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DRUG-INDUCED SHAPE CHANGE IN ERYTHROCYTES CORRELATES WITH MEMBRANE POTENTIAL CHANGE AND IS INDEPENDENT OF GLYCOCALYX CHARGE

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Abstract—The influence of the anionic drugs indomethacin, barbitone, salicylate and the cationic drugs chlorpromazine and tetracaine on the morphology of human erythrocytes suspended in solutions of different chloride concentration (thus altering cell membrane potential) and constant osmolality, has been examined. As expected, the anionic and cationic drugs produced echinocytes and stomatocytes respectively in 145 mM NaCl. The cationic drugs induced fewer stomatocytes in 60 mM chloride than in 145 mM chloride at 37°. Tetracaine induced echinocytes in 60 mM chloride at 20°. Indomethacin and barbitone produced echinocytes in 145 mM chloride and stomatocytes in 60 mM chloride. Salicylate no longer produced echinocytes when the chloride concentration was reduced. Cells exposed to salicylate in 60 mM chloride were less cupped than the control cells. We suggest that the distribution of the charged form of the drug across the membrane is in equilibrium with the distribution of chloride ions. Changes in the intracellular drug concentration when the extracellular chloride is varied could then account for the observed shape changes in a manner which is consistent with the bilayer couple hypothesis for drug–membrane interactions.

Cell glycocalyx charge depletion did not affect the shape induced by either the cationic or anionic drugs. The result suggests that any sialic acid dependent electric potential at the bilayer surface is small compared to the cell's zeta potential.

A large number of chemically unrelated compounds are capable of altering the shape of normal erythrocytes. It has been observed that the amphipathic drugs that change the biconcave disc shape into a cupped shape are exclusively cationic whereas erythrocyte crenators (echinocyte formers) are, as a rule, anionic, non-ionized or neutral compounds [1]. Sheetz and Singer [2] suggested that the effect of drugs on erythrocyte morphology arose from differential uptake of drug in the two monolayers of the bilayer. They proposed that cationic drugs interacted preferentially with the cytoplasmic face of the membrane bilayer, because of its content of negatively charged lipid head groups, rather than the uncharged, or comparatively low charged, outer face of the bilayer. This preferential interaction would expand the inner compared with the outer monolayer to give a "bilayer couple" which would produce a cup shape. Conversely, repulsion of anionic drugs from the negatively charged inner monolayer would lead to preferred expansion of the outer monolayer to give echinocytic cells.

With the exception of a brief comment by Deuticke [1], previous reports have dealt with the shape change in erythrocytes exposed to drugs in saline solutions in the range 130–150 mM [2, 3]. The erythrocyte membrane potential, E, is given (in mV) at 37° with reasonable accuracy [4] by the Nernst equation

$$E = 61.5 \log_{10}(r) \quad (1)$$

where r is the ratio of activities of intracellular to extracellular chloride. In the present study we examine the effect, on drug induced morphological change, of reducing the extracellular chloride concentration significantly while maintaining the ionic strength and the osmolality of the suspending solution constant.

Sheetz and Singer [2] found that sialic acid depletion of the erythrocyte glycocalyx did not detectably change the response of the cell shape to chlorpromazine at 37°. Recently we have shown [5–8] that the fragmentation pattern of erythrocytes at the denaturation temperature (49.5°) of the cytoskeletal protein, spectrin, is a much more sensitive indicator of the influence of morphogens than is the erythrocyte morphology at 37°. Here we study the fragmentation pattern of heated erythrocytes to investigate if this sensitive system can detect any effect of glycocalyx charge (and related surface potential) on the morphogenic effects of a number of anionic and cationic drugs.

MATERIALS AND METHODS

Preparation of cell suspending media. Solutions of the cationic drugs chlorpromazine hydrochloride and tetracaine hydrochloride (Sigma Ltd.) and the anionic drugs indomethacin (Sigma Ltd.) and barbitone (BDH) were prepared separately in media of different salt composition with 5 mM Hepes (hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, Sigma Ltd.). The pH was adjusted by dropwise addition of 10% w/v NaOH.

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The membrane was depolarized, as required, by decreasing the extracellular chloride concentration $[Cl_o^-]$. The cell suspending media were designed to maintain constant cell volume and constant intracellular chloride concentration $[Cl_i^-]$ when $[Cl_o^-]$ was reduced. Chloride and hydrogen ions are in Donnan equilibrium across the erythrocyte membrane [4]. Consequently any reduction of $[Cl_o^-]$ at constant extracellular pH and osmolarity would alter the intracellular pH and haemoglobin charge. Chloride and water would then leave the cell. In order to avoid such cell volume change in the present work the extracellular hydrogen ion concentration $[H_o^+]$ was adjusted when $[Cl_o^-]$ was decreased so that the product $[H_o^+]$. $[Cl_o^-]$ remained constant. The cell suspending phases were buffered at pH 7.24, 7.11, 6.94 and 6.72 when $[Cl_o^-]$ was 120, 90, 60 and 40 mg ion/l respectively. The osmolality, measured with a freezing point osmometer, was maintained constant by addition of sodium gluconate or sorbitol to the solutions of reduced chloride ion content. The human erythrocyte membrane is impermeable to sorbitol and to Na gluconate [9]. We have shown elsewhere [8] that these procedures maintain $[Cl_i^-]$ and cell volume constant.

Preparation of blood suspension. Blood was obtained by finger prick into a buffered solution of appropriate chloride ion concentration. The cell suspension was centrifuged at 3500 *g* for 5 min. The pellet was washed twice before resuspension in the chloride solution (2 ml) containing the drug.

Morphological observations of cells exposed to drugs. Following exposure of the cells to drug for a selected time at a given temperature the cells were fixed by addition of glutaraldehyde in buffer to a final concentration of 0.1%. After 3 min a sample of the fixed cell suspension was drawn by capillarity into a 50 μm pathlength glass microslide (Camblab Ltd.) and examined by differential interference microscopy under ×100 oil immersion. The erythrocyte shapes were characterized according to the criteria of Fujii *et al.* [3]. Over 100 cells were examined for each sample and the percentage of the cells in different shape categories was calculated.

Cellular uptake of drugs. The absorbance (A_1) of the charged drug species in solutions, or dilutions of solutions, of barbitone, chlorpromazine, tetracaine and indomethacin in saline were measured at 240, 254, 310 and 320 nm respectively prior to exposure of cells to the drugs. Out-dated (1–5 weeks) blood, from a blood bank, was washed as described above and resuspended to 15% haemotocrit in the drug/chloride solutions. The cells were exposed to the drug solution for 2 min at 37°. At the end of the exposure time the cell suspension was centrifuged at 1500 *g* for 5 min at 37° and the supernatant collected. The absorbance of the supernatant (A_2) was measured against a blank of the supernatant of a cell suspension which had not been exposed to drug. The amount of drug (S) seqestered by the cells per unit extracellular charged species was

$$S = (A_1 - A_2)/A_2 \qquad (2)$$

Scanning electron microscopy. Cells exposed to drug were fixed by addition of 0.5% v/v of glutaraldehyde in buffer for 1 hr at 4°. The cell suspension was washed four times and post fixed by addition of 1% w/v of osmium tetroxide in buffer at 4° for 1 hr. The cells were dehydrated with increasing concentration of alcohol (30% to 95%) at 4° for 15 min and with 100% alcohol for four times at room temperature for 10 min and dried in a Samdri 780 critical point dryer (Tousimist Research Corporation, U.S.A.) for 1 hr. The cells were mounted on stubs and coated with gold in an Emscope sputter coater (Emscope, U.K.) The preparation was then observed under a Phillips electron microscope 400T fitted with a scanning electron detector (SED) and a scanning transmission electron microscope.

Examination of the fragmentation patterns of heated erythrocytes. The technique for heating erythrocytes at 0.5 K/sec through the thermal denaturation of spectrin in a microscope slide and the video recording of the fragmentation pattern have previously been described [6, 10]. At the spectrin denaturation temperature cells suddenly, within 1 sec, either round up and internalize membrane at the cell dimple or else develop a surface wave on the cell rim. Vesicles can develop from the crests of the growing surface wave [5, 7]. The average number of waves ($\bar{W}$) per cell rim on membrane externalizing cells and the percentage (I) of cells internalizing membrane were scored.

Surface charge depletion by neuraminidase. The removal of glycocalyx sialic acids by neuraminidase treatment and the measurement of erythrocyte electrophoretic mobility were as described previously [5, 8].

RESULTS

Drug-induced cell shape change as a function of chloride concentration

Figure 1 shows the shape change, from biconcave to highly echinocytic forms, which occurs when 5 mM indomethacin is added to cells in 145 mM NaCl, pH 7.32. Control cells in 60 mM NaCl, 85 mM Na gluconate, pH 6.94, are slightly cupped while the presence of 5 mM indomethacin in a cell suspension leads to highly internalized forms (Fig. 2). The drug-treated cells in Figs 1 and 2 were exposed to the drug for 2 min before fixation. It can be seen that the crenating effect of indomethacin was reversed to a stomatocytic effect when the chloride ion concentration was reduced from 145 to 60 mg ion/l. A similar result to that in Fig. 2 was obtained when drug-exposed cells in 60 mg ion/l chloride were (i) buffered at pH 7.32 or (ii) in a suspending phase where 170 mM sorbitol replaced the 85 mM Na gluconate, showing that the change in pH or change in ionic strength of the suspending phase were not responsible for the contrasting results in Figs 1 and 2. The same cell morphologies as those shown in Figs 1 and 2 were observed when unfixed erythrocytes were examined by light microscopy.

Figure 3 records the change in morphology of erythrocytes exposed in 145 mM buffered saline (pH 7.32) to 5 mM indomethacin at 37° for different times. The cells were initially highly crenated (Stage III echinocytes [3]) but the degree of crenation decreased with time. Figure 3 also shows the time dependence of the morphology of cells exposed to

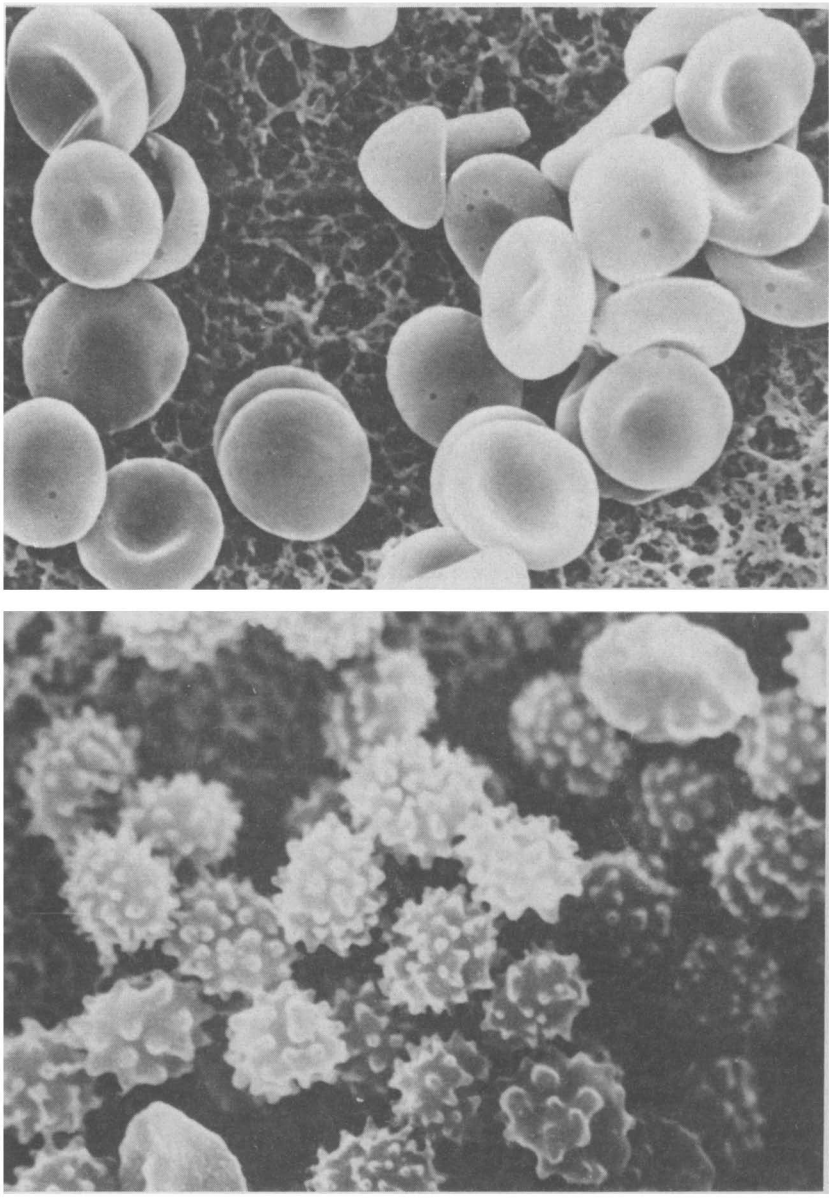

Fig. 1. Scanning electron micrographs of cells in 145 mM buffered NaCl, pH 7.32; control cells (upper micrograph) and cells exposed to 5 mM indomethacin.

5 mM indomethacin in 60 mM NaCl, 85 mM Na gluconate, pH 6.94. The latter cells become Stage III stomatocytes immediately on suspension in the low chloride ion concentration medium and maintained that shape during 1 hr incubation.

Erythrocytes which were echinocytic after 30 min incubation at 37° with 5 mM indomethacin in 145 mM NaCl became Stage III stomatocytes immediately on resuspension, following brief centrifugation, in 60 mM NaCl, 85 mM Na gluconate containing 5 mM indomethacin. Cells which were Stage III stomatocytes following 30 min incubation with 5 mM indomethacin in 60 mM NaCl with Na gluconate became Stage III echinocytes over a 1 min period on resuspension, following a brief centrifugation, at 37° in 145 mM NaCl with drug. These results showed that the effects, on cell morphology, of altering the extracellular chloride concentration were essentially reversible.

Indomethacin is not fully soluble when the nominal concentration is 5 mM. We have determined that, when indomethacin is dissolved to an initial concentration of 1.0 mM, the optical density of that solution and of dilutions of it are linearly dependent on drug concentration indicating that the critical micelle concentration of the drug is in excess of 1.0 mM. Cells in 1.0 mM indomethacin in 145 mM NaCl for 30 min were slightly echinocytic compared to control cells while cells in 60 mM NaCl 85 mM Na gluconate were more cupped than the appropriate

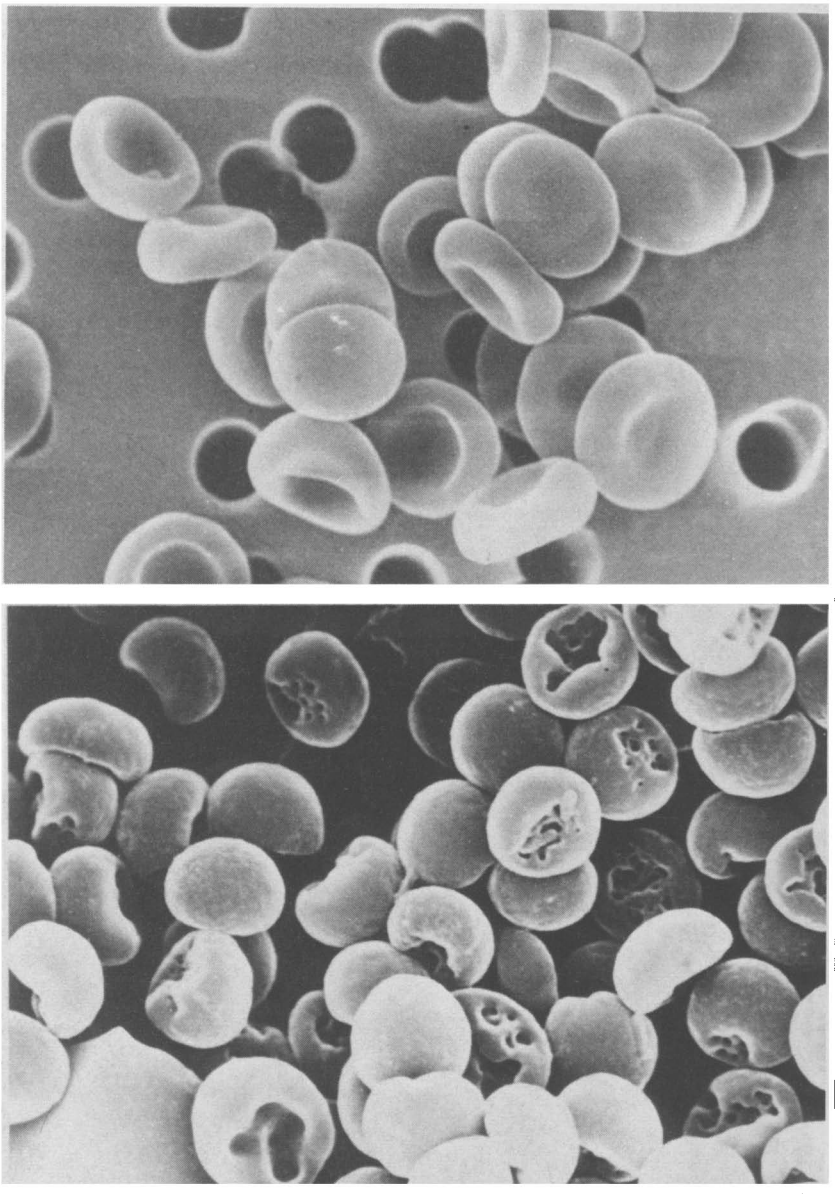

Fig. 2. Scanning electron micrographs of cells in 60 mM NaCl, 85 mM Na gluconate, pH 6.94; control cells (upper micrograph) and cells exposed to 5 mM indomethacin.

control cells. The result shows that the extent of drug solubility was not the determinant of the drug-induced shapes in Figs 1 and 2.

Table 1 describes the effect of $[Cl_o^-]$ on the dominant cell shape after 30 min exposure to 5 mM indomethacin. The membrane potential corresponding to each chloride ion concentration is also given. The shapes became less echinocytic when the membrane was depolarized. The membrane potential at the transition point through the biconcave disc shape is close to 5 mV. It has been found that the incidence of biconcave shapes, immediately on exposing cells to drug solutions, is a maximum at a membrane potential between 5 and 16 mV (closer to 5 mV).

Figure 4 shows that cells resuspended at 37° in 50 mM barbitone with 145 mM NaCl, pH 7.32, were largely Stage III echinocytes. Cells resuspended in 50 mM barbitone, 60 mM NaCl and 85 mM Na gluconate at pH 6.94 showed a low incidence of Stage III stomatocytes. Many Stage I and Stage II stomatocytes were observed in these samples. Cells suspended in 10 mM barbitone showed the same types of shape (but at Stage I) as cells in 50 mM barbitone at the two chloride ion concentrations referred to above.

Salicylate (20 mM) induced Stage II echinocytes in cells in 145 mM saline. Control cells in 60 mM saline were slightly cupped. These latter cells were more like discocytes when 20 mM salicylate was present in the cell suspending phase, i.e. a full reversal of the direction of shape change did not occur when the chloride ion concentration was decreased from 145 to 60 mM.

The shape dependence of erythrocytes suspended

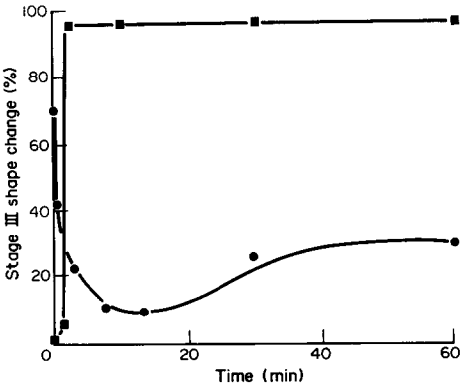

Fig. 3. The percentage of Stage III (i) echinocytic cells in 145 mM NaCl, 5 mM indomethacin, 5 mM HEPES, pH 7.32 (—●—); (ii) stomatocytic cells in 60 mM NaCl, 85 mM Na gluconate, 5 mM indomethacin, 5 mM HEPES, pH 6.94 (■), as a function of time of exposure to drug. The results are averages from five experiments at each chloride concentration.

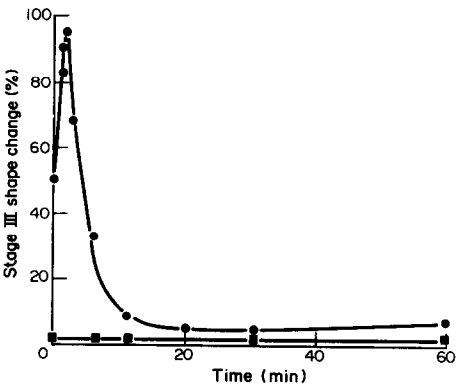

Fig. 4. The percentage of Stage III echinocytes (—●—) in cells exposed to 50 mM barbitone in 145 mM NaCl, 5 mM HEPES, pH 7.32 at 37°. A low incidence of Stage III stomatocytes (—■—) in 50 mM barbitone, 60 mM NaCl, 85 mM Na gluconate, 5 mM HEPES at pH 6.94 is also shown. The results are averages from five experiments at each chloride concentration.

at 37° in 4 mM solutions of the cationic drug tetracaine in 145 or 60 mg-ion/l chloride is shown in Fig. 5. The cells in the lower chloride ion concentration (depolarized cells) were much less stomatocytic than were the cells in 145 mg-ion/l. The reversal of shape type found when cells exposed to indomethacin and barbitone (Figs 1–4) were exposed to the lower chloride concentration was not observed in cells exposed to tetracaine at 37°. However, cells in 4 mM tetracaine at 20° were stomatocytic in 145 mM NaCl but showed some Stage III echinocytic forms in 60 mM chloride.

The shape of erythrocytes to 100 μM chloropromazine at 37° is shown as a function of time and chloride ion concentration in Fig. 6. Cells in 60 mM chloride were less stomatocytic than cells in 145 mM saline. There was a further decrease in the incidence of stomatocytic shapes when the chloride concentration of the resuspending phase was reduced to 40 mg-ion/l but no echinocytic forms were observed either at 20 or 37°

Dependence of drug uptake on chloride concentration. The uptake of drug by erythrocytes was measured as described in the Methods. The ratio (R) of the sequestration of charged species per unit extracellular concentration at 145 mM chloride to that at 60 mM is calculated (see equation 2) as S_{60}/S_{145} for anions and as S_{145}/S_{60} for cations. Table 2 gives the values of R for each drug.

Drug-induced shape in neuraminidase treated cells. Cells in 145 mM saline, pre-treated with neuraminidase so that the electrophoretic mobility (which is proportional to the glycocalyx sialic acid content [11]) was reduced to 15% of control values, were exposed at 37° to the concentrations of the four drugs (indomethacin, barbitone, tetracaine and chlorpromazine) which could induce Stage III shape change. There was no difference, detectable by light microscopy, in the appearance of charge depleted cells and control cells which were exposed to the same drug concentration. This observation is in agreement with and extends to anionic drugs the previous observation of Sheetz and Singer [2] that cell surface charge depletion by neuraminidase did not modify the erythrocyte shape change induced by chlorpromazine.

Figure 7 shows the results of an examination of the thermal fragmentation patterns of control and of surface charge depleted cells in a range of concentrations of chlorpromazine. At zero drug concentration charge depletion increases the sudden incidence of membrane internalisation at the spectrin denaturation temperature compared with the situa-

Table 1. The dependence of the shape of human erythrocytes, in 5 mM indomethacin for 30 min at 37°, on extracellular chloride ion concentration. The membrane potential (equation 1) at each chloride ion concentration is also shown. The cell shapes were scored, as described by Fujii *et al.* [3].

Sodium chloride (mM)	Membrane potential (E, mV)	Stage 1 + 11 + 111 stomatocytic (%)	Bioconcave (%)	Stage 1 + 11 + 111 echinocytic (%)
145	−7.1	0	10	90
120	−2.1	0	62	38
90	5.6	25	75	0
60	16.4	100	0	0

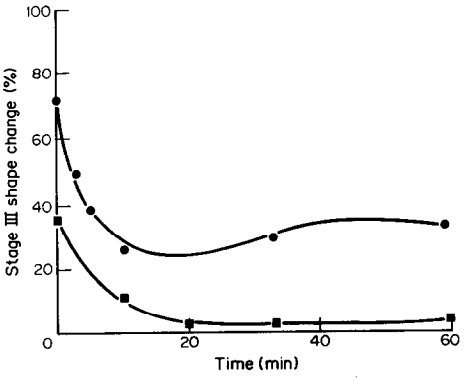

Fig. 5. The percentage of Stage III stomatocytes in cells exposed to 5 mM tetracaine in: 145 mM NaCl, 5 mM HEPES, pH 7.32, at 37° (—●—); 60 mM NaCl, 85 mM Na gluconate, 5 mM HEPES, pH 6.94 (—■—) for different times. The results are the averages from seven experiments at each chloride concentration.

tion for control cells, in agreement with previous results [5, 8]. The percentage, I, of cells which internalised membrane at the cell dimple was determined at each drug concentration. The least-squares best-fit straight lines are shown in Fig. 7. Table 3 gives the values of the regression coefficients b for control and charge depleted cells exposed to 0–30 μM chlorpromazine or to a 0–250 μM concentration of tetracaine. The table shows that there is little difference between the regression coefficients for the control and charge-depleted cells exposed to either cationic drug.

Figure 8 shows that $\bar{W}$, the average number of surface wave crests which develop suddenly around the rim of an erythrocyte heated through the thermal denaturation temperature of spectrin, increases with increasing concentration of the anionic drug indomethacin for both control and charge depleted cells. The curves in Fig. 8 describe the relationship

$$\bar{W}_A - \bar{W} = (\bar{W}_A - \bar{W}_O).\exp(-kc) \qquad (3)$$

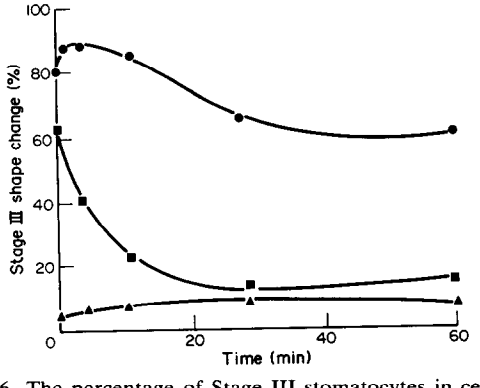

Fig. 6. The percentage of Stage III stomatocytes in cells exposed at 37° to 0.10 mM chlorpromazine with 5 mM HEPES in: 145 mM NaCl, pH 7.32 (—●—); 60 mM NaCl, 85 mM Na gluconate, pH 6.94 (—■—); 40 mM NaCl, 105 mM Na gluconate, pH 6.72 (—▲—). The results are averages from six experiments at each chloride concentration.

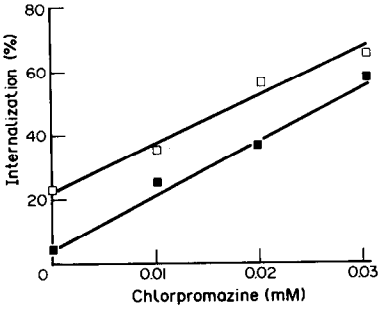

Fig. 7. The percentage (I) of cells which internalize membrane at the cell dimple as cells in chlorpromazine with 145 mM NaCl are heated through the denaturation temperature of spectrin; (i) control cells (—■—); (ii) neuraminidase treated charge depleted cells (—□—).

where $\bar{W}_A$, an asymptotic value of $\bar{W}$, is taken as 12.5, c is the drug concentration and k is the rate constant for change of $\bar{W}_a - \bar{W}$ with drug concentration. A least squares curve fitting routine (Nag Library EO4 FCF) gave values of k and $\bar{W}_A - \bar{W}_O$ which minimized the sum of squares of the residuals ($\bar{W}_O$ is the point at which the curve intercepts the $\bar{W}$ axis when $c = 0$). The values of k for indomethacin and barbitone are shown in Table 3.

For cell populations suspended in cationic drugs $\bar{W}$ decreases, as the drug concentration decreases, with a rage constant k to an asymptotic value of 4.7 as previously shown for tetracaine [6].

The results in Table 3 show that there is little difference in the values of k and b for charge depleted cells compared with the values for control cells. Thus the effects of drug concentration and surface charge density depletion on the thermal fragmentation patterns of the cells (Figs 7 and 8, Table 3) appear to be independent and additive.

DISCUSSION

The observation that there was no detectable difference between drug-induced shape change in control and in neuraminidase-treated cells at 37°

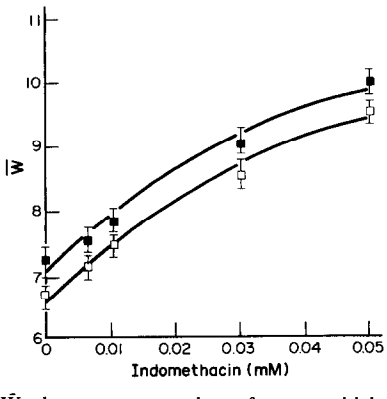

Fig. 8. $\bar{W}$, the average number of waves which grew per cell rim as cells were heated through the spectrin denaturation temperature, as a function of indomethacin concentration for control cells in 145 mM NaCl, 5 mM HEPES (—■—) and neuraminidase treated charge deplected cells (—□—).

Table 2. The influence of extracellular chloride concentration on the sequestration of drugs by cells suspended in 145 mM chloride or in 60 mM chloride. The ratio R is calculated as S_{60}/S_{145} for anions and S_{145}/S_{60} for cations. S is calculated from equation 2

Drug	Concentration (mM)	R
Tetracaine	0.05	2.83
Tetracaine	4.0	1.84
Chlorpromazine	0.1	1.79
Indomethacin	0.125	2.1
Indomethacin	5.0	2.0
Barbitone	0.5	1.94
Barbitone	50.0	2.23

confirmed the previous result of Sheetz and Singer [2] for chlorpromazine and extended it to the case of a second cationic drug, tetracaine, and to the anionic indomethacin and barbitone. The results of Table 3, particularly for those cases where standard deviations were small (as in the regression coefficients for change in I with concentration change for chlorpromazine and tetracaine and the value of k for chlorpromazine and indomethacin) show that the thermal fragmentation indices b and k of erythrocytes exposed to lower drug concentrations than those required to induce shape change at 37° showed little dependence on glycocalyx charge.

Until comparatively recently it has been the practice to measure cell electrophoretic mobility and calculate, from the Smoluchowski equation [12] a zeta potential as an estimate of the potential at the surface of a cell. This calculated zeta potential has a value of about −15 mV for human erythrocytes [8]. The Smoluchowski equation is based on the assumption that surface charge is distributed uniformly, in a zero thickness layer, over the cell surface. Recent theoretical treatments [13–15] which consider the erythrocyte charge as being distributed through a finite thickness glycocalyx lead to a potential profile which is steep close to the outer surface of the glycocalyx and then remains relatively constant on approaching the bilayer. The maximum value of this theoretical potential profile is significantly less than the Smoluchowski zeta potential. For a potential V at the outer face of the bilayer the concentration of monovalent cations of charge e at that face will be $\exp(-e.V/kT)$ times the concentration in the bulk phase. If V were equal to the Smoluchowski zeta potential of −15 mV then the cation and anion concentrations at the outer face of the bilayer would be 1.8 and 0.55 times the bulk concentrations respectively. The absence of a glycocalyx charge effect on cell shape in the present work leads to the conclusion that the potential V is much less than the Smoluchowski zeta potential. This conclusion supports the theoretical treatments which suggest rather low potential values through the glycocalyx.

The morphological change observed on exposing erythrocytes to drugs is dependent on the extracellular chloride ion concentration (Figs 1–6, Table 1). It would be expected, in the light of the application of measurements of the distribution of permeant ions across a membrane to determine membrane potentials in a range of systems [16, 17], that a Donnan equilibrium between the chloride ions and charge drugs would be set up across the cell membrane. Such an equilibrium would, for constant extracellular concentration of charge drug, increase the concentration of anionic drug and decrease the concentration of cationic drug within the cell as the extracellular chloride ion concentration is decreased. It can easily be shown that the ratio (R) of uptakes, as defined in Table 2 should then have the value 145/60, i.e. 2.42. The values of R in Table 2 give general support to the suggestion of Donnan equilibrium between the drugs and chloride. Some departure of the values of R from ideal behaviour might be expected because, *inter alia*, the presence of membrane permeant drugs will (still assuming that the drug concentrations employed do not significantly modify the permeability of the erythrocyte membrane to sodium or potassium) modify the membrane potential equation to

$$E = 61.5\,\mathrm{Log}_{10}\{(P_{Cl}[Cl_o^-] + P_D[D_o^-])/(P_{Cl}[Cl_i^-] + P_D[D_i^-])\} \quad (4)$$

for anionic drugs, with appropriate changes for cationic drugs. In equation (3) P_{CL} and P_d are the permeability coefficients for chloride and drug respectively.

Table 3. The values of the rate constant k (as in eqn. 2 for anionic drugs and as in reference 6 for cationic drugs) for the change in waviness with increasing drug concentration for control and charge depleted cells. The values of the regression coefficient, b, for change in I with cationic drug concentration are also included

Drug	Cells	$k \pm$ S.D. (mM^{-1})	$b \pm$ S.D. (%/mM)
Chlorpromazine	Control	29.4 ± 3.0	1410 ± 50
	Charge depleted	30.6 ± 2.8	1540 ± 140
Tetracaine	Control	3.91 ± 0.37	211 ± 0.25
	Charge depleted	6.88 ± 1.89	228 ± 13
Indomethacin	Control	17.4 ± 2.7	N.A.*
	Charge depleted	14.4 ± 0.5	N.A.
Barbitone	Control	0.052 ± 0.014	N.A.
	Charge depleted	0.036 ± 0.01	N.A.

* Not applicable.

The Sheetz and Singer [2] bilayer couple hypothesis for drug-induced morphological change is that stomatocytes are formed when there is a greater interaction of drug with the inner monolayer compared with the outer monolayer, and crenation occurs when the greater interaction is with the outer monolayer. A membrane-potential-induced change in intracellular drug concentration when the extracellular drug concentration is constant could, in the light of the bilayer couple hypothesis, explain in general terms the differences in morphology in the cells with different extracellular chloride ion concentrations.

The effects of change of intracellular charged drug concentrations have not always been explicitly considered when the consequences of the use of hyperpolarizing potentials to relieve local anaesthetic nerve block are considered [18].

Control cells in 60 mM chloride are slightly cupped showing a small number of Stage I stomatocytes. In contrast to the case for indomethacin and barbitone, which produced Stage III and Stage II stomatocytes respectively in 60 mM NaCl, the anionic salicylate, which induced Stage II echinocytes in 145 mM saline, showed a slight return of the mildly cupped control cells to the discocyte form in 60 mM chloride. Salicylate has a higher solubility than indomethacin or barbitone. However, the ability of 4 mM tetracaine (critical micelle concentration 70 mM [19]) to produce shape reversal with change in chloride concentration at 20° indicates that the dependence of cell morphology on chloride ion concentration is not due to the extent of drug solubility.

Deuticke [1] produced stomatocytes on maintaining cells in low chloride ion isotonic solution. He commented that the stomatocytic effect of low chloride ion concentration could be reversed by agents which crenate cells. The invaginating agents employed were not specified.

The anionic drugs indomethacin and barbitone and the cationic chlorpromazine all produce stomatocytic forms at a membrane potential of 16 mV (60 mM chloride). When cells are exposed to indomethacin or barbitone at 37° or to tetracaine at 20° the class of shape change induced is different at different chloride concentrations. These observations indicate that the generalization [1, 3] that cationic drugs produce stomatocytes and anionic drugs usually produce echinocytes holds only over a restricted range of membrane potential.

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