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DETERMINATION OF THE ELECTRIC POTENTIAL AT THE EXTERNAL AND INTERNAL BILAYER-AQUEOUS INTERFACES OF THE HUMAN ERYTHROCYTE MEMBRANE USING SPIN PROBES

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Partitioning of oppositely charged amphipathic spin probes indicates that the electric potential at the external bilayer-aqueous interface of the human erythrocyte is insignificant, and that protruding sialic acids do not contribute to this potential. This potential at the surface is distinguished from the electrokinetic potential due to all charged groups within the hydrodynamic surface of shear. By contrast, using inside-out erythrocyte membrane vesicles, a substantial potential is observed at the cytoplasmic membrane surface. This can be attributed to the asymmetric distribution of acidic phospholipids on the two sides of the erythrocyte membrane bilayer.

Electrical surface potentials are useful concepts in bilayer membranes having relatively simple and well-defined surfaces. On the other hand, biological membranes usually have highly irregular surface topographies. Their lipid and protein components contain diverse charge groupings so that both lateral and transverse heterogeneities are to be expected in the surface charge distributions and hence the electrical potential profiles. Thus far, only average, operational surface potentials have been measured for biological membranes and no estimate of heterogeneity of lateral or transverse surface charges has been achieved.

Until recently the most popular method of estimating the surface charge or electric potential of cell surfaces and vesicles has been conventional microelectrophoresis [1–7]. This method, however, actually evaluates the so-called zeta or electrokinetic potential ζ, i.e., the value of the potential at the hydrodynamic surface of shear of the membrane relative to the bulk aqueous environment [8–13]. The absolute value of ζ is generally smaller than that of the potential Ψ due to charges at the membrane surface because ζ includes electrostatic contributions from all charges residing within the surface of shear. In human erythrocytes, this includes the important contribution of sialic acids [4,14,15] which, by nature of their branched polymeric structure, may extend well beyond the bilayer-aqueous interface. Although various models, for example, using Debye-Hückel or Gouy-Chapman assumptions, have been used to relate the value of ζ to Ψ [8,9,11,16], none is unequivocal.

Considerable evidence supports the notion that erythrocyte membrane phospholipids are asymmetrically distributed between the two halves of the bilayer [17–19]. In particular, acidic phos-

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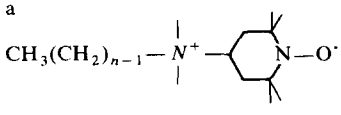
Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; phospho-TEMPO, 2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride.

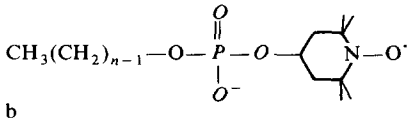
pholipids which tend to carry a net negative charge appear to be located entirely in the inner monolayer. Since this results in an asymmetric distribution of surface charges [20], it is important to contrast the external surface potential with that of the cytoplasmic side of the membrane.

Measurement of the surface potential and the possible effect of sialic acids on the external potential necessitates using a probe which senses the electric potential in the immediate vicinity of the bilayer-aqueous interface; that is, it must 'see' what the phospholipid headgroups 'see'. This has been accomplished by charged amphipathic EPR probes which partition between the lipid membrane and aqueous phase in response to the surface potential [21–25].

In this investigation the cationic and anionic spin probes, generally denoted CAT_n and AN_n respectively (where n indicates the number of carbon atoms in the acyl chain), have been selected (Fig. 1). Their amphipathic structures assure that they insert into a lipid bilayer in a predictable manner. The acyl chains are expected to reside in the lipophilic interior of the membrane, while the charged groups and nitroxide spin labels (2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)) are expected to reside among the polar phospholipid headgroups. The charged group senses the electric potential, and the spin label senses whether the probe is in the lipid bilayer or in the more mobile aqueous environment. The presence of the charged group in an electric field alters the partitioning of the probe, favouring the lipid phase when the surface charge and charged group are of opposite polarity, and shifting towards the aqueous phase when they are of like polarity. Equilibration of partitioning between these environments is essentially instantaneous for all practical purposes.

a





b

Fig. 1. Structure of (a) cationic CAT_n and (b) anionic AN_n spin probes. Analogous structures consist of n-carbon hydrophobic tail, quaternary amine or phosphate charge group and the TEMPO nitroxide radical.

The CAT_n and AN_n probes differ only in the quaternary amine versus phosphate which give the probes their cationic and anionic charges. The probes may be used singly with a reference calibration at a known potential (usually the essentially zero-potential at very high ionic strengths). However, in this case, each membrane suspension requires a separate calibration because the observed lipid-aqueous partitioning ratio is dependent on membrane concentration. Alternatively, a dual probe method, using CAT_n and AN_n in pairs, makes derivation of the surface potential independent of membrane concentration. The amphipathic structures of the two probes are sufficiently analogous that differences in their zero-potential lipid aqueous partition coefficients and electron paramagnetic resonance (EPR) spectral line amplitudes can be accounted for by a linear calibration factor K in the dual probe method.

The measurement of outer surface potentials requires that the probes exhibit negligible flip-flop across the membrane bilayer over the short periods of time during which EPR spectra are obtained [23,25]. Use of fresh human erythrocytes assures that the membranes are sealed and that only the potential of the external surface is being measured. To measure the internal surface potential, erythrocyte ghost vesicle populations which are largely inside-out were prepared. In this case, no assumptions are made regarding resealing of the vesicles. However, sialic acid assays of these inside-out vesicles suggest that approximately half of them are resealed [20]. Surface potential measurements on such vesicles should, therefore, be at least indicative of the polarity and magnitude of the cytoplasmic membrane surface charge.

The principles of charged amphiphatic spin probe partitioning as a means of estimating the surface potential have been detailed [22,23]. The dual probe method [23] has been shown to be useful within a range of at least $\pm$ 80 mV.

Materials and Methods

Preparation of erythrocyte suspensions

Fresh whole human blood was taken in acid/citrate/dextrose to inhibit clotting. The blood was cooled to 7°C and ethylenediaminetetraacetic acid (EDTA) added to 10 mM. The blood was centrifuged at $1200 \times g$ for 5 min, and the supernatant and most of the buffy coat removed. The cells were washed twice more in isotonic saline (150 mM sodium chloride) buffered in 5 mM sodium phosphate in the range pH 7.4–7.8. The cells were resuspended in 1 vol. of isotonic saline, and neuraminidase (from *Vibrio cholerae*) was added to half of the samples to a final concentration of up to 10 IU/ml. Both the neuraminidase-treated and untreated control samples were incubated at 37°C for 1.5 to 2 h. The neuraminidase and liberated sialic acids were washed out in isotonic sucrose and the resulting pellets retained. Into each 1-ml plastic centrifuge tube with a tapered bottom (e.g., Eppendorf microcentrifuge tubes), 200 μl of packed cells were placed and mixed with 400 μl of salt solution. These solutions contained varying concentrations of KCl and $CaCl_2$ with isotonicity maintained by appropriate amounts of sucrose (e.g., 10 mM KCl plus 280 mM sucrose maintains an osmolarity of 300 mosM), and were buffered to pH 7.0–7.8 by 5 mM sodium phosphate or up to 5 mM Tris in the case of $CaCl_2$. The cell suspensions were again spun down and 80–85% of the supernatant removed to ensure a sufficient membrane suspension density for obtaining optimal EPR spectra.

Preparation of inside-out erythrocyte vesicle suspensions

White erythrocyte ghosts were prepared from the washed erythrocyte suspensions by hypotonic lysis in 5 mM sodium phosphate at 4–7°C (pH 7.4–7.8) according to the procedure of Dodge et al. [26]. The ghost pellets were then resuspended for 1 h in 15 vols. of 10 μM $CaCl_2$ buffered in up to 5 mM Tris to pH 7.0–7.7. Vesiculation was accomplished by expelling each suspension through a 27-gauge needle thrice at 4–7°C using a 2 ml glass syringe under continuous manual pressure. This procedure has been shown to produce a population of predominantly, but not entirely, inside-out vesicles [20]. In some experiments the inside-out vesicles were purified by removing contaminating right-side-out vesicles by antibody sedimentation. This added procedure appeared to have little effect on EPR results, confirming that the membranes were primarily inside-out to start with. The vesicles were centrifuged at $23000 \times g$ for 10 min and the pellet resuspended in solutions of varying concentrations of KCl and $CaCl_2$. The vesicles were again spun down and 80–85% of the supernatant removed to ensure a sufficient membrane density for EPR spectroscopy.

Spin probes and EPR measurements

The cationic probes, CAT_n, were synthesized as described previously [23]. The anionic probes were prepared by condensing 2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl (phospho-TEMPO) which had been synthesized according to Weiner [27] with the appropriate primary alcohols with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) [28]. For example, to synthesize AN_9, 1 g of phospho-TEMPO and 3 g of 1-nonanol were dissolved in 30 ml of dry pyridine containing 1 g of Molecular Sieves type 3A (Linde Div., Union Carbide Corp., South Plainfield, NJ). 5 g of TPS were added to this solution with rapid stirring and the reaction was allowed to proceed at room temperature for 6 h. The reaction mixture was filtered (Whatman No. 1 paper) and 2 ml of water were added to the pyridine solution. The volume of this solution was reduced by rotary evaporation and the desired product was purified by TLC with a 50 : 25 : 8 : 4 chloroform/methanol/pyridine/water mixture as the running phase. The probes used in this study were CAT_6, CAT_8, CAT_{10}, CAT_{11}, CAT_{12}, AN_{10}, AN_{11}, and AN_{12}. Working solutions of each spin probe were prepared to concentrations of 1.0–2.0 mM in Tris buffer.

The actual measurement of surface potentials was accomplished by taking 50 μl aliquots of each final cell or vesicle suspension, mixing in 1.0 μl of appropriate probe solution, and placing the sample into 50 μl microcapillary tubes sealed at one end and suitable for insertion into the EPR sample holder. Spectra were recorded on a Varian E 109-E EPR Spectrometer at a microwave power of 10 mW, klystron frequency of 9.13 GHz or 9.51 GHz, field setting of 3225 G or 3390 G, modulation

amplitude of 1.0 G, and sample temperature of 21.4–21.5°C. Recording of spectra was completed within 10 min of the time the probe was added to the cell or vesicle suspension.

Calculation of surface potential by the dual probe method

Each probe with valence z has a lipid-aqueous partitioning ratio P whose dependence on the surface potential is given by the Boltzmann distribution:

$$P = P_0 e^{-\frac{zF\psi}{RT}} \propto \frac{h_l}{h_a} \tag{1}$$

where P_0 is the lipid-aqueous partition coefficient of the probe at zero potential for a given membrane, F is the Faraday constant (96 487 C/equiv.), R is the ideal gas constant (8.3143 J/K per mol), and T is the absolute temperature. The relation between P and the lipid and aqueous EPR spectral line amplitudes, h_l and h_a, respectively, is dependent on membrane concentration. Combining the equations for cationic and anionic probes gives us a way of relating the potential to the lipid and aqueous amplitudes of each probe which is independent of membrane concentration. The membrane concentration dependences cancel out:

$$e^{-\frac{2F\psi}{RT}} = K'\frac{P^+}{P^-} = K\frac{h_l^+ h_a^+}{h_a^+ h_l^-} \tag{2}$$

where $K' = P_0^-/P_0^+$ and K is for practical purposes a calibration coefficient which takes into account the differences in the proportionality factors for the two probes which relate EPR amplitudes to concentration. It also accounts for any differences in zero-potential lipid-aqueous partitioning (i.e., K' is included in K). The + and − superscripts refer to cationic and anionic probes, respectively. The value of K must be determined experimentally at one or more known values of ψ. As in the case of the single probe method, this can best be done at very high ionic strengths where $\psi \simeq 0$. But unlike the single probe method in which calibration must be done for each membrane suspension to account for variations in membrane concentration, the dual probe method requires only one calibration of K for a given pair of probes and a given type of membrane.

Results

To confirm that the interaction of the CAT_n and AN_n spin probes was limited to the outer red cell surface the rate of transmembrane equilibration (flip-flop) of AN_{12} was determined. It is shown in Fig. 2 that AN_{12} becomes considerably more immobilized when bound to bovine serum albumin than when bound to red cell membranes and that bovine serum albumin rapidly removes AN_{12} from the outer membrane surface. However, after prolonged incubation of AN_{12} with red cells, a substantial membrane signal remained after addition of bovine serum albumin, indicating that flip-flop to the inner membrane surface had

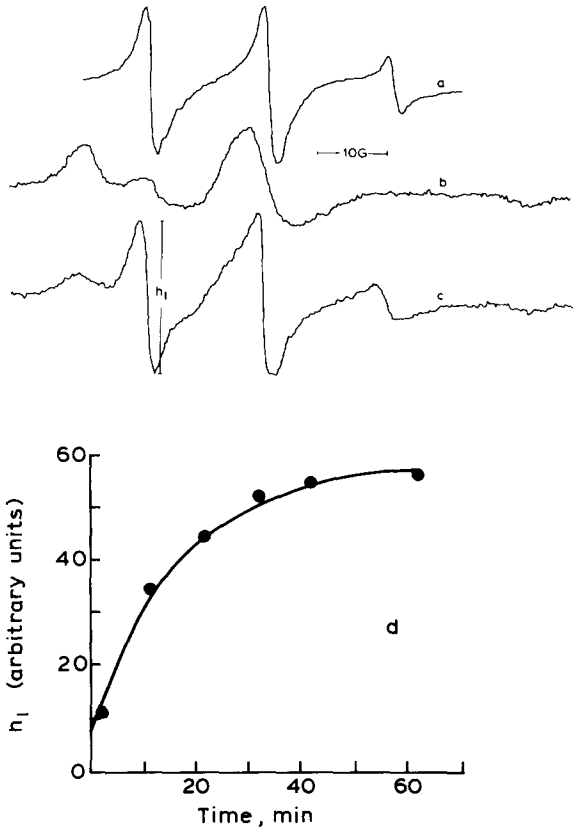


Fig. 2. EPR spectra of 150 μM AN_{12} in (a) red cells, 45% hematocrit, gain $8 \cdot 10^3$, (b) red cells with 25 mg/ml bovine serum albumin added 2 min after addition of the probe, 45% hematocrit, gain $3.2 \cdot 10^4$, (c) same as (b) but bovine serum albumin was added 70 min after addition of the probe. EPR scans were obtained immediately after albumin addition. A spectrum of bovine serum albumin with AN_{12} in the absence of red cells is essentially identical to (b). (d) Time course of the height of the low-field membrane resonance line.

occurred. This inner membrane signal becomes converted to the bovine serum albumin-type signal after about 1 h of incubation in the presence of bovine serum albumin, consistent with outward flip-flop of the AN_{12} over that time period. The time-dependence of the increase in the inner-membrane monolayer signal of AN_{12} (Fig. 2d) shows that about 85% of this probe's EPR signal resides on the outer membrane surface within the first 5 min of AN_{12} addition to red cells. We therefore performed all of our experiments with red cells within 5 min of probe addition to ensure that only the surface potential of the outer monolayer was being measured. No correction was made for the small fraction of probes arising from the inner membrane surface.

The flip-flop rate of the CAT_n probes was also investigated. Unfortunately, these probes do not bind to bovine serum albumin, hence another method was developed to study their flip-flop characteristics. A series of probes, CAT_6, CAT_8 and CAT_{10}, were added to red cells together with 40 mM ferricyanide. Shorter chain lengths were chosen to facilitate detection of aqueous spectral components inside the cells. The ferricyanide ion is impermeable and broadens the extracellular aqueous, but not membrane-bound or cytoplasmic, signals of the CAT_n probes. This method is analogous to an EPR volume-measurement technique which has been used successfully in many different cell and vesicle preparations [29]. No cytoplasmic aqueous signal was observable for these three probes after more than 1-h incubation with red cells, implying that the cationic probes flip-flop at a much slower rate than the corresponding anions.

The external surface potential of whole human erythrocytes was measured with and without neuraminidase treatment. Data were read from composite EPR spectra in which the magnitude of both the lipid and aqueous components could be clearly quantified (Fig. 3). As shown in Fig. 4, the surface potential was found to be consistently less than 5 mV in magnitude except at very high concentrations of $CaCl_2$. This is within an approx. ± 5 mV calibration uncertainty relative to the real zero-potential. Thus, for all practical purposes, the actual surface potential was insignificant. This was found to be true regardless of whether the cells

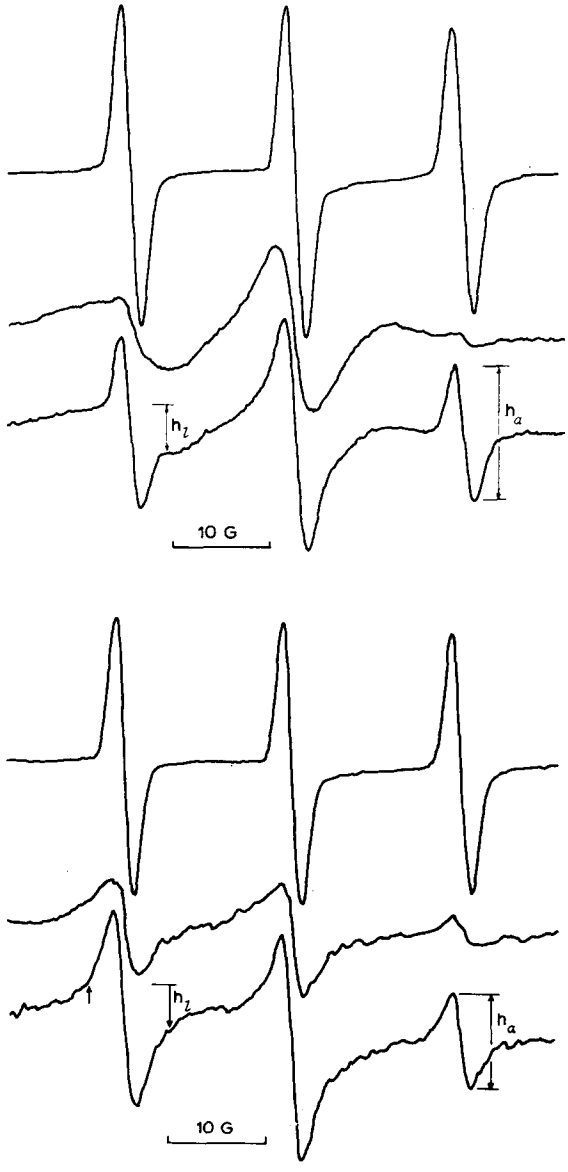


Fig. 3. Sample three-line EPR spectra. (a) CAT_{12} spin probe aqueous signal in buffer (top), mostly lipid signal in packed membranes (middle), and typical composite spectrum (bottom) due to significant partitioning into both the aqueous and lipid phases. Aqueous line height, h_a, and lipid line height, h_l, are read as shown on the composite spectrum. (b) AN_{12} spin probe aqueous signal (top), lipid signal (middle), and typical composite spectrum (bottom). Aqueous line height is read as in the case of CAT_n. Lipid line height on the composite spectrum is read by choosing consistent points (e.g., at arrows on left resonance line) which on the pure aqueous spectrum would sit just to either side of the low-field peak, and by recording the difference in height.

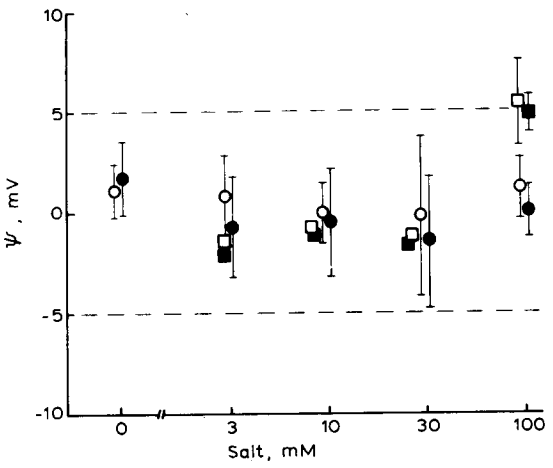


Fig. 4. External surface potentials of whole human erythrocyte as functions of neuraminidase treatment and cation concentration. The external surface potential at bulk aqueous pH 7.0–7.8 is shown for untreated cells retaining their sialic acids in KCl (●) and $CaCl_2$ (■), and neuraminidase-treated cells stripped of sialic acids in KCl (○) and $CaCl_2$ (□). Potentials are plotted as functions of KCl and $CaCl_2$ concentrations [M], excluding the 5 mM sodium phosphate used with KCl or Tris buffer (up to 5 mM) used with $CaCl_2$. Isotonicity was maintained by sucrose. Vertical bars indicate standard errors.

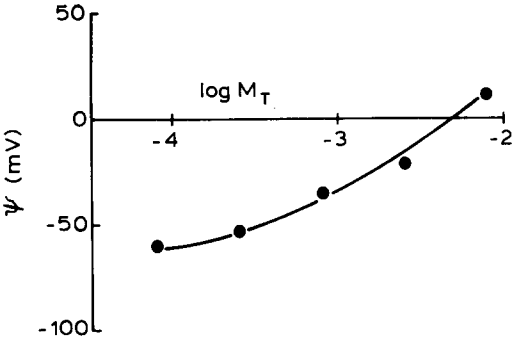


Fig. 5. Surface potentials of largely inside-out populations of human erythrocyte ghost vesicles as a function of calcium concentration M_T at bulk aqueous pH 7.0–7.8.

had been treated with neuraminidase. In other words, sialic acids appeared to make no detectable contribution to the electric potential at the external bilayer-aqueous interface, i.e., at the surface defined by the phospholipid headgroups.

To eliminate any doubt that what was being measured was in fact a potential of near-zero magnitude, the surface potential was measured in the presence of various concentrations of K^+ and Ca^{2+}. If the surface potential becomes more positive with rising cation concentration, this would suggest that either cations are being adsorbed to the surface or the rising ionic strength is screening out a negative surface potential. On the other hand, if it remains the same at all cation concentrations and ionic strengths, there is good reason to believe that there is little cation adsorption to the bilayer surface and little actual surface potential to be screened. The experimental results plotted in Fig. 4 provide compelling evidence that the latter situation prevails. Since there is no change in ionic strength inside the cell, these results also imply that possible contamination by probes on the interior surface does not alter the conclusion that the surface potential at the outer membrane surface is insignificant. The very slight Ca^{2+} adsorption suggested by the small positive surface potential at 100 mM Ca^{2+} is consistent with the relatively low binding affinity between Ca^{2+} and neutral phospholipids.

Fig. 5 summarizes the results for inside-out vesicles at varying concentrations of K^+ and Ca^{2+}. Since the suspensions contain some right-side-out vesicles as well, the effect of averaging the surface potential over both inside-out and right-side-out subpopulations would be to underestimate the magnitude of the true surface potential for a pure population of sealed inside-out vesicles. A very substantial negative surface potential is measured, generally decreasing in magnitude with increasing ionic strength as cations are adsorbed to the surface and screen out more of the surface potential. In the case of Ca^{2+}, the surface potential curve appears to be biphasic with a cross-over point to positive potential at around 5.0 mM. In contrast to the external surface of the membrane, the cytoplasmic surface exhibits a very definite and substantial surface potential, and hence net surface charge.

Discussion

Since, according to Gouy-Chapman diffuse double-layer theory (e.g. Refs. 13,30,31), a very small surface charge density can give rise to a rather large surface potential at low ionic strengths (well over 100 mV in magnitude), the failure to

detect a significant electric potential at the external bilayer-aqueous interface suggests that there is essentially no net charge at this interface. It further suggests that the negative charge of sialic acids generally resides well beyond this interface and protrudes into the aqueous phase. This is entirely consistent with their branched polymeric structure and the fuzzy external coat so commonly seen in thin section electron micrographs of the human erythrocyte, the distinction between the electrokinetic potential due to all structures within the hydrodynamic surface of shear and the actual surface potential is indeed a fundamental one.

These results are also consistent with evidence that the external half of the human erythrocyte membrane contains no acidic (anionic) phospholipids, but rather only neutral phospholipids (principally phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) [17–19,32] which neither make a contribution to surface charge at physiological pH nor have much affinity for binding metal cations [33–35]. This is in contrast to the large surface potential and significant net surface charge of the interior half of the bilayer which probably results from the presence of acidic phospholipids (mostly phosphatidylserine, phosphatidylinositol, and phosphatidic acid) localised on this inner surface.

It is also interesting that membrane proteins exposed on the external surface appear to make no appreciable contribution on the surface potential as measured by this method. This is consistent with previous evidence that following removal of sialic acids the human erythrocyte is stripped of most of its net negative charge [1,14,15] and hence has little true external surface charge whether due to intrinsic proteins or phospholipids.

The dependence on cation concentration of the surface potential of the cytoplasmic surface of the membrane is entirely consistent with Gouy-Chapman double-layer theory. The character of the Ca^{2+} surface potential curve is consistent with the three different binding stoichiometries between Ca^{2+} and acidic phospholipids: 0 : 1 (no binding), 1 : 2 (Ca^{2+} cross-bridging of two adjacent acidic phospholipids), and 1 : 1 (one Ca^{2+} binding to one acidic phospholipid) [20]. Thus, as Ca^{2+} concentration increases, the surface charge changes continuously from negative to net neutrality to positive, while screening of the surface potential continuously increases. This system has been quantitatively modelled by a modification of the Gouy-Chapman formulation applied to divalent cations [36].

Acknowledgements

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