15], then the saturation binding of chlorpromazine · HCl is $4 \cdot 10^7$ molecules per membrane. The total quantity of chlorpromazine · HCl bound per membrane thus appears to be about forty times as great as the amount of electrophoretically detectable chlorpromazine · HCl bound at the exterior membrane surface.

One additional calculation that can be made from the present data is the fraction of membrane surface area occupied by electrophoretically detectable chlorpromazine · HCl binding sites. If the chlorpromazine · HCl molecule is considered to be spherical with a van der Waals molecular volume of 186 $cm^3 \cdot mol^{-1}$ [6], then the surface area occupied per bound chlorpromazine · HCl molecule is $5.52 \cdot 10^{-7}$ $\mu m^2$. Using the above estimate that 7620 molecules of chlorpromazine · HCl are bound per $\mu m^2$ of membrane surface, approximately 0.4% of the human erythrocyte surface area is occupied by chlorpromazine · HCl molecules under these saturation conditions.

Results presented here demonstrate the applicability of cell electrophoresis for analysis of the binding of phenothiazine derivatives at the erythrocyte surface. In extending these studies, it will be of interest to determine the effect on

cellular surface charge of phenothiazine derivatives that induce echinocyte transformation, as compared with the effect of those derivatives that induce transformation to a stomatocyte form. The former group of compounds include both cationic and anionic forms of phenothiazine, whereas the latter group of phenothiazine derivatives are exclusively cationic compounds [1]. By determining phenothiazine-induced changes in surface charge densities of inside-out and right-side-out erythrocyte ghosts [16], it should also be possible to analyze the relative binding of these drugs at the inner and outer aspects of the membrane. Such studies may have utility in testing the hypothesis of Sheetz and Singer [7] that crenating agents bind preferentially at the outer membrane surface, while stomatocyte-inducing agents bind preferentially at the inner surface.

## Acknowledgments

We thank Professor G.V.F. Seaman for helpful suggestions regarding this work. Research support was received from U.S. Department of Energy contract W-7405-eng-48 with the Lawrence Berkeley Laboratory, from Public Health Service Research Grant CA15184 awarded by the National Cancer Institute, and by National Institutes of Health Biophysics Training Grant GM00829.

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