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CHARGE-INDEPENDENT EFFECTS OF DRUGS ON ERYTHROCYTE MORPHOLOGY

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Abstract—The effects of chlorpromazine, tetracaine, indomethacin, barbitone and benzyl alcohol on human erythrocyte shape have been examined. Cationic and anionic drugs produced stomatocytes and echinocytes respectively as expected for cells in isotonic saline. Particular attention has been directed here to some features of drug induced morphology change which are independent of the charge of the drug. It was found that (i) the direction (increase or decrease) of the extent of morphological change as temperature was increased from 20 to 37°, (ii) the exposure time for maximum shape change (0–2 min), and (iii) the time course of cell morphology (0–30 min) were different for similarly charged drugs. The influence of low concentrations of the drugs on the thermal fragmentation patterns of the cells has been determined. A single index has been derived which allows comparison of the morphological effects of cationic and anionic drugs.

It was concluded that, while the type (stomatocyte or echinocyte) of shape change observed was dependent on the charge of the drug, cell morphology at drug concentrations high enough to produce marked shape change at 37° was strongly influenced by charge independent drug-specific effects.

Many chemically unrelated amphipathic drugs can induce shape changes in human erythrocytes [1–3]. In physiological saline cationic amphipaths give rise to stomatocytic (cupped) erythrocytes while anionic and uncharged drugs generally produce crenated (echinocytic) cells. The type of cell shape observed with charged drugs can alter, in a manner consistent with a membrane potential dependent repartition of the drug across the membrane, when the chloride ion content of the cell suspending phase is changed [4]. The above studies of cell–drug interactions have concentrated on the relationship between drug charge and the category (stomatocytic or echinocytic) of shape change.

In the present study we identify some characteristics of drug-induced shape change which can be different for drugs of identical charge.

We also extend previous investigations [4, 5] of the influence of drugs on heated erythrocyte fragmentation [6]. If an amphipath interacts principally with the membrane bilayer then the cytoskeletal network [7] may act against the expression, as a shape change, of the amphipath-induced stress in the bilayer when cells are maintained at 37°. Erythrocyte shape change is much more sensitive to cell surface charge depletion [8, 9] intrinsic electric fields [9], divalent cations [10] and low concentrations of tetracaine [5] at the denaturation temperature (50° [11, 12]) of the cytoskeletal protein, spectrin, than is the case at 37°. The thermal fragmentation patterns of cells have been examined here at drug concentrations which are lower than those required to produce a detectable shape change at 37°.

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MATERIALS AND METHODS

(i) Preparation of blood samples. Human erythrocytes were freshly collected by finger prick into a buffered saline solution made up of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, Sigma Ltd.) in 145 mM sodium chloride, pH 7.4. The collected cells were centrifuged at 3500 *g* for 5 min. The pellets were washed twice in buffer and resuspended to a cell concentration of approximately 5×10^7 cells/ml.

(ii) Chemicals. The final HEPES buffered resuspension contained a concentration known to cause morphological effects, of either (a) the cationic drug chlorpromazine hydrochloride or tetracaine hydrochloride (Sigma Ltd), (b) the anionic amphipath indomethacin (Sigma Ltd) or baritone (BDH) or (c) neutral benzyl alcohol (BDH).

(iii) Morphological observations of cells exposed to drug at 20° or at 37°. One millilitre of a cell suspension (5×10^7 cells/ml in drug solution) and glutaraldehyde in buffer (0.5%, v/v) were maintained at the same temperature of either 20° or 37°. Following exposure of the cells to drug for a known time 0.2 ml of the glutaraldehyde solution was added to 1.0 ml of cell suspension. The cell suspension was then allowed to stand at the appropriate temperature for at least 3 min. A sample of the fixed cell suspension was drawn by surface tension into 5.0 cm long glass microcapillaries of rectangular cross section, 0.2 mm pathlength and 1.2 mm width (Microslides, Camlab Ltd) and observed using DIC (differential interference contrast) with a ×100 oil immersion objective on a Nachet 400 microscope. The cells were examined immediately after loading into the microcapillaries and about 45 min later. No change was detected between these two examina-

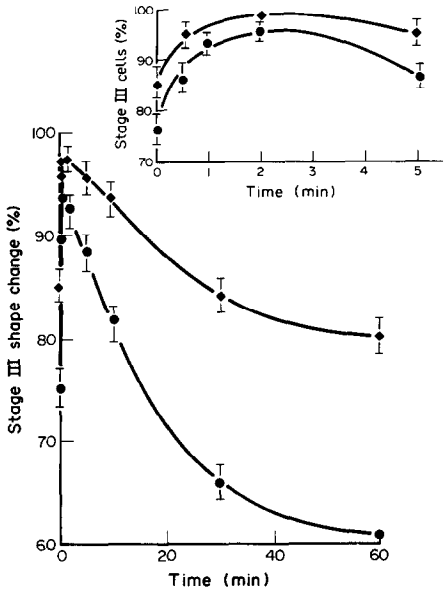

Fig. 1. The time dependence of the incidence of Stage III internalized cells in 0.08 mM chlorpromazine at 20° (—◆—) and at 37° (—●—). The insert shows the time dependence of cell morphology over the first five minutes of incubation. The results are the average values from seven experiments at each temperature.

tions. The initial erythrocyte shape change (0 min) was determined either by addition of cell suspension to the drug solution followed by addition of glutaraldehyde as quickly as possible (within 2–5 sec) or by bringing drug in saline solution to the filled end of the loaded microcapillaries (with unfixed cells) mounted on the microscope stage. The erythrocyte shapes were characterised according to the criteria proposed by Fujii *et al.* [3]. The number of erythrocytes in each shape category was counted and expressed as a percentage of the total number of cells scored. For clarity of presentation, the percentage of Stage III shape changes [3] was calculated and presented in the results section.

(iv) Heating and examination of the fragmentation patterns of heated erythrocytes. The apparatus and method for heating cells in a rectangular glass microcapillaries at a rate of 0.5 K/sec from room temperature through the thermal denaturation temperature of cytoskeletal protein, spectrin, has previously been described [13, 14]. The morphological changes [4, 5, 9, 14] which suddenly develop on cells at the spectrin denaturation temperature were scored for cells heated in a range of low concentrations of the cationic and anionic drugs. The number of wave crests per cell rim was scored and $\underline{W}$, the average number of waves per wavy cell, was calculated.

RESULTS

A. Influence of amphipaths on cell morphology at 20° and at 37° for times up to 60 min

(i) Chlorpromazine. Figure 1 shows the time and temperature dependence of the incidence of Stage III stomatocytic cells in erythrocytes maintained with 0.08 mM chlorpromazine at 20° and at 37°. The percentage of Stage III internalized cells increased over the first 2 min from 70% and 80% at 37° and 20° respectively to a maximum and then fell gradually with time. In a previous study, confined to chlorpromazine, Roth and Jay [15] reported that many cells which had cupped an initial exposure to chlorphromazine had reverted to a discoid form after 30 min. Erythrocytes at 20° had a higher incidence of Stage III internalized forms than cells at 37° for the same exposure time (Fig. 1).

Erythrocytes in 0.05 mM chlorpromazine at 20° had a much more pronounced dimple compared with cells at 37°. The morphology of erythrocytes at either 20° or 37° in 0.05 mM chlorpromazine did not change detectably for up to 1 hr of exposure to the drug.

(ii) Tetracaine. The percentage of Stage III internalized cells in tetracaine (4.0 mM) had maxima of 66 and 72% immediately on exposure to drug at 20° and 37° respectively. The incidence of internalized cells decreased rapidly with continued exposure, reaching values of 6 and 30% at 20° and 37° after 30 min as previously reported [4]. Erythrocytes in 1.0 mM tetracaine showed a Stage I internalized form and the cell shape did not change detectably during a one hour exposure to tetracaine at 20° or 37°.

(iii) Indomethacin. Figure 2 shows that the percentage of Stage III crenated cells in 5 mM indomethacin was highest immediately following resuspension of erythrocytes in the drug solution at both 20° and 37°. The high incidence of externalization fell rapidly within the first 2 min of exposure of cells to the drug (Fig. 2). Subsequently erythrocytes at 20° maintained a constant shape with increasing time while cells at 37° showed an increased tendency towards Stage III externalized forms. The morphology of erythrocytes in 1.0 mM indomethacin was

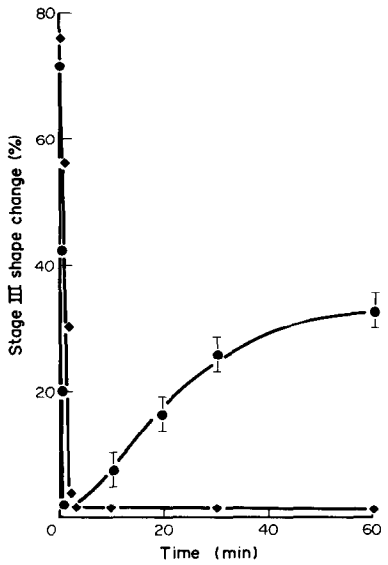

Fig. 2. The incidence of Stage III externalized cells in 5.0 mM indomethacin at 20° (—◆—) and 37° (—●—) for different times. The results are the average values from seven experiments at each temperature.

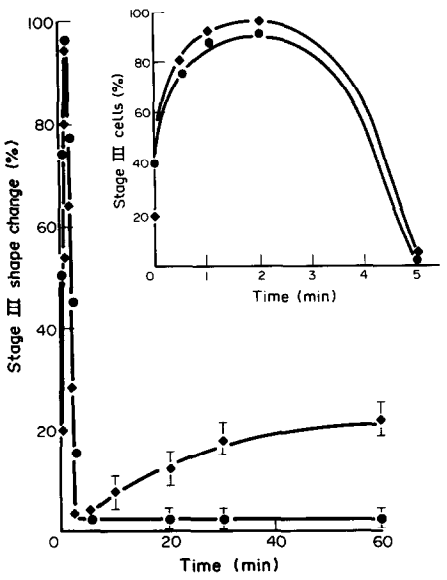

Fig. 3. The time dependence of Stage III externalized cells in 50 mM barbitone at 20° (—◆—) and 37° (—●—). The insert shows the time dependence of cell morphology over the first five minutes of incubation. The results are the average values from seven experiments at each temperature.

also examined as a function of both exposure time and temperature. Erythrocytes in 1.0 mM indomethacin at 20° had a mixture of stage I externalized shapes and discocytes. Cells at 37° had a mixture of discocytes and Stages I, II and III externalized cells. These cells did not change shape with time at 20° or at 37°.

(iv) Barbitone. The incidence of Stage III externalized cells in 50 mM barbitone increased with time over the first 2 min following resuspension of erythrocytes to the drug at 20° and at 37° and then fell gradually with time (Fig. 3). Erythrocytes at 37° maintained a constant shape with increasing time while cells at 20° showed an increased tendency towards the Stage III externalized forms. Erythrocytes in 10 mM barbitone at 20° showed a mixture of discocytes and Stage I externalized cells while cells at 37° were essentially discoid forms. The erythrocytes in 10 mM barbitone did not change their shape during a one hour exposure to the drug at either 20° or 37°.

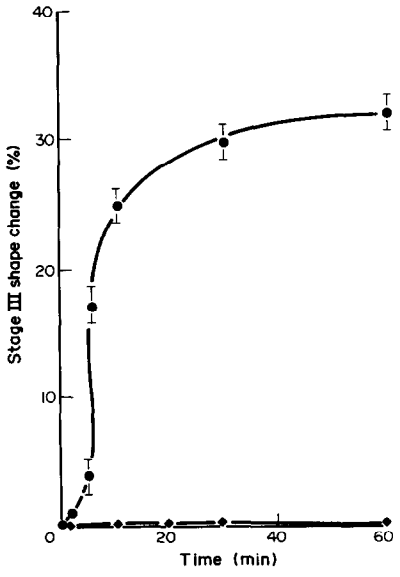

Fig. 4. The incidence of Stage III externalized cells in 50 mM benzyl alcohol in 145 mM NaCl at 20° (—◆—) and 37° (—●—) for different times. The results are average values from five experiments at each temperature.

(iv) Benzyl alcohol. Figure 4 shows that the initial (0–2 min) high incidence of Stage III externalized forms which were observed when first exposing cells to charged drugs were not found with 50 mM benzyl alcohol at 20° or at 37°. The percentage of Stage III externalized cells at 37° increased gradually with time, whereas erythrocytes at 20° showed a low incidence of Stage III crenated forms. On the other hand, cells maintained with 10 mM benzyl alcohol were essentially discocytes at the two temperatures of 20° and 37°. The latter cells did not change shape with time at 20° and at 37° during a 1 hr exposure.

B. Fragmentation pattern of heated erythrocytes in the presence of added amphipathic drugs.

(i) Anionic drugs. $\overline{W}$, the average number of waves per cell rim as the erythrocytes are heated through the thermal denaturation temperature (50°) of spectrin, is shown in Fig. 5 as a function of increasing concentration of indomethacin in the range 0–0.1 mM. The curve in Fig. 5 represents the relationship:

$$\overline{W}_A - \overline{W} = (\overline{W}_A - \overline{W}_0) \exp(-kc) \qquad (1)$$

where $\overline{W}_A$, an asymptotic value of $\overline{W}$, is taken to be 12.0 (cells in 1.0 mM indomethacin and below did not fragment by membrane externalization involving growth of more than 12 waves) and c is the drug concentration. k is a constant of proportionality for change of $\overline{W}_A - \overline{W}$ with change in drug concentration. The curve in Fig. 5 was derived from a Gauss–Newton routine (NAG Library EO4 FCF)

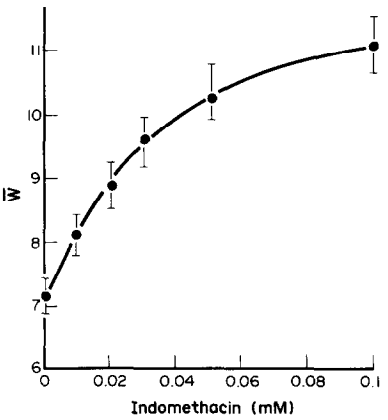

Fig. 5. The average number ($\overline{W}$) of waves per wavy cell for cells heated, in different concentrations of indomethacin, at a rate of 0.5 K/sec.

Table 1. The values, for each drug, of the proportionality constant k (equations 1 and 2) for changes in $\overline{W}$ with increase in drug concentrations as erythrocytes are heated at 50° (The drug concentration range studied is also given; the sign of the drug charge is in parentheses after the drug name.)

Drug	Concentration range (mM)	$k \pm$ S.D. (mM^{-1})
Indomethacin (−)	0–0.1	23.3 ± 2.3
Barbitone (−)	0–50	0.030 ± 0.013
Chlopromazine (+)	0–0.05	29.4 ± 3.0
Tetracaine (+)	0–0.25	3.91 ± 0.37

which, for a given value of $\overline{W}_A$, selected values of $\overline{W}_A - \overline{W}_0$ and k to minimize the sum of squares of the residuals. $\overline{W}_0$ is thus the point at which the curve of equation 1 intercepts the abscissa at $c = 0$. The values of k for indomethacin and barbitone (which was also obtained by equation 1) are given in Table 1.

6.4% of control cells internalized membrane following spectrin denaturation. No internalized cells were recorded in cells heated in indomethacin concentrations of 0.05 mM and above. Similarly no internalized cells were seen in cells heated in 20 mM barbitone and above.

(ii) Cationic amphipaths. Figure 6(a) shows that $\overline{W}$ decreased with increasing chlorpromazine concentration. The curve in Fig. 6 is described by equation 2, a relationship already described for tetracaine (4). $\overline{W}_B$, an asymptotic value of $\overline{W}$ is taken as 4.7.

$$\overline{W} - \overline{W}_B = (\overline{W}_0 - \overline{W}_B) \exp(-kc) \qquad (2)$$

The values of k for chlorpromazine and for tetracaine are given in Table 1.

From equation 1, the rate of change of $\overline{W}$ with anion concentration is given, as concentration goes towards zero, by

$$(d\overline{W}/dc)_{c=0} = k.(\overline{W}_A - \overline{W}_0) \qquad (3)$$

while for cations, from equation 2,

$$(d\overline{W}/dc)_{c=0} = k.(\overline{W}_0 - \overline{W}_B) \qquad (4)$$

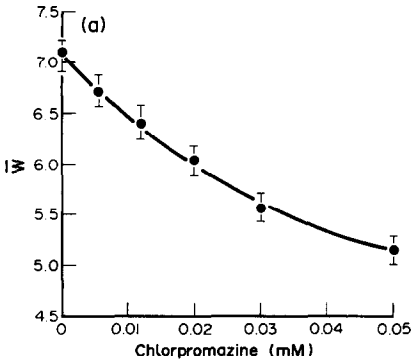

Fig. 6. The average number ($\overline{W}$) of waves per wavy cell for cells heated at 0.5 K/sec in different chlorpromazine concentrations.

The parameter $(d\overline{W}/dc)_{c=0}$ provides a single index (independent of amphipath charge) for comparing the effects of drugs of similar or of different charges.

Cells heated in high concentrations of the anionic amphipaths indomethacin and barbitone as well as cells heated in high concentrations of the cationic amphipaths chlorpromazine and tetracaine [4, 5] gave externalization at high drug concentration and at high temperature. The results show that the morphological influence of cationic drugs at high concentrations and at high temperatures was qualitatively different from the influence of high concentrations at 37° or of cells in low cationic drug concentrations at 50°.

DISCUSSION

The observation above (Figs. 1–3) that cationic drugs internalize and anionic drugs crenate human erythrocytes in HEPES-buffered saline for 30 min are in agreement with previous reports [1–4]. However, the results collected and presented in Table 2 show that (i) the time to reach the maximum percentage of Stage III shape forms (for the cationic drugs and for the anionic drugs tested), (ii) the temperature which gives the greater incidence of Stage III cells at any time, and (iii) the direction of change of shape with temperature increase for cells incubated for 30 min, can vary for similarly charged drugs. For erythrocytes in chlorpromazine, alone among the charged drugs tested, the incidence of Stage III forms did not fall to low values within the first few minutes of exposure and never fell below 60%.

The above results indicate that the hydrophobic region or the neutral species of a drug may also influence erythrocyte shape. This suggestion is supported by the observation that the morphology of erythrocytes maintained in uncharged benzyl alcohol is affected differently at 20° and at 37° (Fig. 4).

If the hypothesis advanced by Sheetz and Singer [2], that drug induced shape changes in erythrocytes occur because of a differential distribution of drug at the inner and outer faces of the membrane is correct, and since there was no immediate crenation on adding chlorpromazine or tetracaine to cell suspensions, the equilibrium of the drugs across the membrane must occur in times less than the time taken to fix the cells. The time delay before observation of the initial shape change was not greater than 5.0 sec. The rapid distribution of drug in the

Table 2. The human erythrocyte shape changes induced by amphipathic drugs in 145 mM NaCl at 20° and at 37°

Drug concentration (mM)	Time of maximum Stage III shape change at both 20° and 37° (min)	Change of shape with temperature increase at constant drug concentration*	Temperature (20° or 37°) at which greater shape change from biconcave form was observed after 30 min
Chlorpromazine (+) 0.08	2	towards biconcave	20 > 37
Indomethacin (−) 5.0	0	away from biconcave	37 > 20
Barbitone (−) 50	2	towards biconcave	20 > 37
Tetracaine (+) 4.0	0	away from biconcave	37 > 20
Benzyl alcohol (0) 50	>10	away from biconcave	37 > 20

* Cells video-recorded during heating of cells on a microscope slide from room temperature to 48°.

Table 3. The drug concentrations dI and dIII for Stage I and Stage III shape change respectively at 37° and their products with $(d\overline{W}/dc)_{c=0}$

Drug	Concentration dI for Stage I shape change (mM)	Concentration dIII for Stage III shape change (mM)	$dI.(d\overline{W}/dc)_{c=0}$	$dIII.(d\overline{W}/dc)_{c=0}$
Indomethacin (−)	0.08	5.0	8.8	600
Barbitone (−)	0.10	50	1.6	8
Chlorpromazine (+)	0.04	0.08	2.9	5.9
Tetracaine (+)	0.5	4.0	4.9	39.0

present system implies that the 2 min delay before a maximum number of Stage III cells is observed (with chlorpromazine or barbitone) is due to secondary changes in the membrane or cytoskeleton or a combination of both. There is also a marked delay to crenation in cells in benzyl alcohol at 37° (Fig. 4).

The values of $(d\overline{W}/dc)_{c=0}$, from equations 3 and 4, were determined from data obtained at drug concentrations below those required for a Stage I shape change. If the mechanisms which determine $(d\overline{W}/dc)_{c=0}$ at 50° are closely related to those which influence cell shape (Stage I) at higher drug concentration (dI) at 37°, then the product $dI.(d\overline{W}/dc)_{c=0}$ should be similar for drugs of the same charge. Likewise the product $dIII.(d\overline{W}/dc)_{c=0}$, where dIII is the drug concentration for a Stage III shape, should be similar for drugs of the same charge. Table 3 shows much closer agreement between the values of $dI.(d\overline{W}/dc)_{c=0}$ than between those of $dIII.(d\overline{W}/dc)_{c=0}$ for both the cationic and anionic drugs. This result suggests that the mechanisms responsible for Stage I shape and for the variation of thermal fragmentation pattern of erythrocytes are similar but that new drug-specific mechanisms determine the progression to Stage III shape. It supports the conclusion from Figs 1–4 and from Table 2 that, at high concentrations, drug-specific charge independent effects influence erythrocyte morphology.

The generalization that the type (stomatocyte or echinocyte) of drug-induced shape change depends on drug charge has provided valuable insights to drug membrane interactions [1–4]. The success of this simple generalization appears to have obscured the possibility that charge-independent drug-specific properties might also influence the shape change. We have drawn attention above to some features of the shape change which are independent of drug charge.

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