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Microcalorimetric Study for the Binding of Ionic Drugs to Human Erythrocytes and the Ghost Membranes

HATSUMI AKI AND MAGOBEI YAMAMOTO

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Fukuoka 814-01, Japan

Abstract—The binding of phenothiazine derivatives (as cationic drugs) and non-steroidal anti-inflammatory drugs (as anionic drugs) to human erythrocytes and ghost membranes has been compared with respect to their thermodynamic characteristics, by flow microcalorimetry at pH 7·4 and 37°C. From enthalpy-entropy correlation, it was shown that anionic and cationic drugs are bound to different binding sites on the membranes. Phenothiazines bind to a single common site of the erythrocyte membranes with relatively high binding affinities ($K = 10^4$–10^5 M^{-1}). The binding is entropy-driven and characterized by a small negative enthalpy (ΔH) and a positive entropy change (ΔS), reflecting hydrophobic interactions. However, the binding reaction for the intact erythrocytes shows large negative values for both ΔH and ΔS. The values of K for the membranes and ΔH for the intact erythrocytes increased with the increase of the hydrophobic character of the substituent group at the C-2 position of the phenothiazine nucleus ($H < Cl < CF_3$). The results indicate that phenothiazines bind and/or penetrate to the inner membranes of the erythrocytes and react with intracellular components such as haemoglobin, while anti-inflammatory drugs bind to the surface protein on the membranes with a lower affinity ($K = 10^3$ M^{-1}) than phenothiazines, reflecting the small negative ΔH and positive ΔS for the interaction with intact erythrocytes.

In contrast to numerous studies on the binding of drugs to human plasma proteins, less attention has been given to the interaction of drugs with intact erythrocytes or other components in the blood system. Quantitative studies of drug binding to blood cells have been made by equilibrium dialysis (McArthur et al 1971; Bickel 1975), the dynamic dialysis technique (Cruze & Meyer 1976), the uptake into erythrocytes or ghost membranes (Kanaho et al 1981; Nishihata et al 1984; Tamura et al 1987), and estimation from the unbound fraction in plasma and the blood to plasma concentration ratio of the drug (Lund 1980; Horie et al 1981). The application of classical binding methods to the study of drug binding to erythrocytes or whole blood is recognized as being difficult; the dialysis may cause haemolysis of erythrocytes or denaturation of other blood components, and competition for drug binding may also occur when numerous blood components are present. Therefore, a more direct measurement of drug binding to erythrocytes is needed.

We have studied drug interactions in the blood system using microcalorimetric methods (Yamamoto & Aki 1988; Aki & Yamamoto 1990) and reported on the thermodynamic characteristics of the binding of phenothiazines to plasma proteins (Aki & Yamamoto 1989). The flow microcalorimetric measurement was not affected by such side effects as non-specific adsorption to dialysis membranes or gel filtration materials, or influenced by the separation of free and bound fractions. Since optically clear solutions are not required, a large concentration range of materials or suspensions can be measured without purification steps. In addition, the direct analysis of calorimetric titration curves allows the determination of an apparent binding constant, the number of binding sites, and the change in binding enthalpy, as well as the evaluation of free energy and entropy. In the present work, the binding interactions between human erythrocytes and ionic drugs have been investigated using microcalorimetry.

Correspondence to: M. Yamamoto, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-Ku, Fukuoka 814-01, Japan.

Materials and Methods

Materials

Fluphenazine dimaleate (FPZ), perphenazine dimaleate (PPZ), trifluoperazine dimaleate (FPRZ), prochlorperazine dimaleate (CPRZ) and perazine dimaleate (PRZ) were obtained from Yoshitomi Pharmaceuticals Co. Ltd (Osaka, Japan). Flufenamic acid, mefenamic acid, ibuprofen and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, USA) and flurbiprofen was a gift from Kaken Pharmaceutical Co. Ltd (Tokyo, Japan).

Human erythrocytes, supplied by the Red Cross Blood Centre (Fukuoka, Japan), were washed three times in phosphate-buffered isotonic saline (PBS) (10 mM sodium phosphate, 140·5 mM NaCl, pH 7·4) by centrifugation at 1000 *g* for 10 min and then resuspended in the same buffer as a stock solution. Before use, erythrocytes were washed 2–3 times with PBS until the supernatant was clear and colourless, to obtain packed cells with a 100% erythrocyte concentration. Haemoglobin-free ghosts were prepared by haemolysing 1 volume of packed erythrocytes in 20 volumes of cold 30 mOsm phosphate buffer, pH 7·4, according to the procedure of Dodge et al (1963) and were resuspended in PBS. The protein concentration in ghost cell solutions was determined by the method of Lowry et al (1951), using human serum albumin (Sigma, HSA Fraction V) as a standard. The number of erythrocyte or ghost cells in each experimental suspension was measured by a Coulter Counter Model TA-2 (Hialeah, FL, USA).

Microcalorimetry

The calorimetric experiments were performed at 37·0 ± 0·05°C by a twinned titration flow calorimeter (Yama-

moto & Aki 1988) into which the reaction solutions were introduced at equal flow rates (0·12 mL min^{-1}) through Tygon tubing using a four-channel peristaltic pump (Gilson Minipuls 2, Villers-Le-Bel, France). A base line was established by directing PBS solution and erythrocyte or ghost cell suspension into each flow line in both the reaction and reference cells of the calorimeter. Drug solutions at different concentrations were then introduced sequentially into the PBS flow line in the reaction cell. The heat of mixing was proportional to the recorded steady-state value.

Calculation of binding and thermodynamic parameters

The experimental heat evolved (ΔQ_{exp}) during the mixing of protein and drug solutions is related to the sum of the binding heat (ΔQ), and the dilution heat of protein ($\Delta Q_{dil,p}$) and drug ($\Delta Q_{dil,d}$):

$$\Delta Q_{exp} = \Delta Q + \Delta Q_{dil,p} + \Delta Q_{dil,d} \quad (1)$$

The differential measurements correct for the heat of dilution of the protein and the thermal effect due to friction and turbulence by the flowing erythrocyte suspension. Since the heat of dilution of the drug was measured separately and subtracted, the value of ΔQ is proportional to the quantity of the complex formed with the total protein concentration fixed at P_t, as follows:

$$\Delta Q = \Delta H \cdot D_b \cdot F_r \quad (2)$$

Where ΔH is the binding enthalpy per mole of a drug and D_b is the bound concentration of drug at the constant flow rate of F_r. Solutions were prepared in PBS and the total concentrations of the drugs in the final calorimetric solutions were measured by UV absorption after measuring $\Delta Q_{dil,d}$.

In the case of drug binding to protein with only one class of binding site, the equilibrium aspect of such interactions was correlated through the mass law, yielding the familiar equation:

$$\frac{D_b}{P_t} = \frac{n \cdot K \cdot D_f}{1 + K \cdot D_f} \quad (3)$$

where D_f is the concentration of free drug related to the equation,

$$D_t = D_b + D_f \quad (4)$$

where D_t is the total concentration of the drug. Thus, equation 2 can be expressed as a function of D_t by using equations 3 and 4, as follows:

$$\Delta Q = \frac{\Delta H \cdot F_r}{2}(A - \sqrt{A^2 - 4n \cdot P_t \cdot D_2}) \quad (5)$$

where

$$A = 1/K + n \cdot P_t + D_t \quad (6)$$

The best fitted values of the binding and thermodynamic parameters, K, n, and ΔH, can be computed directly using the non-linear least squares regression method. An initial value of ΔH was estimated from the slope of the initial linear plots of the titration curve.

Results

Calorimetric titrations of ghost membranes

In Fig. 1 the heat of binding of drugs to erythrocyte ghosts,

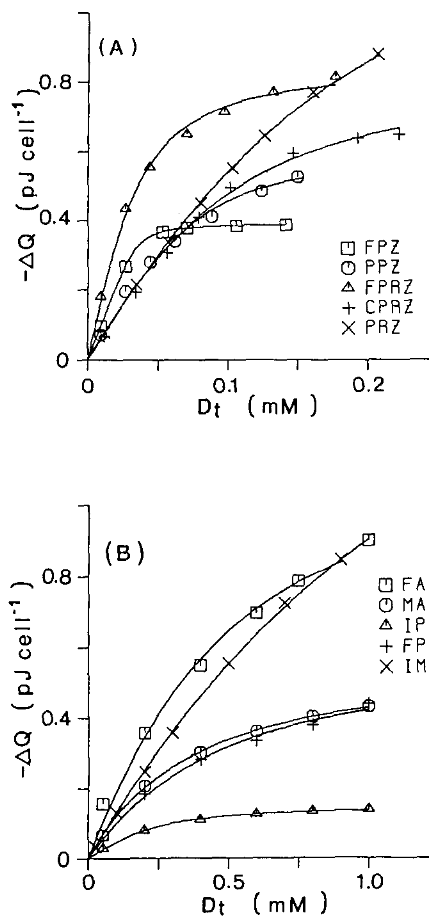

FIG. 1. Calorimetric titration curves for binding of phenothiazines (A) and anti-inflammatory drugs (B) to ghost membranes at pH 7·4 and 37°C. The final concentration of erythrocyte ghosts was in the range 1·84 – 2·53 × 10^{-5} M by the method of Lowry et al (1951) with HSA as a standard. Points show experimental values calculated from a single titration. Solid lines represent computer-generated best-fit curves assuming a one-class binding model with binding parameters given in Table 1. (FA flufenamic acid, MA mefenamic acid, IP ibuprofen, FP flurbiprofen, IM indomethacin.)

expressed in picojoules per cell of erythrocyte ghosts (ΔQ, pJ $cell^{-1}$), are summarized as a function of D_t in the final calorimetric solution. The concentrations of the drugs used were in the range of 0·01 – 0·2 mM for phenothiazines (Fig. 1A) and 0·05 – 1 mM for anti-inflammatory drugs (Fig. 1B). The initial concentration of erythrocyte ghosts was 40% (v/v). The protein concentration in the final calorimetric solution was estimated to be 2·084 ± 0·213 × 10^{-5} M. The solid lines drawn through the points represent the computer-generated best fit curves assuming a one-class binding model (eqn 5). The curves were fitted to the actual calorimetric data with correlation coefficients exceeding 0·99. For these experiments overall precision was within ±5%. The estimated values of the binding and thermodynamic parameters are listed in Table 1. The free energy changes (ΔG) were calculated from the K values in the table, according to the equation $\Delta G = -RT \ln K$, where R is the gas constant and T is the temperature in Kelvin. The entropy changes (ΔS) were then calculated from ΔG and ΔH using the relationship: $\Delta S = (\Delta H - \Delta G)/T$. The parameter, ΔH_{cell} in Table 1 means

Table 1. Binding and thermodynamic parameters of drug binding to human erythrocyte ghost membranes for a one-class binding model at pH 7·4 and 37°C.

Drug	K ($\times 10^{-4}$, M^{-1})	n (fmol $cell^{-1}$)	$-\Delta H$ (kJ mol^{-1})	$-\Delta H_{cell}$ (pJ $cell^{-1}$)	$-\Delta G$ (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
Fluphenazine	79·78	1·9	14·25	0·413	35·02	67
Perphenazine	10·65	3·4	11·03	0·700	29·83	61
Trifluoperazine	17·67	3·3	18·52	0·989	31·14	34
Prochlorperazine	2·238	4·2	13·81	0·908	25·81	39
Perazine	1·221	7·3	13·60	1·550	24·25	35
Flufenamic acid	0·3124	3·9	20·85	3·428	20·74	−0·4
Mefenamic acid	0·3447	2·1	18·28	1·550	20·99	9
Ibuprofen	0·5799	3·9	2·836	0·201	22·33	63
Flurbiprofen	0·2701	5·1	7·711	0·667	20·36	41
Indomethacin	0·1988	3·5	6·334	3·608	19·57	43

the enthalpy change per cell of the erythrocyte and the values are estimated from the plateau value (ΔQ_{max}) of the titration curve by using the equation: $\Delta Q_{max} = \Delta H_{cell} \cdot F_r \cdot E$, where E is the number of erythrocyte cells.

Phenothiazines were bound to a single class of binding site on the ghost membranes with high affinity ($K = 10^4 - 10^5$ M^{-1}) and high capacity ($n = 1{\cdot}9 - 7{\cdot}3$). The values of K were enhanced by halogen atom(s) at position C-2 in the phenothiazine nucleus ($H < Cl < CF_3$). The binding was characterized by the negative ΔH and positive ΔS values. Nevertheless, anti-inflammatory drugs were bound with lower affinities ($K = 10^3$ M^{-1}) than were phenothiazines. All values of ΔG were almost equal at $20{\cdot}80 \pm 0{\cdot}90$ kJ mol^{-1}. The molecular structure of the drugs contributed to the thermodynamic parameters of ΔH and ΔS; ibuprofen, flurbiprofen and indomethacin were bound to ghosts with small negative ΔH and large positive ΔS values, whereas flufenamic acid and mefenamic acid were bound with large negative ΔH and negligible ΔS values.

Calorimetric titrations of intact erythrocytes

The heat of binding of drugs to intact erythrocytes was measured as a function of D_t in the same concentration range as the binding to the ghosts. The results are shown in Fig. 2. The initial concentration of erythrocyte suspension was 4% (v/v). Each datum point was an average of three measurements and solid lines represented the fitted curves for one class of binding site. The best-fit values of the binding and thermodynamic parameters are tabulated in Table 2. The binding reactions between drugs and intact erythrocytes proceeded exothermically with the increase of drug concentration. There was neither any destruction of the intact erythrocytes in the calorimeter nor any haemolysis caused by drugs.

For the binding reactions between phenothiazines and intact erythrocytes, values of both ΔH and ΔS were strongly negative (from $-65{\cdot}10$ to $-119{\cdot}1$ kJ mol^{-1} and -128 to -308 J mol^{-1} K^{-1}, respectively), and their absolute values decreased in the order FPZ > FPRZ > PPZ > CPRZ > PRZ. Thus, the interactions are influenced by the substituted halogen atom(s) at position C-2 in the phenothiazine nucleus. However, the free energy changes calculated from the K values remained constant ($-25{\cdot}08 \pm 1{\cdot}51$ kJ mol^{-1}). On the other hand, the binding reactions of anti-inflammatory drugs were characterized by the slightly negative ΔH, positive ΔS and ΔG values estimated to be $-20{\cdot}02 \pm 0{\cdot}46$ kJ mol^{-1}. The heat effect and ΔH_{cell} were smaller than those obtained for phenothiazine binding.

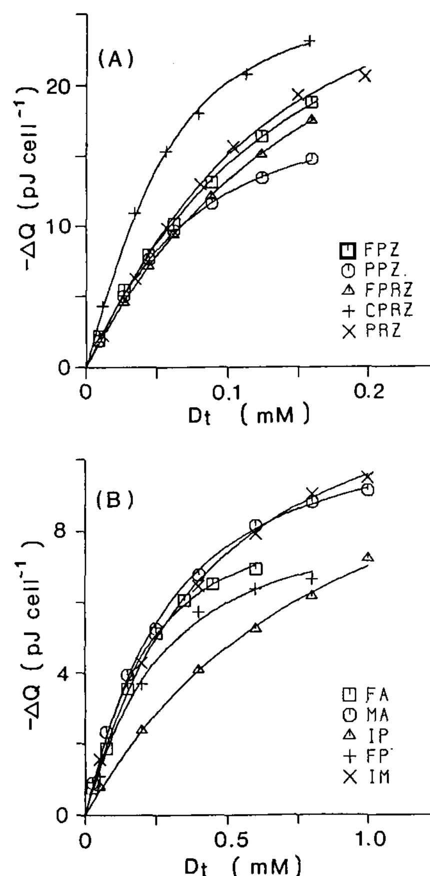

FIG. 2. Calorimetric titration curves for binding reactions of phenothiazines (A) and anti-inflammatory drugs (B) with intact human erythrocytes at pH 7·4 and 37°C. Human erythrocytes were suspended in PBS solution to make an initial haematocrit value of 4% (v/v). Each point shows the mean value of three measurements. Solid lines represent computer-generated best-fit curves assuming a one-class binding model with binding parameters given in Table 2.

Relationship between ΔH and ΔS

In the drug-erythrocyte binding systems, enthalpy-entropy correlation analyses were examined by plotting ΔS against ΔH. Good linear relationships are shown in Fig. 3. These relationships indicate that the dependence of ΔG on the

Table 2. Binding and thermodynamic parameters of drug binding to intact human erythrocytes for a one-class binding model at pH 7·4 and 37°C.

Drug	K ($\times 10^{-4}$, M^{-1})	n (fmol $cell^{-1}$)	$-\Delta H$ (kJ mol^{-1})	$-\Delta H_{cell}$ (pJ $cell^{-1}$)	$-\Delta G$ (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
Fluphenazine	0·941	0·286	119·1	33·70	23·58	−308
Perphenazine	1·992	0·207	99·50	20·49	25·51	−239
Trifluoperazine	0·852	0·331	101·8	33·30	23·33	−253
Prochlorperazine	4·241	0·303	93·01	28·11	27·46	−211
Perazine	2·004	0·435	65·10	29·26	25·53	−128
Flufenamic acid	0·282	0·580	17·32	9·97	20·48	10
Mefenamic acid	0·177	0·809	13·86	11·09	19·28	18
Ibuprofen	0·209	1·503	7·125	10·61	19·70	41
Flurbiprofen	0·256	0·744	11·85	8·75	20·23	27
Indomethacin	0·275	0·699	16·51	11·70	20·41	13

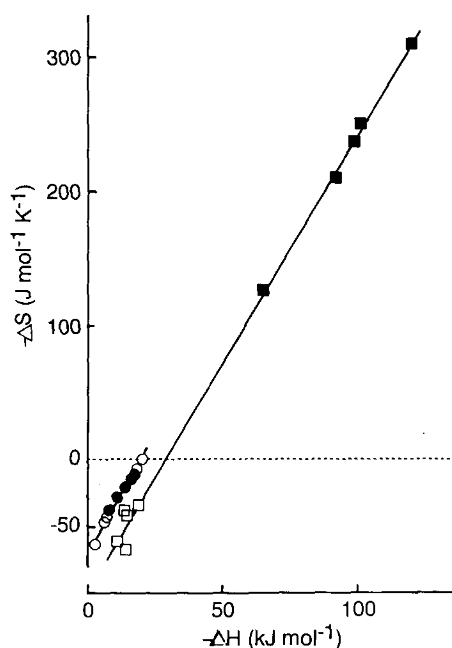

FIG. 3. Correlation between ΔH and ΔS for drugs binding to intact human erythrocytes and ghost membranes. ■ and □, phenothiazine binding to intact erythrocytes and ghosts, respectively; ● and ○, anti-inflammatory drug binding to intact erythrocytes and ghosts, respectively.

binding of drugs to the erythrocytes is much smaller than that of ΔH or TΔS. The plots are described by equations 7 and 8 with excellent correlation coefficients (r = 0·9981 and 0·9911).

Phenothiazines:

$$\Delta H\ (J\ mol^{-1}) = 298 \cdot \Delta S\ (J\ mol^{-1}\ K^{-1}) + 28{\cdot}3 \times 10^3 \quad (7)$$

Anti-inflammatory drugs:

$$\Delta H\ (J\ mol^{-1}) = 300 \cdot \Delta S\ (J\ mol^{-1}\ K^{-1}) + 20{\cdot}2 \times 10^3 \quad (8)$$

Equations 7 and 8 imply that all the ΔG values have to be identical at 298 and 300°K for phenothiazines and anti-inflammatory drugs binding to the human erythrocytes, respectively, and that anionic and cationic drugs bind to different binding sites on the erythrocyte cell.

Discussion

Calorimetric titration of human erythrocytes with drugs has been a useful technique to determine thermodynamic characteristics as well as the binding stoichiometry. The existence of ΔH − ΔS compensation has been shown to imply that a single mechanism predominates in the binding process investigated (Tomlinson 1983), and it is tempting to rationalize the linearities on the basis of the nature of a common binding site for drugs on the erythrocyte cells. As to the extrathermodynamic relationship (isoequilibrium relationship or compensation law, or effect) reviewed by Tomlinson (1983), Krug et al (1976) stated that ΔH vs ΔS plots often lead to an erroneous conclusion due to propagation of experimental error when ΔH is calculated from van't Hoff plots, and demonstrated that the error arises from the experimental temperature range. Therefore, as a method of choice for calorimetric data, ΔH is more accurate than ΔS and consequently the plots of ΔG against ΔH would provide a safe conclusion as stated by Exner (1973). In our experimental techniques, however, calorimetric heat (ΔQ) is measured directly to generate protein-ligand titration curves and to determine all binding parameters (K, n, ΔH and ΔS) from the curves. At a controlled temperature of 37° ± 0·05°C, K and thus ΔG obtained with different drugs was almost constant; −25·08 ± 1·51 kJ mol^{-1} for five cationics and −20·02 ± 0·46 kJ mol^{-1} for five anionics. It is not necessary to estimate the temperature dependence of K to obtain ΔH. The ΔH vs ΔG plots (Krug's data) are linear, because the variation of ΔH is larger compared with the nearly constant ΔG. Since errors in ΔG (or K) can be neglected, the overall fit is estimated by means of the correlation coefficient r = 0·9981 and 0·9911, and the graphical representation (Fig. 3) of the isoequilibrium relationships in the coordinates ΔH and ΔS clearly shows that each of the linear correlations is fulfilled within the experimental errors.

The data presented in Table 1 demonstrate that the binding of the cationic phenothiazines to ghost membranes is an entropy-driven reaction associated with a small negative ΔH and positive ΔS, which can be taken as evidence of a large degree of hydrophobic interaction (Ross & Subraminian 1981). The introduction of halogen atom(s) into the phenothiazine nucleus results in a significant increase in the binding affinity of the drug to the membrane of human

erythrocytes, reflecting their hydrophobic characteristics in an increasing order of $H < Cl < CF_3$. This result is consistent with the order of the binding affinity of phenothiazines obtained by Kanaho et al (1981) and that of surface activity of the drugs reported by Zografi & Munshi (1970). On the other hand, the affinity of the anionic anti-inflammatory drugs for the membrane is remarkably lower than that of the cationic drugs. The thermodynamic parameters varied widely from $-2{\cdot}8$ to $-20{\cdot}9$ kJ mol^{-1} (ΔH) and $-0{\cdot}4$ to 63 J mol^{-1} K^{-1} (ΔS), suggesting that other interacting forces were acting to mitigate the hydrophobic effect. This is because negative enthalpy and entropy changes arise from van der Waals' interaction and hydrogen bonding formation in low dielectric media. It has also been pointed out that positive entropy change may be a manifestation of electrostatic forces, in which case, the enthalpy change is expected to be very small or near zero for a purely electrostatic interaction (Klotz 1973). According to the report by Cousin & Motais (1982), hydrophobic regions of the flufenamate molecule play a leading part but hydrophilic —COO^- and —NH— groups which can readily form intramolecular hydrogen bonds (—NH . . . O) also contribute to the binding. It is therefore suggested that the binding site of flufenamate exhibited positively charged group(s) close to the hydrophobic surface with electron-donor groups on the erythrocyte membrane. Upon binding, the negative charge of the carboxylate anion of the drug is neutralized by a cationic locus on the protein; the complexed anion behaves then as a neutral molecule with respect to hydrophobic interactions.

The thermodynamic parameters for the binding reaction of drugs with the intact erythrocytes determine the contributions of the overall reactions with the erythrocyte cells containing haemoglobin. In the case of phenothiazines, the values of ΔH, ΔH_{cell} and ΔS were negatively larger than those binding to the ghosts (Table 2). The absolute values increased when the binding affinities for the membranes were increased. These results suggest either that the drugs may be inserted into erythrocyte cells instead of remaining on the membrane surface or that the drugs react directly with the intra- or inter-cellular constituents of erythrocytes such as haemoglobin. This is because the more the drug enters the potential surface of the erythrocyte binding sites, the more strongly it is bound (the more negative the ΔH of binding), and the more its rotational and translational freedom will be restricted (the more negative the ΔS). This result agrees with the conclusion that the phenothiazines have an expanding effect on erythrocyte membranes similar to the manner in which lipid mono-layers are penetrated by these constituents (Seeman & Kwant 1969; Ogiso et al 1986). In contrast, the results of anti-inflammatory drugs with intact erythrocytes indicate that the binding affinities, ΔH and ΔS values are the same as the ghosts, implying very little binding of the drug to haemoglobin in the cell. It is therefore suggested that the binding site of these drugs is on the surface rather than in a pocket of the erythrocyte cells. Thus, these results support the bilayer couple hypothesis for drug-induced morphological changes (Sheetz & Singer 1974); cationic drugs intercalate mainly into the inner monolayer, thus inducing invagination whereas anionic drugs interact with the outer monolayer and cause the cell to crenate.

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