Effect of Ion Adsorption on the Electrokinetic Properties of Erythrocytes

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Abstract. The microelectrophoresis technique was used to determine the dependence of human erythrocyte surface potential on the concentration of various cations and anions. The interpretation of the results is based on the Gouy—Chapman—Stern theory. Values of pK, characterizing the binding of ions to the external surface of erythrocytes, as well as numbers of binding sites per unit area were determined. The affinities of ions for the red cell membrane were shown to decrease in the sequence: $H^+ > Ca^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+} > Li^+ > Na^+ \simeq \simeq K^+ \simeq NH_4^+$ and trinitrophenol $> IO_4^- > ClO_4^-$ > salicylate $\simeq I^- > > SCN^- > H_2PO_4^- > Br^- > Cl^- > HPO_4^{2-}$. Changes in the ionic strength of the medium resulted in changes in numbers of exposed ion-binding sites. This phenomenon is interpreted in terms of ionic strength-dependent structural transformations of the cell surface coat.

Key words: Erythrocyte — Microelectrophoresis — Surface potential — Ion binding

Introduction

There is strong evidence that red blood cells carry net negative charge at their external surface (Abramson 1934; Furchgott and Ponder 1941; Bangham et al. 1958; Seaman and Heard 1960; Eylar et al. 1962; Jan and Chien 1973a, b; Donath and Lerche 1980; Snabre and Mills 1985; Pasquale et al. 1986). It was concluded based on early electrophoretic studies of red cells and lipid particles that this charge was due to acidic phospholipids of erythrocyte plasma membranes (Furchgott and Ponder 1941; Bangham et al. 1958; Ponder and Ponder 1960). Dolowy and Godlewski (1980) suggested that the experimentally measured values of red cell surface charge density could be attributed to acidic lipids of cell membranes. However, Lin et al. (1983) demonstrated that the outer surface of the human erythrocyte bilayer membrane was electrically neutral,

whereas the cytoplasmic surface of the membrane was negatively charged. This finding is consistent with the fact that acidic lipids are located predominantly in the inner leaflet of the human erythrocyte plasma membranes (Gordesky and Marinetty 1973; Op den Kamp 1979; McEvoy et al. 1986).

The treatment of red blood cells with influenza virus, neuraminidase, or other proteolytic enzymes substantially decreased the cell electrophoretic mobility (Hanig 1948; Cook et al. 1961; Eylar et al. 1962; Seaman and Uhlenbruck 1963; Hydon and Seaman 1967). The release of neuraminic acid from red cell membranes under the action of neuraminidase showed that the enzyme is an α-glycosidase (Klenk and Lempfrid 1957; Klenk and Uhlenbruck 1958; Eylar et al. 1962). These data, as well as a linear correlation between the amount of liberated neuraminic acid and the decrease in cell electrophoretic mobility, clearly suggested that the net negative charge of the red cell outer surface is derived from carboxyl groups of neuraminic acid integrated in cell surface glycoproteins (Heard and Seaman 1960; Seaman and Heard 1960; Cook et al. 1960; Seaman and Cook 1965; Markosian et al. 1977; Nordt et al. 1978; Walter et al. 1983). It was shown that almost all of the surface neuraminic acid in human erythrocytes is represented by N-acetylneuraminic acid (NANA, or sialic acid), the carboxyl groups of which contribute 60 to 65 % to the total surface charge of these cells (Cook et al. 1961; Cook 1962; Eylar et al. 1962; Haydon and Seaman 1967; Mehrishi 1972; Seaman 1973; Viitala and Järnefelt 1985). On the other hand, some investigators reported that treatment with neuraminidase degraded 70 to 100 % of NANA and abolished erythrocyte electrophoretic mobility (Nordt et al. 1978; Donath and Lerche 1980; Walter et al. 1983).

A number of studies have shown that the erythrocyte surface charge is affected by selective adsorption of ions to the cell plasma membrane. The competitive binding of calcium, barium, and hydrogen cations, as well as chloride, phosphate, and acetate anions to red cell membranes was observed as early as in 1921 (Coulter 1921). The differential effects of cations on the erythrocyte surface potential (Abramson 1934; Jandl and Simmons 1957; Seaman and Pethica 1964) and the suppression of Ca^{2+} binding by mono-, di-, and trivalent cations (Gent et al. 1964; Long and Mouat 1971; Mikkelsen and Wallach 1974; Cohen and Solomon 1976) provided evidence for selective binding of cations to red blood cell membranes. However, quantitative data on cation binding to erythrocyte membranes are largely inconsistent; e.g. the following values for Ca^{2+} binding constant have been reported: 11.4 l/mol (Seaman et al. 1969), 3.6×10^3 l/mol (Gent et al. 1964; Long and Mouat 1971), and up to 2×10^5 l/mol (Cohen and Solomon 1976).

Wieth (1970a, b) observed that monovalent anions increased cation (Na^+, K^+) permeability and inhibited anion (Cl^-, SO_4^{2-}) permeability of red cell

membranes. It is rather surprising that no comprehensive data have since been reported on anion binding to erythrocyte membranes.

Based on the above, the present work was undertaken to quantitate selective binding of various cations and anions to human erythrocytes and to examine the effect of ion binding on red cell surface electrostatic potential.

Cell electrokinetic properties have mostly been described by the classical Gouy-Chapman and Helmholtz-Smoluchowski theory. In recent years an improved theory has been put forward; it claims that the cell electrophoretic mobility depends on volume, rather than surface, charge density of the glycocalyx, as well as on the glycocalyx thickness and the hydrodynamic drag exerted by the cell surface coat on the electroosmotic flow (Pastushenko and Donath 1976; Donath and Pastushenko 1979, 1980; Levine et al. 1983; Snabre and Mills 1985; Snabre et al. 1986; Donath and Voigt 1986; Pasquale et al. 1986). This new theory, however, fails to overcome difficulties in describing the experimental dependence of cell mobility on salt concentration. In particular, a deviation between experimental and theoretical curves occurs below 75 mmol/l for 1 : 1 electrolytes and below 50 mmol/l for 2 : 2 electrolytes (Levine et al. 1983; Snabre and Mills 1985). Snabre et al. (1986) attempted to overcome this by using the nonlinearized Poisson—Boltzmann equation and found that at ion concentrations lower than 20 mmol/l the theory still predicted values of electrophoretic mobility exceeding the experimentally obtained results by up to 30 %. This was explained, as in the previous case (Levine et al. 1983; Snabre and Mills 1985), by glycocalyx swelling due to decreased charge shielding at low ionic strengths. On the other hand, the new theory provided highly divergent values for the main parameters of the human erythrocyte surface coat, i.e. the glycocalyx thickness and the mean glycoprotein segment radius: 7.5 nm and 0.7 nm. (Levine et al. 1983) and 4 nm and 0.45 nm, respectively (Snabre and Mills 1985).

Hence the use of the new, more complicated multiparameter theory obviously makes little sense. Therefore we used the classical Gouy-Chapman-Stern theory to describe ion binding and screening effects.

Materials and Methods

Human erythrocytes were obtained from fresh peripheral blood of two healthy male donors. The blood was derived by fingerpuncture and immediately diluted with isotonic solutions of the respective ionic composition. The cell electrophoretic mobility was measured at 37 °C using a Parmoquant-2 device (Carl Zeiss Jena, GDR). Values of the zeta potential (electrostatic potential at the slipping plane) were determined by the Helmholtz—Smoluchowski equation:

$$\zeta = \eta u / \varepsilon_0 \varepsilon \tag{1}$$

where ζ is the zeta potential, u is the electrophoretic mobility, η and ε are the dynamic viscosity and

the dielectric constant of the medium, respectively, $\varepsilon_0 (= 8.85 \times 10^{-12}$ F/m) is the permittivity of free space. The mobilities of 100 to 300 red cells from each donor were measured and averaged. Usual standard deviations were 3 to 7% of the respective mean values, and mobilities of cells from different donors were indistinguishable within the range of experimental error. Sodium-potassium phosphate buffer containing (mmol/l) 53.36 Na_2HPO_4 and 13.34 KH_2PO_4 (pH 7.4) or 317 sucrose and 5 Tris-HCl (pH 7.4) was used as initial suspending medium. The measurements of pH-dependence of red cell electrophoretic mobility were carried out at two ionic strengths: (a) in solutions containing (mmol/l) 140 NaCl, 5 KCl, and 27 sucrose, and (b) 17.5 NaCl, 0.625 KCl, and 280.75 sucrose. The pH of the medium was changed by adding 145 mmol/l HCl or NaOH + 27 mmol/l sucrose (a), or 18.125 mmol/l HCl or NaOH + 280.75 mmol/l sucrose (b). To vary the anion composition of the suspending medium containing phosphate buffer, isotonic (173 mmol/l) salt solutions were added up to a final concentration of 100 mmol/l. This changed the pH of the medium by no more than 0.2 pH units. In experiments designed to study the effect of the ionic strength, isoosmolality was maintained by the addition of sucrose. The dynamic viscosity of all the solutions used was carefully measured at room temperature, then extrapolated to 37°C using Tables of Temperature—Dependence of the Viscosity of Sucrose Solutions (Handbook Biochem. Mol. Biol., 1976). The bulk values of η and $\varepsilon = 74$ at 37°C were used in Eq. (1).

The curves of the salt concentration-dependence of zeta potential were described by the equation:

$$\sigma_{ads}^{(+)} - \sigma_{ads}^{(-)} - \sigma_0 = \text{sign}\,\zeta[2\varepsilon_0 \varepsilon R T \sum C_i (e^{z_i x} - 1)]^{1/2} \tag{2}$$

where $\sigma_{ads}^{(+)}$ and $\sigma_{ads}^{(-)}$ are the contributions to the cell surface charge density determined by the binding of cations and anions, respectively, σ_0 is the charge density irrespective of ion binding, C_i is the bulk concentration of the ith ion, z_i is the valence number of the ith ion taken with its sign, $x = F\psi_0/RT$, ψ_0 is the electrostatic potential of the outer Helmholtz plane, F is the Faraday constant, R is the gas constant, T is the absolute temperature. Eq. (2) was used on the assumption that $\psi_0 = \zeta$. The theoretical curves were simulated using a CM-3 computer (USSR).

Results

Curves of pH-dependence of the erythrocyte zeta potential at two different ionic strengths are shown in Fig. 1. Let us assume, for the sake of simplicity, that protons are the only ions to be bound to the cell surface and that this binding is characterized by a single pK value. Then, making use of the Langmuir-Stern adsorption isotherm and taking $\sigma_{ads}^{(-)} = 0$, we get instead of Eq. (2):

$$\frac{N^{(+)}}{1 + e^x/K_H C_H} - \sigma_0 = \text{sign}\,\zeta \sqrt{8\,\varepsilon_0 \varepsilon R T I}\,\text{sh}\frac{x}{2} \tag{3}$$

where $N^{(+)}$ is the number of titrable groups per unit area, K_H is the proton binding constant ($pK = \log K$), I is the ionic strength. The experimental curves shown in Fig. 1 corresponding to $I = 145$ mmol/l suggests that the value of pK is in the range between 3 and 4. Consequently, at a pH range from 9 to 11 the protonable sites under consideration are fully dissociated, i.e. the first term in

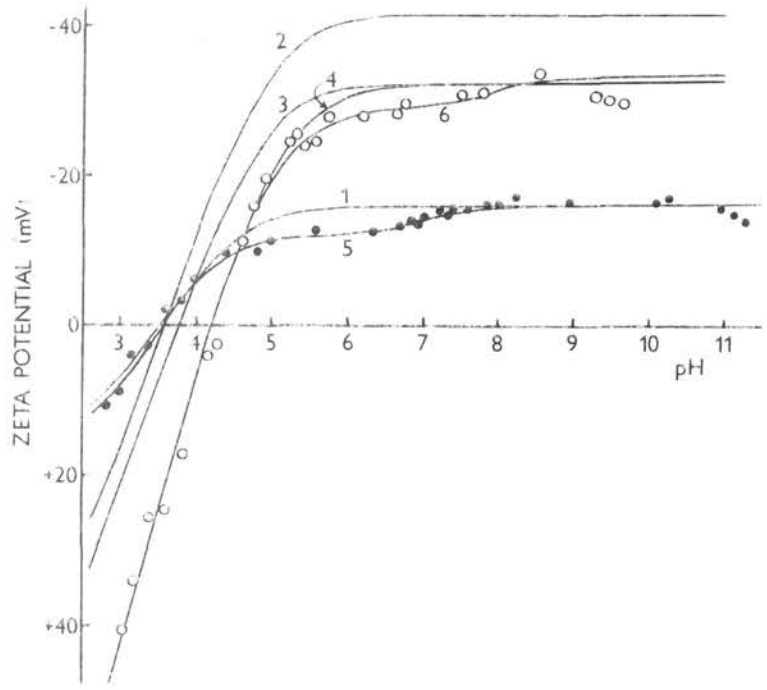

Fig. 1. Dependence of the red cell zeta potential on pH at ionic strengths of 145 (closed circles) and 18.125 mmol/l (open circles). For explanation of the theoretical curves, see the text.

Eq. (3) is zero. In this pH range the zeta potential is -16 mV. By Eq. (3) we get $\sigma_0 = 13.47\,\mathrm{mC/m^2}$. The parameters $N^{(+)} = 28\,\mathrm{mC/m^2}$ and $K_H = 3.4 \times 10^3\,\mathrm{l/mol}$ (pK = 3.53) are then derived from the best fit of curve 1 with the corresponding experimental curve. The same values of σ_0, $N^{(+)}$, and K_H at $I = 18.125\,\mathrm{mmol/l}$ yield curve 2 (Fig. 1) which by no means describes the experimental results. Obviously, lowering of the ionic strength results in a change in at least one of these three parameters. As a matter of fact, at $I = 18.125\,\mathrm{mmol/l}$ the zeta potential is approximately -32.5 mV in the pH range from 8 to 9 (Fig. 1). This corresponds to $\sigma_0 = 10.13\,\mathrm{mC/m^2}$. Curve 3 (Fig. 1) was calculated by Eq. (3) using $\sigma_0 = 10.13\,\mathrm{mC/m^2}$, $N^{(+)} = 28\,\mathrm{mC/m^2}$, and $K_H = 3.4 \times 10^3\,\mathrm{l/mol}$. The discrepancy between the calculated and the experimentally obtained curves suggests that in addition to σ_0 some other parameter is changed at low ionic strength. Curve 4 (Fig. 1) was constructed with $\sigma_0 = 10.13\,\mathrm{mC/m^2}$, $N^{(+)} = 59.6\,\mathrm{mC/m^2}$, $K_H = 3.4 \times 10^3\,\mathrm{l/mol}$ and it satisfactorily describes the experimental data.

A careful analysis of the results shown in Fig. 1 indicates that significant

deviations of the zeta potential exist not only at pH 3 to 5 but also at pH 6.5 to 8.0. This might be explained by the existence of a second titrable group at the cell surface. On the other hand, Sanui and Pace (1962) and Sanui et al. (1962) demonstrated that Na^+ and K^+ ions are able to compete with protons for titrable groups on the surface of human erythrocytes. It is therefore reasonable to describe the experimental data (Fig. 1) by assuming two types of cation-binding groups with distinct binding constants for H^+, Na^+, and K^+ ions. Hence, the following equation is to be used:

$$\sum_{i=1}^{2} \frac{N_i^{(+)}}{1 + e^{x}[K_{i,Na}(C_{Na} + C_K) + K_{i,H} C_H]^{-1}} - \sigma_0 = \text{sign}\,\zeta \sqrt{8\varepsilon_0 \varepsilon R\, TI}\, \text{sh}\frac{x}{2} \quad (4)$$

As will be shown later, Na^+ and K^+ ions have the same binding constant denoted $K_{i,Na}$ in Eq. (4). Curves 5 and 6 (Fig. 1) were calculated by Eq. (4) implicating ionic strength-independent binding constants: 0.917, 1.8×10^2, 3.4×10^3, and 3.54×10^8 l/mol for $K_{1,Na}$, $K_{2,Na}$, $K_{1,H}$, and $K_{2,H}$, respectively. The corresponding pK values are -0.037, 2.25, 3.53, and 8.55. The parameters $N_1^{(+)}$, $N_2^{(+)}$, and σ_0 were shown to depend on the ionic strength. Their values were 28, 202, and 217 mC/m^2 at $I = 145$ mmol/l (curve 5) and 66, 26, and 38 mC/m^2 at $I = 18.125$ mmol/l, respectively (curve 6). It should be noted that these data as deduced using the new approach (two independent cation-binding sites and competition between H^+, Na^+, and K^+ ions) are principally in no contradiction to the results obtained with the simplier approach (single type of titrable groups and no Na^+ and K^+ binding); in both cases, the values for pK and the surface density of low-affinity titrable groups are in good agreement.

Fig. 2 illustrates the dependence of the erythrocyte zeta potential on the concentration of alkaline-earth metal chlorides. Since the experiments were performed at different ionic strengths, appropriate concentrations of sucrose were added to maintain the isoosmolality. Although the four experimental curves shown in Fig. 2 differ from each other significantly (which is a convincing evidence for selective interactions of the cations with the red cell membrane), we attempted describing the results by a theoretical curve calculated by Eq. (2). In doing so we assumed that no ion binding took place ($\sigma_{ads}^{(+)} = \sigma_{ads}^{(-)} = 0$) and used $\sigma_0 = 6.654$ mC/m^2 as derived from the zeta potential of -39.5 mV in absence of divalent cations and in the presence of 317 mmol/l sucrose and 5 mmol/l Tris-HCl. Interestingly enough, this curve (curve 1, Fig. 2*A*) satisfactorily describes the experimentally observed dependence of the zeta potential on Ca^{2+} and/or Sr^{2+} concentration, although it deviates from both experimental curves at low ion concentrations. If the differences between the four experimental curves are due to selective binding of the cations, then $K \simeq 0$ for Ca^{2+} and Sr^{2+} implies that $K > 0$ for Mg^{2+} and Ba^{2+}. This should have resulted in more positive

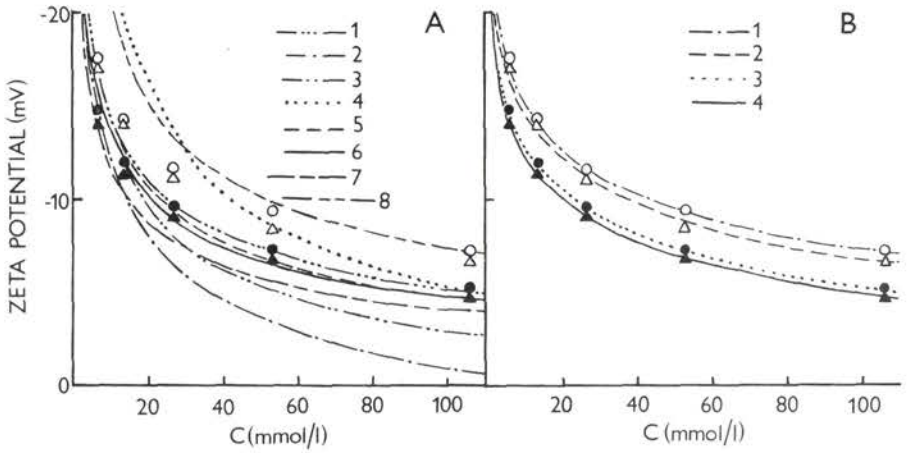

Fig. 2. Dependence of the red cell zeta potential on the concentration of $BaCl_2$ (open circles), $MgCl_2$ (open triangles), $SrCl_2$ (closed circles), and $CaCl_2$ (closed triangles) in the presence of 5 mmol/l Tris-HCl (pH 7.4) and of varying sucrose concentrations. Parameters used to calculate curves 1—8 in panel A are given in the text. The binding constants used to calculate curves 1—4 in panel B are shown in Table 1.

values of the zeta potential in the presence of the latter cations. However, the opposite is shown by the experiment. Hence, the no-ion-binding hypothesis seems to be invalid. To account for the selective binding of divalent cations, Eq. (2) will be rewritten in the form:

$$\frac{2N^{(+)}}{1 + e^{2x}/K_M C_M} - \sigma_0 + [2\varepsilon_0 \varepsilon R T \sum C_i (e^{-z_i x} - 1)]^{1/2} = 0 \qquad (5)$$

where K_M and C_M are binding constants and the concentration of the divalent cation, respectively. Let us construct a curve via Eq. (5) using $N^{(+)} = 28\,mC/m^2$ (the same as curve 1, Fig. 1), $\sigma_0 = 6.654\,mC/m^2$, and a very low binding constant, e.g. $K_M = 1$ l/mol. This curve (curve 2, Fig. 2*A*) does not describe any of the experimentally obtained dependences. Evidently, the situation can be improved by lowering $N^{(+)}$. Suppose that two titrable groups bind one divalent cation, i.e. $N^{(+)} = 14\,mC/m^2$. Curve 3 in Fig. 2*A* was constructed using this value of $N^{(+)}$ and the above values of K_M and σ_0 (see curve 2, Fig. 2*A*), and is as useless as curve 2. Seaman et al. (1969) reported that the binding of Ca^{2+} ions to human red cells was characterized by parameters $K_M = 11.4$ l/mol and $N^{(+)} = 8.01\,mC/m^2$. We attempted to describe the Ca^{2+}-curve in Fig. 2 by these parameters and $\sigma_0 = 16.63\,mC/m^2$, chosen to fit the experimental curve at high

Ca^{2+} concentrations (curve 4, Fig. 2*A*). The failure of this curve to describe experimental data does not indicate discrepancy between our results and those of Seaman et al. (1969) since the latter authors derived their parameters from experiments carried out at constant ionic strength. Curve 5 in Fig. 2*A* was calculated by Eq. (5) using $K_M = 1\,l/mol$, $N^{(+)} = 2.43\,mC/m^2$, and $\sigma_0 = 6.654\,mC/m^2$. This curve, similarly as curve 1, fits reasonably well the Ca^{2+}- and/or Sr^{2+}-curves at high ion concentrations. A better fitting at low ionic strengths can presumably be achieved by increasing K_M and by lowering $N^{(+)}$. A 500-fold increase in K_M gives a curve (curve 6, Fig. 2*A*, $K_M = 500\,l/mol$, $N^{(+)} = 0.343\,mC/m^2$, $\sigma_0 = 6.654\,mC/m^2$) which describes rather well the C_{Ca}-dependence of the cell surface potential at $C_{Ca} > 20\,mmol/l$. It should be stressed that the experimental values of zeta potential shown in Fig. 2 were highly reproducible and exhibited trifling deviations, therefore we demand exact coincidence between the experimental and theoretical curves. Curve 7 in Fig. 2*A* was calculated by Eq. (5) with parameters: $K_M = 500\,l/mol$, $N^{(+)} = 0.778\,mC/m^2$, $\sigma_0 = 6.654\,mC/m^2$. The value of $N^{(+)}$ was derived from exact fitting of this and the experimental Ca^{2+}-curve at $C_{Ca} < 20\,mmol/l$. As expected, this curve predicts more positive values of the zeta potential at $C_{Ca} > 20\,mmol/l$. As shown above, the value of $\sigma_0 = 6.654\,mC/m^2$ cannot be used to describe the experimental curves for Mg^{2+} and Ba^{2+} ions. To describe the Ba^{2+}-curve, let us try to use $\sigma_0 = 13.47\,mC/m^2$, taken from Fig. 1 ($I = 145\,mmol/l$). This curve (curve 8, Fig. 2*A*, $K_M = 400\,l/mol$, $N^{(+)} = 2.07\,mC/m^2$, $\sigma_0 = 13.47\,mC/m^2$) also fails to describe the experimental data at low Ba^{2+} concentrations. We shall not present here the results of further attempts to theoretically describe the experimental curves in Fig. 2 using constant (independent of ionic strength) parameters. Instead, we shall confine ourselves to say that such parameters yield theoretical curves steeper than the experimental curves. Evidently, additional factors are to be considered. In particular, as suggested by Fig. 1, the number of the cation-binding sites may depend on the ionic strength. On the other hand, as shown by subsequent results, the binding of Cl^- anions to erythrocyte membranes also accounts for some peculiarities of the electrokinetic behavior of the cells. The ionic strength-dependence of the number of high-affinity cation-binding sites, $N_2^{(+)}$, can be written as $N_2^{(+)} = 1.4I$, where $N_2^{(+)}$ and I are expressed in the units of mC/m^2 and mmol/l, respectively (see Fig. 1). To describe the experimental data in Fig. 2 we use Eq. (2) written in the form:

$$\frac{2N^{(+)}}{1 + 1/K_M C_M e^{-2\chi}} - \frac{N^{(-)}}{1 + 1/K_{Cl} C_{Cl} e^{\chi}} - \sigma_0 = \text{sign}\,\zeta[2\varepsilon_0 \varepsilon RT \sum C_i (e^{-z_i\chi} - 1)]^{1/2} \tag{6}$$

where $N^{(-)}$ is the surface density of the anion-binding sites. The theoretical

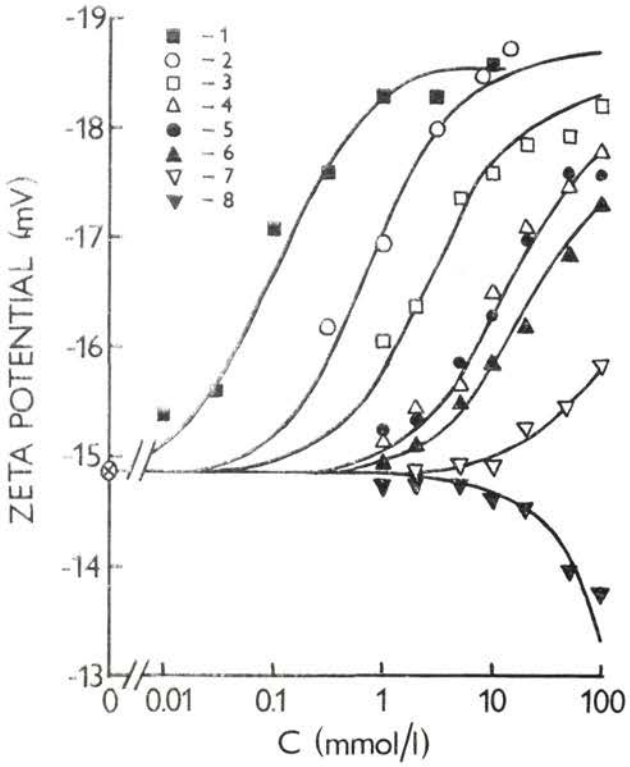

Fig. 3. The dependence of the red cell zeta potential on the concentration of trinitrophenol, periodate, perchlorate, salicylate, iodide, thiocyanate, bromide, and chloride (curves 1—8, respectively). All the anions were used as potassium salts, except for salicylate, which was used as sodium salt. The initial medium contained 53.36 mmol/l Na_2HPO_4 and 13.34 mmol/l KH_2PO_4 (pH 7.4). The symbol at zero concentration corresponds to the mean zeta potential of cells in non-diluted phosphate buffer. For explanation of the theoretical curves, see the text and Table 1.

curves in Fig. 2*B* were calculated by Eq. (6) on the assumption that $N^{(+)}$ depends on the ionic strength similarly as does $N_2^{(+)}$, i.e. $N^{(+)} = 1.4\,I$. Values of $N^{(-)} = 5.5\,\mathrm{mC/m^2}$ and $K_{Cl} = 0.51/\mathrm{mol}$ were used (see Fig.3). Futhermore, the experimental results could be described satisfactorily only assuming that σ_0 also depends on the ionic strength. The theoretical curves turned out to be extremely sensitive to the coefficient of the linear dependence of σ_0 on I, and the relation $\sigma_0 = 2.7881\,I$ appeared to be proper. Then the best fit of the theoretical to the experimental curves in Fig. 2B was achieved by choosing the values of divalent cation binding constants. Thus, the data in Figs. 1 and 2 indicate that ion binding to red cell membranes under conditions of varying ionic strength can be quantified when the dependence of both the surface charge density of the cell and the number of ion-binding sites on the ionic strength are taken into account.

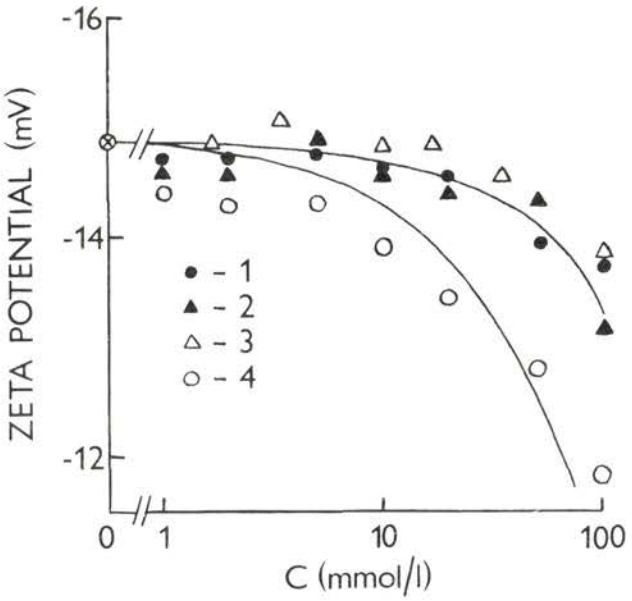

Fig. 4. Erythrocyte zeta potential as a function of KCl, NaCl, NH_4Cl, and LiCl concentration (curves 1—4, respectively). The symbol at zero concentration shows the mean zeta potential of cells in non-diluted phosphate buffer (53.36 mmol/l Na_2HPO_4 and 13.34 mmol/l KH_2PO_4, pH 7.4). The theoretical curve (1—3) is identical with curve 8 in Fig. 3. For the calculation of curve 4, see the text.

The data in Fig. 3 show that replacement of the phosphate buffer anions for other anions (with the exception of Cl^-) results in an increase in the negative zeta potential of erythrocytes. In contrast, an increase in Cl^- concentration in the medium with concomitant lowering of phosphate concentration is accompanied by a reduction of the cell surface potential. These effects are obviously due to selective binding of anions to the red cell outer surface. The medium initially contained 53.36 mmol/l Na_2HPO_4 and 13.34 mmol/l KH_2PO_4 (pH 7.4), which corresponds to an ionic strength of $I = 3C_1 + C_2$, where C_1 and C_2 are the concentrations of HPO_4^{2-} and $H_2PO_4^-$, respectively. Note that $C_1 = 4C_2$. A dilution of the phosphate buffer with 173 mmol/l solution of the salt of ith anion changes C_2 and C_1 to $C_2' = C_2 - C_i/13$ and $C_1' = 4C_2'$, where C_i is the ith anion concentration. Considering these relations, and with $C_K = C_2' + C_i$ and $C_{Na} = 2C_1'$, Eq. (2) can be written as:

$$\frac{N^{(-)}[(C_2 - C_i/13)(8K_1 e^x + K_2) + K_i C_i] e^x}{1 + [(C_2 - C_i/13)(4K_1 e^x + K_2) + K_i C_i] e^x} + \sigma_0 = \tag{7}$$

$$= \{2\varepsilon_0 \varepsilon RT[(C_2 - C_i/13)(4e^{2x} + e^x + 9e^{-x} - 14) + C_i(e^x + e^{-x} - 2)]\}^{1/2}$$

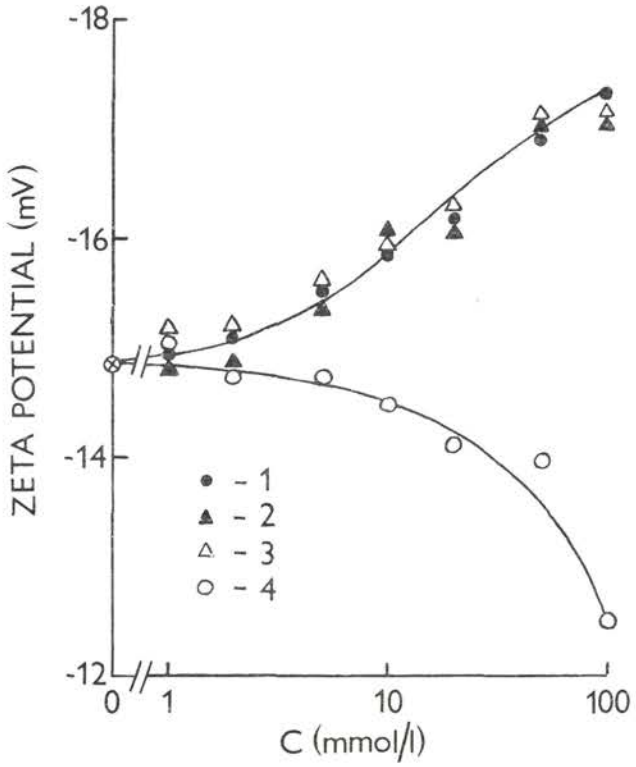

Fig. 5. Erythrocyte zeta potential as a function of KSCN, NaSCN, NH_4SCN, and LiSCN concentration (curves 1—4, respectively). The theoretical curve (1—3) is identical with curve 6 in Fig. 3. For the calculation of curve 4, see the text. Other conditions as in Fig. 4.

where K_1, K_2, and K_i are the binding constants of HPO_4^{2-}, $H_2PO_4^-$, and the ith anion, respectively. In deriving Eq. (7) the term $\sigma_{ads}^{(+)}$ (see Eq. (2)) was omitted because the change in the anion composition was not associated with significant changes in the cation composition of the medium. This implies that the share in the cell surface charge density of cation binding is included into σ_0. This in turn suggests that the term σ_0 has distinct meanings in Eqs. (4)—(7). The curves shown in Fig. 3 were calculated by Eq. (7) using $\sigma_0 = 10.5\,mC/m^2$ and the parameters K and $N^{(-)}$ given in Table 1. The data presented in Fig. 3 are described in terms of competitive binding of the anions HPO_4^{2-}, $H_2PO_4^-$, and the ith anion, replacing the phosphates. Thus, both the sign and the extent to which the zeta potential of the cells is changed, depend on the relative values of the binding constants of these three anions. The decrease in the zeta potential upon the replacement of the phosphate anions with Cl^- is obviously due to the fact

that $K_{Cl} \simeq K_1 \ll K_2$ (see Table 1). The relation $K_1 < K_{Br} < K_2$ is reflected in the moderate rise in zeta potential with increasing Br^- concentration. The progressive elevation of the cell surface potential accompanying the replacement of phosphates with the other anions tested is a result of higher affinity of the latter ions to erythrocyte membranes, i.e. $K_1 < K_2 < K_i$.

Figs. 4 and 5 illustrate the dependence of the red cell zeta potential on the concentration of chlorides and thiocyanates of univalent cations, respectively. Data of these figures clearly indicate that the effects of Na^+, K^+, and NH_4^+ ions are approximately the same, whereas Li^+ ions markedly suppress the cell surface potential. In terms of the binding constants, this may be expressed as $K_{Na} \simeq K_K \simeq K_{NH_4} < K_{Li}$. It is important to note, however, that a much steeper reduction of the negative zeta potential was observed with lithium thiocyanate (Fig. 5) than with lithium chloride (Fig. 4). This phenomenon can be explained suggesting that Li^+ ions are able to compete not only with other cations but also with SCN^- anions. A comparison of the curves for LiCl (Fig. 4) and LiSCN (Fig. 5) shows that Li^+ ions compete with SCN^- ions for only a part of the "anion-binding" sites, $\gamma N^{(-)}$, with $0 < \gamma < 1$. Considering the above conclusions, Eq. (2) can be rewritten as:

$$\frac{N^{(+)}}{1 + e^{x}[9K_{Na}(C_2 - C_i/13) + K_{2,Li}C_i]^{-1}} -$$

$$\frac{\gamma N^{(-)}\{[(8K_1 e^{x} + K_2)(C_2 - C_i/13) + K_1 C_i]e^{x} - K_{1,Li}C_i e^{-x}\}}{1 + [(4K_1 e^{x} + K_2)(C_2 - C_i/13) + K_1 C_i]e^{x} + K_{1,Li}C_i e^{-x}} -$$

$$\frac{(1-\gamma)N^{(-)}[(8K_1 e^{x} + K_2)(C_2 - C_i/13) + K_1 C_i]e^{x}}{1 + [(4K_1 e^{x} + K_2)(C_2 - C_i/13) + K_1 C_i]e^{x}} - \sigma_0 + \tag{8}$$

$$\{2\varepsilon_0 \varepsilon RT[(C_2 - C_i/13)(4e^{2x} + e^{x} + 9e^{-x} - 14) + C_i(e^{x} - e^{-x} - 2)]\}^{1/2} = 0$$

where K_1, K_2, and K_i have the same meaning as in Eq. (7), $K_{1,Li}$ is the Li^+ binding constant to the sites $\gamma N^{(-)}$, which bind competitively Li^+ and SCN^- ions, $K_{2,Li}$ is the Li^+ binding constant to the cation-binding sites, $N^{(+)}$.

Curves 4 in Figs. 4 and 5 were constructed according to Eq. (8) using $K_{Na} = 180$ l/mol, $K_1 = 0.387$ l/mol, $K_2 = 85.11$/mol, $K_{2,Li} = 350$ l/mol, $N^{(+)} = 203$ mC/m^2, $N^{(-)} = 5.54$ mC/m^2, and $K_i = 0.5$ and 128 l/mol, $K_{1,Li} = 0.0$ and 50 l/mol, $\gamma = 0.0$ and 0.35, respectively. Note that the values for K_{Na} and $N^{(+)}$ agree with those characterizing the high-affinity Na^+-and/or K^+-binding sites at physiological ionic strength as derived from data in Fig. 1. Figs. 4 and 5 show that the same parameters apply to NH_4^+ ions. The values of K_i (the subscript i stands for Cl^- in Fig.4, and for SCN^- in Fig. 5), K_1, K_2, and $N^{(-)}$ were derived

Table 1. The values of binding constants (K), pK, surface densities of ion-binding sites (N), and the mean surface area per ion-binding site (S), characterizing the binding of ions to human erythrocyte outer surface at ionic strength of 145 mmol/l (cations) and 173 mmol/l (anions)

Ion	K (l/mol)	pK	N (mC/m^2)	Sn (m^2)
H^+	$K_1 = 3.40 \times 10^3$	3.53	28	5.71
	$K_2 = 3.54 \times 10^8$	8.55	202	0.79
Li^+	$K_1 = 5.00 \times 10^{1}$ [a]	1.70 [a]	1.94 [a]	82.5 [a]
	$K_2 = 3.50 \times 10^2$	2.54	203	0.79
Na^+ [b]	$K_1 = 9.17 \times 10^{-1}$	−0.037	28	5.71
	$K_2 = 1.80 \times 10^2$	2.25	202	0.79
Ba^{2+}	4.00×10^2	2.60	203	0.79
Mg^{2+}	4.36×10^2	2.64	203	0.79
Sr^{2+}	6.00×10^2	2.78	203	0.79
Ca^{2+}	6.30×10^2	2.80	203	0.79
HPO_4^{2-}	3.87×10^{-1}	−0.41	5.500	29.1
Cl^-	5.00×10^{-1}	−0.30	5.502	29.1
Br^-	2.80×10^1	1.45	5.623	28.4
$H_2PO_4^-$	8.51×10^1	1.93	5.500	29.1
SCN^-	1.28×10^2	2.11	5.585	28.6
I^-	2.16×10^2	2.33	5.603	28.5
salicylate	2.16×10^2	2.33	5.603	28.5
ClO_4^-	1.22×10^3	3.08	5.425	29.5
IO_4^-	4.24×10^3	3.63	5.586	28.6
trinitrophenol	3.20×10^4	4.50	5.443	29.4

[a] Parameters characteristic for the sites involved in competitive binding of Li^+ and SCN^- ions.
[b] K^+ and NH_4^+ binding is described by the same parameters.

from Fig. 3. Thus, the parameters determined from Figs. 4 and 5 are $K_{1,Li}$, $K_{2,Li}$, and γ. The ion-binding sites $\gamma N^{(-)}$ are presumably represented by electrically neutral residues which are able to bind either cations or anions depending on the relative polarizabilities or other physical properties of the ions. The possible existence of such sites on the surface of phosphatidylcholine membranes has recently been reported (Tatulian 1987).

Discussion

It is well documented that, in addition to anionic carboxyl groups, the erythrocyte membranes bear at their outer surface cationic amino, imido, and guanidino groups, belonging to the amino acids of the band 3 protein and responsible for the exceptionally high anion selectivity of red cell membranes (Passow 1969; Schnell 1972; Cabantchik and Rothstein 1972, 1974; Ho and Guidotti 1975;

Schnell et al. 1977; Wieth and Bjerrum 1982). Some of these groups, e.g. ε-amino group of lysine with pK 10.5 and arginyl guanidino group with pK 12.5, are positively charged at pH values as high as 10 (Nenitescu 1963). Thus, we can write for the red cell surface charge density at pH $\simeq$ 10

$$\sigma_0 = \sigma^{(-)} - \sigma^{(+)} \tag{9}$$

where $\sigma^{(-)}$ and $\sigma^{(+)}$ are the surface densities of negatively and positively charged groups at pH 10, respectively. Furthermore, $\sigma^{(-)}$ is likely to be composed of the cation-binding sites $N_1^{(+)}$ and $N_2^{(+)}$, i.e.

$$\sigma^{(-)} = N_1^{(+)} + N_2^{(+)} \tag{10}$$

Relationships (9) and (10) together with the values of σ_0, $N_1^{(+)}$, and $N_2^{(+)}$ (see Eq. (4)) yield values of $\sigma^{(-)} = 230\,\text{mC/m}^2$ and $\sigma^{(+)} = 13\,\text{mC/m}^2$ at $I = 145$ mmol/l and $\sigma^{(-)} = 92\,\text{mC/m}^2$ and $\sigma^{(+)} = 54\,\text{mC/m}^2$ at $I = 18.125$ mmol/l. This implies that at physiological conditions the ratio $\sigma^{(+)}/\sigma^{(-)}$ is 0.056 and agrees with other reports (Haydon and Seaman 1967; Weiss et al. 1972; Seaman 1983).

We have shown here that lowering of the ambient ionic strength from 145 to 18.125 mmol/l results in a considerable increase in the number of low-affinity cation-binding sites $N_1^{(+)}$ and in a reduction of the number of high-affinity groups $N_2^{(+)}$. This is paralleled by a drop of $\sigma^{(-)}$ from 230 to 92 mC/m^2 and an elevation of $\sigma^{(+)}$ from 13 to 54 mC/m^2; this becomes reflected in the suppression of the net negative surface charge density of the cells, σ_0. The changes in quantities of surface chemical groups induced by variations in the ionic strength are evidently brought about by structural changes of glycoproteins and other macromolecules composing the cell surface coat (see Prieve and Ruckenstein 1976; Donath and Lerche 1980; Donath and Pastushenko 1980; Levine et al. 1983; Snabre and Mills 1985; Herrmann et al. 1986). The ionic stregth-dependent structural changes of the glycocalyx can be explained as follows. At physiological ionic strength the negatively charged groups, $\sigma^{(-)}$, are compensated by both the counterions from the diffuse layer and the cell surface cationic groups, $\sigma^{(+)}$. A decrease in ionic strength results in a restricted stabilizing effect of the electrolyte, which makes additional screening of the negative charge by surface basic groups thermodynamically favorable. These phenomena presumably lead to rearrangement of electrokinetically effective chemical groups on the cell surface, and this becomes expressed in a decrease in $\sigma^{(-)}$, increase in $\sigma^{(+)}$, and consequently, in a reduction of the net negative surface charge density of the cell.

The lowering of the ionic strength was shown to cause an increase in the isoelectric point (pI) of erythrocytes (Heard and Seaman 1960, 1961; Seaman and Heard 1960; Donath and Steidel 1980), polymorphonuclear leukocytes (Wilkins et al. 1962), and viruses (Molodkina et al. 1986). Following pI values

have been reported for human red cells at physiological conditions: 1.7 (Furchgott and Ponder 1941), 2—2.3 (Ponder and Ponder 1960; Heard and Seaman 1960, 1961), 3.5—4.7 (Abramson 1934), 3.5 (Ambrose 1966), 4.6 (Coulter 1921). The value of pI = 3.52 measured in this work for human erythrocytes at $I = 145$ mmol/l is bracketted by those enumerated. The considerable inconsistencies in pI values measured by different investigators obviously reflect the instability of the erythrocyte surface potential at pH < 4.5, when the cells begin to lyse rather extensively (Ponder and Ponder 1960; Heard and Seaman 1960; Seaman and Cook 1965). As a result of the hemolysis, the positively charged hemoglobin molecules with a pK of 7.1 (Boggs 1983) release from the lysed cells and adsorb to the surface of intact calls thereby reducing progressively the erythrocyte negative surface charge (Seaman and Heard 1960; Eylar et al. 1962). This is more pronounced at lower ionic strengths (Heard and Seaman 1960; Seaman 1973). To minimize the influence of these phenomena on the measured values of electrophoretic mobility our measurements were made within a few minutes after suspending the cells in media with pH < 5, as well as with pH > 9.

Donath and Pastushenko (1980) explained the rise in the isoelectric point of red cells with decreasing ionic strength by suggesting that the chemical groups with higher pK values are located deeper in the glycocalyx and are exposed at low ionic strengths as a result of glycocalyx swelling. Prieve and Ruckenstein (1976) concluded that lowering of the ionic strength results in an increase in pK of red cell surface acidic groups and a reduction in their surface density. We explain the increase in erythrocyte pI from 3.52 to 4.22 induced by a drop of ionic strength from 145 to 18.125 mmol/l by an enhancement in the number of protonable groups $N_1^{(+)}$ with a constant (ionic strength-independent) value of pK = 3.53. The latter figure is consistent with pK values reported for human erythrocyte membranes: 3.5 (Sanui et al. 1962), 2.8—3.5 (Seaman and Cook 1965), 3.35 (Haydom and Seaman 1967), 3.2—3.5 (Freedman and Radda 1969), 3.1—3.3 (Todd and Gingell 1980), and 2.6—3.4 (Seaman 1983). We failed to find in the literature any data suggesting the presence of chemical groups with pK 8 to 9 on erythrocyte surface (like $N_2^{(+)}$ with pK 8.55 detected in this work).

Peculiarities of ion binding to erythrocyte membranes have been examined in a number of studies. Seaman et al. (1969) reported that at $I = 145$ mmol/l Ca^{2+} binding to red cell outer surface was characterized by a binding constant of 11.4 l/mol and $N^{(+)} \simeq 0.05\,\mathrm{nm}^{-2}$. Gent et al. (1964) reported a value of $K_{Ca} = 3.6 \times 10^3$ l/mol. Three types of Ca^{2+}-binding sites at the outer surface of human red cells have been detected with binding constants of 6×10^4, 3.6×10^3, and 6×10^2 l/mol; the second group was attributed to NANA (Long and Mouat 1971). Other investigators also revealed three classes of Ca^{2+}-binding sites at the external surface of human erythrocyte membranes and reported binding constants of 2.5×10^5, 1.4×10^4, and 3×10^2 l/mol (Cohen and Solomon 1976) and

3×10^4, 3×10^3, and $\sim 10^2$ l/mol (Moore et al. 1984). The third group of sites found by the latter authors ($K_{Ca} \simeq 10^2$ l/mol) was ascribed to NANA residues. This is consistent with the data of McDaniel and McLaughlin (1985) who reported a Ca^{2+} binding constant to sialoglycolipid membranes of $\leq 10^2$ l/mol.

We obtained a value of $K_{Ca} = 630$ l/mol which is much larger than that (11.4 l/mol) reported by Seaman et al. (1969). Our value of K_{Ca} is embraced by the constants of Ca^{2+} binding to NANA: $\sim 10^2$ l/mol (Moore et al. 1984) and 3.6×10^3 l/mol (Gent et al. 1964; Long and Mouat 1971).

Human erythrocytes have been shown to carry at their outer surface protonable groups with pK 7.1, 6.1, and 3,5, the former two binding also Na^+ and K^+ ions with constants 79.4 and 10.7 l/mol for Na^+, and 139 and 16.6 l/mol for K^+, respectively (Sanui et al. 1962; Sanui and Pace 1962). Our results point to the existence of two types of protonable groups with pK 8.55 and 3.53, which bind Na^+, K^+, and NH_4^+ ions with constants 180 and 0.92 l/mol, respectively.

The affinity of alkaline-earth metal cations to human erythrocystes was shown to decrease in the sequence: $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Seaman and Pethica 1964), $Ca^{2+} > Sr^{2+} > Mg^{2+}$ (Gent et al. 1964), $Ca^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+}$ (Long and Mouat 1971), and $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ (Cohen and Solomon (1976). The sequence described in this paper ($Ca^{2+} > Sr^{2+} > > Mg^{2+} > Ba^{2+}$) agrees with those established by Seaman and Pethica (1964), Gent et al. (1964), and Long and Mouat (1971).

Some authors reported similar values of the electrophoretic mobility of erythrocytes in NaF, NaCl, NaI, and NaSCN solutions and concluded that red cell membranes can hardly bind inorganic anions (Heard and Seaman 1960; Seaman and Heard 1960; Brooks and Seaman 1973). However, certain experimental results can be readily explained by selective binding of anions to red blood cells. For example, the well recognized fact that the electrophoretic mobility of red cells is larger in the presence of phosphates rather than chlorides was interpreted by preferential binding of phosphate anions to the cell membranes (Bangham et al. 1958). Our data, showing lowered electrophoretic mobility of red cells induced by the substitution of phosphates for chlorides, provides confirming evidence for the above suggestion.

Wieth (1970a, b) reported a sequence for anion effectiveness in enhancing Na^+ and K^+ permeability and reducing Cl^- and SO_4^- permeability of red cell membranes: salicylate $> SCN^- > I^- \simeq NO_3^- > Br^- \simeq Cl^-$, and explained this result in terms of selective anion binding to positively charged chemical groups of the cell plasma membranes and subsequent decrease in positive electrostatic potential created by these groups. The differential effect of anions on red cell surface potential observed in this study (see Fig. 3 and Table 1) is in good agreement with these data.

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