Influence of Anions and Cations on the Dipole Potential of Phosphatidylcholine Vesicles: A Basis for the Hofmeister Effect

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ABSTRACT Anions and cations have long been recognized to be capable of modifying the functioning of various membrane-related physiological processes. Here, a fluorescent ratio method using the styrylpyridinium dyes, RH421 and di-8-ANEPPS, was applied to determine the effect of a range of anions and cations on the intramembrane dipole potential of dimyristoylphosphatidylcholine vesicles. It was found that certain anions cause a decrease in the dipole potential. This could be explained by binding within the membrane, in support of a hypothesis originally put forward by A. L. Hodgkin and P. Horowicz [1960, *J. Physiol. (Lond.)* 153:404–412.] The effectiveness of the anions in reducing the dipole potential was found to be $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- > SO_4^{2-}$. This order could be modeled by a partitioning of ions between the membrane and the aqueous phase, which is controlled predominantly by the Gibbs free energy of hydration. Cations were also found to be capable of reducing the dipole potential, although much less efficiently than can anions. The effects of the cations was found to be trivalent > divalent > monovalent. The cation effects were attributed to binding to a specific polar site on the surface of the membrane. The results presented provide a molecular basis for the interpretation of the Hofmeister effect of lyotropic anions on ion transport proteins.

INTRODUCTION

It has long been known that various inorganic and organic salts can change the effectiveness of some membrane-related physiological processes. In both whole muscle (Lillie, 1910; Kahn and Sandow, 1950, 1955) and single muscle fibers (Hodgkin and Horowicz, 1960), it was found that nitrate and other anions can cause an increase in the muscle twitch tension. The effect of the anions was shown to increase in the order $Cl^- < Br^- < I^- < SCN^-$. Hodgkin and Horowicz (1960) noted that this order corresponded to the anions' adsorbability and suggested that the effect might result from adsorption to the surface of the muscle membrane, thus resulting in an alteration in the electric field within the membrane. The order of anion effectiveness is in agreement with the so-called Hofmeister, lyotropic or chaotropic series (Horowicz, 1964; Collins and Washabaugh, 1985; Cacace et al., 1997) first described by Hofmeister (1888, 1890) based on the ability of neutral salts to precipitate a given protein. Anion effects obeying a similar order of effectiveness have since been discovered using other membrane systems. For example, Dani et al. (1983) reported that lyotropic anions reversibly shift the voltage dependence of sodium channels of skeletal muscle. Rychkov et al. (1998) recently discovered a similar effect on the voltage dependence of gating and the blocking potency of the skeletal muscle ClC-1 chloride channel. The fact that such effects are not limited to channels is shown by recent findings of the effects of lyotropic anions on the kinetics of the electrogenic ion pump, the Na^+,K^+-ATPase (Klodos et al., 1994; Suzuki and Post, 1997; Nørby and Esmann, 1997). Because of the large number of diverse systems affected by lyotropic anions, Dani et al. (1983) expressed the opinion that: "... one would expect all membrane properties that are sensitive to the membrane electric field to be shifted by lyotropic anions. The question would not be whether they are shifted, but only how much they are shifted."

As reported by Collins and Washabaugh (1985), anions by far dominate cations in terms of Hofmeister effects. Nevertheless, effects of cations on protein structure, function, and stability have also been found, and Hofmeister, lyotropic, or chaotropic series have been published (Baldwin, 1996; Cacace et al., 1997). Here, it suffices to say that the effects of divalent cations are, in general, greater than those of monovalent ones.

In spite of the large number of investigations of effects of ions on membrane- and surface-related phenomena, the underlying principles are still not well understood. As pointed out above, Hodgkin and Horowicz (1960) hypothesized that lyotropic anions may adsorb to the membrane and alter the intramembrane electric field strength. However, this hypothesis is very difficult to test because of the lack of available methods for detecting electric field strength in membranes. So far, two pieces of electrical evidence supporting Hodgkin and Horowicz's hypothesis have been obtained. First, McLaughlin et al. (1975) found that the lyotropic anions ClO_4^- and SCN^- decrease the carrier-mediated conductance of negative charge and increase that of positive charge across black lipid membranes. Second, the same authors showed that ClO_4^- and SCN^- induce a negative zeta potential on the surface of phosphatidylethanolamine vesicles. The electrophoretic zeta potential method was subsequently applied by Tatulian (1983, 1987) to the investigation of the interaction between alkaline earth

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metal cations and a range of anions with phosphatidylcholine vesicles. Further investigations of the interaction of both cations and anions with phospholipid vesicles have been carried out through the analysis of chemical shifts of ^{1}H-NMR (Jendrasiak, 1972; Hauser et al., 1977) and ^{31}P-NMR spectra (Grasdalan et al., 1977; Westman and Göran Eriksson, 1979; Rydall and Macdonald, 1992) and the analysis of ^{2}H-NMR quadrupole splittings (Rydall and Macdonald, 1992).

The major aim of the present paper is to investigate the origin of the Hofmeister effect by studying the interaction of anions and cations with phospholipid membranes, using a relatively new fluorescence technique for the detection of intramembrane electric field strength. The method involves the application of the voltage-sensitive styryl dyes, N-(4-sulfobutyl)-4-(4-(p-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) and 4-(2-(6-(dioctylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt (di-8-ANEPPS) (see Fig. 1). These dyes, which were originally developed in the laboratories of Grinvald (Grinvald et al. (1982, 1983) and Loew (Hassner et al., 1984; Fluhler et al., 1985; Bedlack et al., 1992), and ones of similar structure are presently attracting great interest as a means of optical imaging of electrical transients in neurons (Grinvald et al., 1988; Loew, 1994) and muscle cells (Müller et al., 1986; Efimov et al., 1994) and for the investigation of the reaction mechanisms of ion pumps, e.g., the Na^+,K^+-ATPase (Klodos and Forbush, 1988; Pratap and Robinson, 1993; Heyse et al., 1994; Fedosova et al., 1995; Kane et al., 1997; Clarke et al., 1998). The dyes respond to a change in the local electric field strength with a shift in their fluorescence excitation spectrum. To quantify the shift, dual wavelength excitation ratiometric fluorescence measurements have been applied by several groups (Montana et al., 1989; Loew et al., 1992; Zouni et al., 1993; Gross et al., 1994; Schulz and Apell, 1995; Clarke and Kane, 1997; Clarke, 1997; Cladera and O'Shea, 1998; Zhang et al., 1998). In a previous paper (Clarke and Kane, 1997), experimental conditions were reported under which the dyes RH421 and di-8-ANEPPS are insensitive to membrane fluidity and respond purely to changes in the local membrane electric field strength.

In principle, the local membrane electric field strength could be influenced by a number of factors: the presence of a membrane potential across the total width of the membrane, the presence of charged residues on the membrane surface, and the presence of dipolar groups within the membrane interior. However, several pieces of evidence support the conclusion that the dyes RH421 and di-8-ANEPPS are particularly sensitive to the membrane dipole potential, ψ_d, which arises from the alignment of dipolar residues of the lipids as well as water dipoles in the region between the aqueous phase and the hydrocarbon-like interior of the membrane (Brockman, 1994; Cafiso, 1995). For example, it has been found that the binding of the dipolar species, phloretin and 6-ketocholestanol, to vesicles made of the zwitterionic phospholipid phosphatidylcholine, in the absence of any total membrane potential, causes significant spectral shifts of both membrane-bound probes (Gross et al., 1994; Clarke and Kane, 1997). Furthermore, changes in the phosphatidylcholine structure (i.e., chain length and saturation) have been found to shift the pK_a of both probes (Zouni et al., 1994; Clarke, 1997), suggesting a change in the electronic environment of the probes. Further convincing evidence that the dyes are, in fact, detecting the dipole potential has come from measurements showing that the fluorescence excitation shifts of the dyes are correlated to the lipid packing density (as would be expected based on the Helmholtz equation for a parallel-plate capacitor), and to the literature values of the dipole potential from electrical measurements on monolayers and bilayers (Clarke, 1997).

If, as proposed by Hodgkin and Horowicz (1960), lyotropic anions bind to the membrane and alter the intramembrane electric field, it is reasonable to expect that they might cause a change in dipole potential, which could be detected using RH421 and di-8-ANEPPS. It will be shown here that significant shifts in the fluorescence excitation spectra of both probes are induced by the interaction of the membrane with a number of the anions and cations studied and that the shifts can be explained by ion adsorption at different depths within the membrane. In particular, the agreement found between the order of anion effects on the dipole potential and the Hofmeister series allows a molecular interpretation of the Hofmeister effect on ion-transporting membrane proteins to be presented. In a subsequent paper (Ganea, Babes, Lüpfert, Grell, Fendler, and Clarke, in preparation), the effect of lyotropic anions on the kinetics of partial reactions of the Na^+,K^+-ATPase will be described.

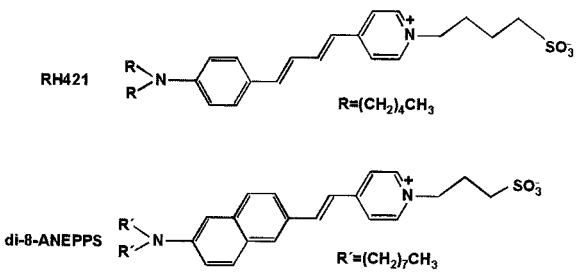

FIGURE 1 Structures of RH421 and di-8-ANEPPS.

MATERIALS AND METHODS

RH421 and di-8-ANEPPS were obtained from Molecular Probes (Eugene, OR). In vesicles and in aqueous solution, both dyes showed a single long wavelength fluorescence emission band regardless of the excitation wavelength. For spectral measurements in the presence of lipid vesicles, 5 μl of an ethanolic dye solution (0.83 mM for RH421 and 1.00 mM for di-8-ANEPPS) was added to 1 ml of the vesicle-containing aqueous solution. The final solutions thus contained a small percentage of 0.5% ethanol. After addition of the dye, the solutions were left overnight to allow for dye disaggregation and incorporation into the membrane. The effect of the small volume of ethanol added to the fluorescence spectra of membrane-bound dye was checked in separate control experiments and found to be negligible.

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere (Zouni et al., 1993, 1994). The final vesicle suspension contained no detectable trace of ethanol, i.e., [ethanol] $\leq 10\ \mu$M, according to a nicotinamide adenine dinucleotide/alcohol dehydrogenase enzymatic assay (Boehringer, Mannheim, Germany). All steps of the vesicle preparation were performed at 30°C, i.e., above the main phase transition temperature. Dialysis tubing was purchased from Medicell International (London, UK). The phospholipid content of the vesicle suspension was determined by the phospholipid B test from Wako (Neuss, Germany).

The vesicles were normally prepared in a buffer containing 10 mM Tris and 1 mM EDTA. In addition, 0.5 M of the salt to be investigated was, except where specified otherwise, added to the buffer medium before preparation of the vesicles by dialysis. This prevents any variation in ionic concentrations on either side of the membrane. In the case of the salt $NaClO_4$, vesicles were prepared using a range of different concentrations, from 0 to 5 M. For each $NaClO_4$ concentration, a new vesicle suspension was prepared, again so that no concentration differences across the membrane were present. The pH of the buffer was, except where specified otherwise, adjusted to 7.2 with HCl. However, in the case of measurements in the presence of $LaCl_3$, the pH was not adjusted because, at neutral pH values, La^{3+} combines with hydroxide ions in solution to form an insoluble white precipitate of $La(OH)_3$. The pH of the buffer containing 0.5 M $LaCl_3$ was measured to be 4.9. However, this is well above the apparent pK_a values of both RH421 and di-8-ANEPPS when bound to DMPC vesicles of 3.1 (Clarke et al., 1995) and <1.8 (Clarke, 1997), respectively. Therefore, at a pH of 4.9, both dyes can still be considered to be predominantly in their basic unprotonated forms.

All solutions were prepared using deionized water. The origins of the various reagents used were as follows. Tris-[(hydroxymethyl)amino]methane (99.9%, Sigma), EDTA (99%, Sigma), HCl (1.0 M Titrisol solution, Merck), ethanol (analytical grade, Merck), NaF (analytical grade, Merck), NaCl (analytical grade, Merck), NaBr (Suprapur, Merck), NaI (analytical grade, Merck), $Na_2SO_4 \cdot 10H_2O$ (analytical grade, Merck), $NaNO_3$ (analytical grade, Merck), NaSCN (>98.5%, Merck), $NaClO_4$ (analytical grade, Merck), KCl (analytical grade, Merck), NH_4Cl (analytical grade, Merck), tetraethylammonium chloride (microselect, Fluka), LiCl (analytical grade, Merck), RbCl (analytical grade, Merck), CsCl (analytical grade, Merck), $MgCl_2 \cdot 6H_2O$ (analytical grade, Merck), $CaCl_2 \cdot 2H_2O$ (analytical grade, Merck), $SrCl_2 \cdot 6H_2O$ (analytical grade, Merck), $BaCl_2 \cdot 2H_2O$ (analytical grade, Merck), and $LaCl_3 \cdot 7H_2O$ (microselect, Fluka).

Absorbance measurements were performed with a Hitachi (Tokyo, Japan) U-3000 spectrophotometer equipped with a head-on photomultiplier to minimize the effects of light scattering. Steady state fluorescence measurements were recorded with a Hitachi F-4500 fluorescence spectrophotometer. To minimize contributions from scattering of the exciting light and higher order wavelengths, a glass cutoff filter was used in front of the emission monochromator. For both dyes, the fluorescence emission was measured at an emission wavelength of 670 nm (+RG645 glass cutoff filter, Schott, Mainz, Germany). For RH421, the fluorescence excitation ratio, R, is defined as the ratio of the fluorescence intensity at an excitation wavelength of 440 nm divided by that at 540 nm. For di-8-ANEPPS, R is defined as the ratio of the fluorescence intensity at an excitation wavelength of 420 nm divided by that at 520 nm. The wavelengths were chosen based on a previous study (Clarke and Kane, 1997) to avoid any effects of membrane fluidity on the measured fluorescence ratios. The temperature of the cuvette holder was thermostatically controlled to 30°C, i.e., above the main phase transition temperatures of DMPC of 23°C (Cevc, 1993), so that the lipid was in its liquid crystalline state.

The association of substrates to lipid vesicles can often be analyzed according to a binding model (Bashford and Smith, 1979; Zouni et al., 1993). The apparent microscopic binding constant, K, is defined, according to the mass action law, by

$$K = \frac{c^*_{XL}}{(nc^*_L - c^*_{XL})(c^*_X - c^*_{XL})}, \tag{1}$$

where c^*_L, c^*_X, and c^*_{XL} represent the total concentrations of lipid, substrate (free and bound), and bound substrate, respectively. Variable n is the number of substrate binding sites per lipid molecule. Under conditions in which there is a large excess of substrate molecules or ions over the available binding sites (the condition of the perchlorate titration to be presented here), i.e., $c^*_X \gg c^*_{XL}$, it can be shown from Eq. 1 that c^*_{XL} is given by

$$c^*_{XL} = \frac{nKc^*_X c^*_L}{1 + Kc^*_X}. \tag{2}$$

In analogy to the theoretical analysis, which has already been applied to the binding of the dipolar species phloretin to lipid vesicles (Clarke and Kane, 1997), the fluorescence ratio, R, of dye in the membrane is assumed to be related to the fraction of binding sites occupied, c^*_{XL}/nc^*_L, by

$$R = R_0 + (R_\infty - R_0) \times \frac{c^*_{XL}}{nc^*_L}, \tag{3}$$

where R_0 is the ratio in the absence of any substrate and R_∞ is the ratio when all of the substrate binding sites are occupied. Substitution of Eq. 2 for c^*_{XL} into Eq. 3 leads to the following equation relating the experimentally measured R value to the total concentration of substrate added:

$$R = R_0 + (R_\infty - R_0) \times \frac{Kc^*_X}{1 + Kc^*_X}. \tag{4}$$

It should be noted that Eq. 4 is only strictly valid in cases in which no cooperativity is involved in the binding process, i.e., the value of K is independent of the amount of substrate that is bound. In the case of charged substrates, however, a negative cooperativity may become apparent as the concentration of bound substrate increases because of charge repulsion. In such cases, the variation in the apparent value of K as a function of the fraction of binding sites occupied can be taken into account by using a Boltzmann relation,

$$K = K_i \exp\left(-\frac{R - R_0}{R_\infty - R_0} \times \frac{ze_0\psi_{max}}{kT}\right), \tag{5}$$

where K_i is the intrinsic microscopic binding constant in the absence of any charge repulsion, ψ_{max} is the maximum additional apparent electrical potential experienced by a charged substrate species in the membrane caused by charge–charge interactions between the charged substrate species within the membrane when all the binding sites are occupied, e_0 is the elementary charge, z is the valence of the charged substrate, k is the Boltzmann constant, and T is the absolute temperature. The term $(R - R_0)/(R_\infty - R_0)$ in Eq. 5 defines, in agreement with Eq. 3, the fraction of the binding sites occupied. Inherent in Eq. 5 is the approximation that the additional apparent electrical potential, ψ, at any particular concentration of the charged substrate increases linearly with the fraction of binding sites occupied. In combination with each other, Eqs. 4 and 5 provide a theoretical adsorption isotherm to which the experimentally determined R values and the total concentration of the charged substrate, c^*_X, can be fitted to obtain values of K_i, ψ_{max}, R_0, and R_∞.

Nonlinear least squares fits of the perchlorate titration data to Eq. 4, excluding charge interactions, were carried out using the commercially available program ENZFITTER (Biosoft, Cambridge, UK). The system Eqs. 4 and 5 can be solved numerically and fitted to the same data. This was carried out using the program MLAB (Civilized Software Inc., Bethesda, MD).

RESULTS

Interaction of anions with DMPC vesicles

Fluorescence excitation spectra have been recorded of RH421 and di-8-ANEPPS bound to DMPC vesicles in the

presence of a variety of different anions. It was found that the observed spectrum shifted depending on the anion used. The shifts have been quantified through a ratiometric method, i.e., the ratio, *R*, of the fluorescence intensities detected at two excitation wavelengths on the blue and red flanks of the excitation spectrum was measured (see Materials and Methods). Initial experiments performed in a buffer containing 10 mM Tris and 1 mM EDTA (adjusted to pH 7.2 with HCl) in the presence and absence of 0.5 M NaCl, showed that NaCl had no effect on the measured *R* value. For RH421, the *R* values were 1.655 (± 0.019) in the absence of NaCl and 1.654 (± 0.022) in the presence of NaCl. For di-8-ANEPPS, the *R* values were 2.090 (± 0.020) in the absence of NaCl and 2.094 (± 0.025) in the presence of NaCl. Na^+ was, therefore, used as a neutral counterion to test the effect of the various anions on the measured *R* value.

The values of *R* obtained in the presence of 0.5 M of the various anions are listed in Table 1. It can be seen that there is a significant decrease in the *R* value when going from SO_4^{2-} to ClO_4^-, from 1.679 (± 0.017) to 0.951 (± 0.016) for RH421, and from 2.200 (± 0.041) to 1.030 (± 0.006) for di-8-ANEPPS. This corresponds to a red shift of the fluorescence excitation spectra of the two dyes and a decrease in the membrane dipole potential (Gross et al., 1994; Clarke and Kane, 1997; Clarke, 1997), according to a calibration of the di-8-ANEPPS spectra, (Clarke, 1997) from 333 (± 128) to 117 (± 83) mV. The shifts of the excitation spectra of the probes induced by ClO_4^- are demonstrated in Fig. 2. The magnitude of the red shift increases in the order $SO_4^{2-} < F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$. This order agrees well with the order of effectiveness of the anions in increasing muscle twitch tension discussed in the Introduction.

In Fig. 3, the measured *R* values for the various anions are plotted against their respective values of the theoretical Gibbs free energy of hydration (Marcus, 1991) for the hypothetical process of transfer of the ion from a vacuum into water. It can be seen that there is a relatively good correlation between the magnitude of the observed effect on *R* and the Gibbs free energy of hydration, i.e., anions with a low free energy of hydration produce the greatest red shifts of the fluorescence excitation spectra of the two probes. The significance of this will be discussed later.

TABLE 1 Effect of anions and cations on the fluorescence excitation ratios, *R*, of RH421 and di-8-ANEPPS bound to DMPC vesicles

Ion*	*R* (RH421)#	*R* (di-8-ANEPPS)#	ΔG^0_{hyd} (kJ mol^{-1})§
—	1.655 (± 0.019)	2.090 (± 0.020)	—
$C1^-$	1.654 (± 0.022)	2.094 (± 0.025)	−270
SO_4^{2-}	1.679 (± 0.017)	2.200 (± 0.041)	−1145
F^-	1.664 (± 0.012)	2.143 (± 0.033)	−345
Br^-	1.604 (± 0.018)	2.024 (± 0.015)	−250
NO_3^-	1.575 (± 0.011)	1.948 (± 0.016)	−275
I^-	1.267 (± 0.029)	1.435 (± 0.022)	−220
SCN^-	1.145 (± 0.009)	1.201 (± 0.015)	−230
$C1O_4^-$	0.951 (± 0.016)	1.030 (± 0.006)	−180
Na^+	1.654 (± 0.022)	2.094 (± 0.025)	−400
TEA^+¶	1.718 (± 0.014)	2.133 (± 0.024)	—
Rb^+	1.695 (± 0.016)	2.123 (± 0.027)	−285
K^+	1.661 (± 0.017)	2.099 (± 0.020)	−305
Cs^+	1.651 (± 0.006)	2.083 (± 0.030)	−245
NH_4^+	1.620 (± 0.023)	2.084 (± 0.024)	−285
Li^+	1.597 (± 0.018)	2.084 (± 0.017)	−510
Ba^{2+}	1.542 (± 0.021)	1.993 (± 0.034)	−1210
Sr^{2+}	1.584 (± 0.030)	1.988 (± 0.029)	−1385
Mg^{2+}	1.480 (± 0.020)	1.962 (± 0.021)	−1940
Ca^{2+}	1.480 (± 0.023)	2.047 (± 0.028)	−1515
La^{3+}	1.340 (± 0.026)	1.823 (± 0.026)	−3090

*In the case of the anions, the counterion was always Na^+; for the cations it was always $C1^-$. The first row in the table refers to values measured in buffer (10 mM Tris, 1 mM EDTA, pH 7.2) in the absence of any additional salt. Na^+ and $C1^-$ were chosen as neutral counterions because the *R* values obtained in buffer containing 0.5 M NaCl were indistinguishable from the values for buffer alone. The concentration of each ion listed in the table was 0.5 M.

#In the case of RH421 the fluorescence ratio, *R*, is defined as the fluorescence intensity of membrane-bound dye measured at an excitation wavelength, λ_{ex}, of 440 nm divided by that measured at 540 nm. For di-8-ANEPPS, *R* is defined as the fluorescence intensity at a λ_{ex} of 420 nm divided by that at 520 nm. In each case, the emission wavelength used was 670 nm (+ RG645 cut-off filter). RH421 (4.14 μM) or di-8-ANEPPS (4.97 μM) were added from ethanolic stock solutions to DMPC vesicles ([DMPC] = 2.5–2.9 mM). The temperature for the measurements was 30°C.

§ΔG^0_{hyd} is the Gibbs free energy of hydration at 298.15 K for the hypothetical transfer of an ion from a vacuum into water. The values given are theoretical values calculated by Marcus (1991).

¶tetraethylammonium.

Interaction of $NaClO_4$ with DMPC vesicles

Because the magnitude of the red shift induced by anions was found to be the greatest with ClO_4^-, the interaction of this ion with lipid vesicles was investigated in more detail. The effects of increasing concentrations of $NaClO_4$ on the fluorescence ratio, *R*, of membrane-bound RH421 and di-8-ANEPPS are shown in Fig. 4. It can be seen that, in both cases, there is a decrease in the *R* value as the $NaClO_4$ concentration increases until, at high concentrations, a saturating value is reached. At first, it was attempted to fit the data to a saturable binding model with a constant value of the microscopic binding constant *K*, as described by Eq. 4. However, as shown in Fig. 4, significant positive and negative deviations of the best fit curve from the experimental points were observed. This simple model was, therefore, discarded. A more complicated model was then tried, in which the apparent microscopic binding constant varies according to the amount of bound perchlorate ions, because of charge repulsion on the membrane. This effect could be taken into account through a Boltzmann relation, as given by Eq. 5. The data were, therefore, fitted to the combination of Eqs. 4 and 5. The more complicated model was found to yield a good description of the observed experimental behavior (see Fig. 4). For RH421, the best fit values of the

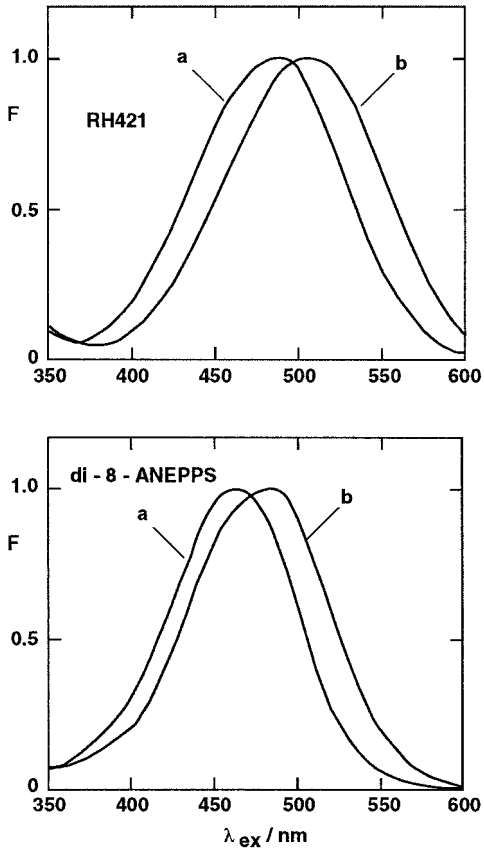

FIGURE 2 Normalized fluorescence excitation spectra of 4.1 μM RH421 (*top*) and 5.0 μM di-8-ANEPPS (*bottom*) in the presence of 3 mM of DMPC in the form of unilamellar vesicles. Curves *a* and *b* indicate the spectra obtained in the absence (*a*) and presence (*b*) of 5 M $NaClO_4$. λ_{em} = 670 nm (+ RG645 cutoff filter), bandwidths = 5 nm, T = 30°C.

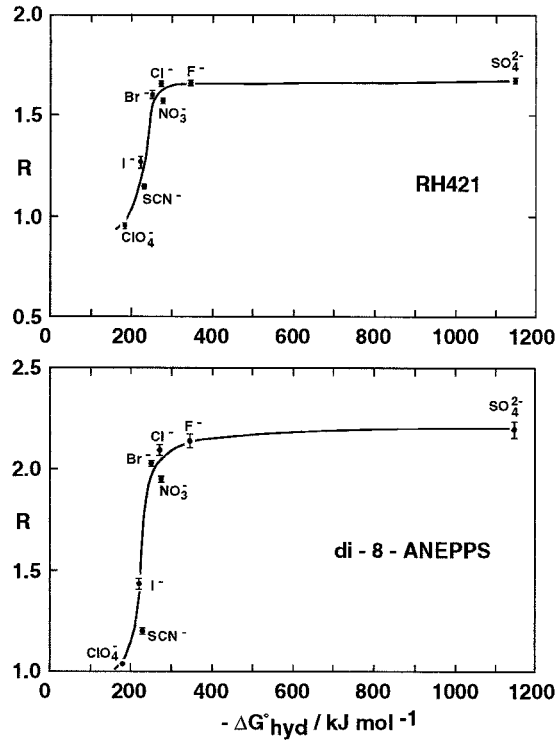

FIGURE 3 Fluorescence intensity ratios, R, of 4.1 μM RH421 (*top*) and 5.0 μM di-8-ANEPPS (*bottom*) in the presence of DMPC unilamellar vesicles (3 mM lipid) and 0.5 M of a range of sodium salts as a function of the Gibbs free energy of hydration of the relevant anions, ΔG^0_{hyd}. The values of ΔG^0_{hyd} are theoretical values calculated by Marcus (1991). The solid line has been drawn to aid the eye of the reader. In the case of both probes, the fluorescence emission was observed at 670 nm (+ RG645 cutoff filter). In the case of RH421, R represents the ratio of the fluorescence intensity produced by excitation at 440 nm to that produced by excitation at 540 nm. In the case of di-8-ANEPPS, the excitation wavelengths were 420 nm and 520 nm. T = 30°C, bandwidths = 5 nm.

parameters calculated were

$$R_o = 1.654\ (\pm\ 0.020), \qquad R_\infty = 0.407\ (\pm\ 0.063),$$

$$K_i = 9.9\ (\pm\ 1.8)\ M^{-1}, \qquad \psi_{max} = -0.058\ (\pm\ 0.014)\ V.$$

The corresponding best fit values for di-8-ANEPPS were

$$R_o = 2.089\ (\pm\ 0.011), \qquad R_\infty = 0.545\ (\pm\ 0.021),$$

$$K_i = 22.7\ (\pm\ 2.1) M^{-1}, \qquad \psi_{max} = -0.061\ (\pm\ 0.006) V.$$

The reciprocal of K_i corresponds to the intrinsic microscopic dissociation constant. This amounts to 101 (± 18) mM for the RH421 measurements and 44 (± 4) mM for the di-8-ANEPPS measurements. Although both values are of the same order of magnitude, di-8-ANEPPS appears to yield a somewhat higher affinity of perchlorate for the membrane than does RH421. This has also been found to be the case for the binding of the dipolar molecule phloretin to DMPC vesicles (Clarke and Kane, 1997). However, the two values of ψ_{max} calculated, −58 (± 14) mV and −61 (± 6) mV using RH421 and di-8-ANEPPS, respectively, are identical within experimental error.

The values of K_i determined here can be compared to values reported in previous studies. Tatulian (1983, 1987), based on the effect of perchlorate ions on the electrophoretic mobility of lipid vesicles, determined K_i values of 222 (± 15) M^{-1} and 70 (± 10) M^{-1} for multilamellar DMPC and egg PC, respectively. From ^2H NMR measurements, Rydall and Macdonald (1992) determined a K_i value of 115 M^{-1} for perchlorate binding to multilamellar POPC vesicles. All three values are significantly greater than those reported here. There could be a number of reasons for this. For example, the value of K_i may depend on the lipid used and on the size and lamellar structure of the vesicles used. The experimental method and the method of data analysis could also play a role. Apart from using experimental techniques very different from that employed here, Tatulian (1983, 1987) and Rydall and Macdonald (1992) also used a somewhat different data analysis procedure. Both have taken charge repulsion into account as done here, by using a Boltzmann relation, but they calculated the electrical potential at the membrane surface from the surface charge density, theoretically, by using an electrical double layer

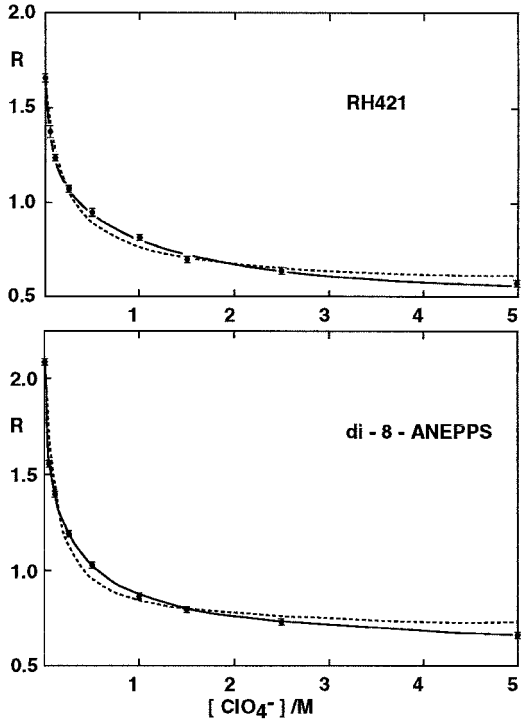

FIGURE 4 Fluorescence intensity ratios, R, of 4.1 μM RH421 (*top*) and 5.0 μM di-8-ANEPPS (*bottom*) in the presence of DMPC vesicles (3 mM lipid) as a function of the $NaClO_4$ concentration included in the buffer medium. The excitation and emission wavelengths were as given in Fig. 3. $T = 30°C$, bandwidths = 5 nm. The *dotted lines* represent fits of the data to a binding model according to Eq. 4, excluding any electrostatic effects. The *solid lines* represent fits of the data to an extended binding model, described by the combination of Eqs. 4 and 5, including electrostatic repulsion effects from bound perchlorate ions.

theory. In contrast, we have preferred to determine the electrical potential directly from the experimental data by including it as a fit parameter, which accounts for the deviation from a pure nonelectrostatic binding model (Langmuir adsorption isotherm). However, it should be pointed out that widely varying intrinsic binding constants have also been reported for other ions. For example, Macdonald and Seelig (1988) reported a K_i for SCN^- binding to POPC vesicles of 12.6 M^{-1}, and, in a later paper, Rydall and Macdonald (1992) determined a value of 80 M^{-1} for the same system. Because of the relatively large variation in reported intrinsic binding constants, Rydall and Macdonald (1992) concluded that one should concentrate on the trends across the Hofmeister or lyotropic series, rather than focusing on the absolute values of the binding constants.

Interaction of cations with DMPC vesicles

Fluorescence excitation spectra have also been recorded of RH421 and di-8-ANEPPS bound to DMPC vesicles in the presence of a variety of different cations. Here, it was also found that the observed spectrum shifted depending on the cation used, although the effects were significantly smaller than those observed by varying the anion. Again, the shifts were quantified using the ratiometric method. Because, as stated earlier, NaCl had no effect on the measured R value, in this case, Cl^- was used as a neutral counterion to test the effect of the various cations.

The values of R obtained in the presence of 0.5 M of the various cations are listed in Table 1. A decrease can be seen in the R value when going from the monovalent cations, which all have values of 1.6–1.7 for RH421 and approximately 2.1 for di-8-ANEPPS, to La^{3+}, which has R values of 1.340 ($\pm$ 0.026) and 1.823 ($\pm$ 0.026) for RH421 and di-8-ANEPPS, respectively. As in the case of the anion effects, this corresponds to a red shift of the fluorescence excitation spectrum of the two dyes and a decrease in the membrane dipole potential from a value of approximately 320 ($\pm$ 120) to 264 ($\pm$ 111) mV.

In Fig. 5, the measured R values for the various cations are plotted against their respective values of the theoretical Gibbs free energy of hydration. There is an approximately linear relationship between R and $-\Delta G^0_{hyd}$ for both dyes. However, in contrast to the results obtained with the anions,

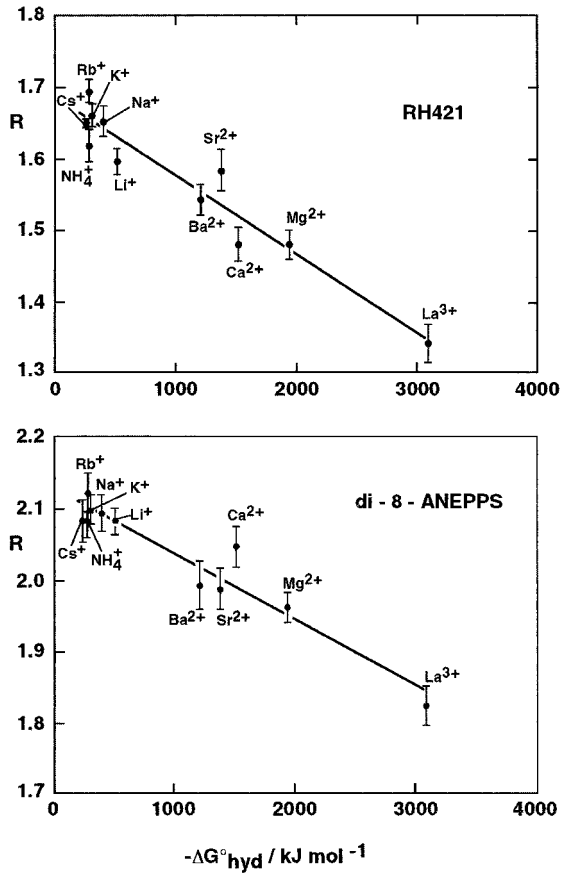

FIGURE 5 Fluorescence intensity ratios, R, of 4.1 μM RH421 (*top*) and 5.0 μM di-8-ANEPPS (*bottom*) in the presence of DMPC unilamellar vesicles (3 mM lipid) and 0.5 M of a range of chloride salts as a function of the Gibbs free energy of hydration of the relevant cations, ΔG^0_{hyd}. The *solid line* represents a fit of the data to a straight line according to Eq. 6. The value of ΔG^0_{hyd} are theoretical values calculated by Marcus (1991). The excitation and emission wavelengths were as given in Fig. 3. $T = 30°C$, bandwidths = 5 nm.

it should be noted that the cation with the highest free energy of hydration, La^{3+}, produces the greatest red shift of the fluorescence excitation spectra of the two probes.

The linear relationship between R and $-\Delta G^0_{hyd}$ (see Fig. 5) is defined by

$$R = m(-\Delta G^0_{hyd}) + c. \quad (6)$$

In the case of both probes, fitting the data according to Eq. 6 yielded a correlation coefficient of 0.96. The best fit values of m derived were $-1.11\ (\pm 0.11) \cdot 10^{-4}$ mol kJ^{-1} and $-0.92\ (\pm 0.09) \cdot 10^{-4}$ mol kJ^{-1} for RH421 and di-8-ANEPPS, respectively. The corresponding values of c were 1.69 ($\pm$ 0.01) and 2.13 ($\pm$ 0.01).

DISCUSSION

The fluorescent styrylpyridinium dyes RH421 and di-8-ANEPPS have been found to show shifts in their fluorescence excitation spectra when bound to DMPC vesicles during interaction with a variety of anions and cations. The shifts have been quantified by using the ratio, R, of the fluorescence intensities detected at two different excitation wavelengths. Based on previous work (Gross et al., 1994; Clarke and Kane, 1997; Clarke, 1997), these shifts can be confidently attributed to changes in the magnitude of the membrane dipole potential (Brockman, 1994; Cafiso, 1995). Theoretical calculations (Flewelling and Hubbell, 1986) and kinetic measurements of the rate of transport of hydrophobic ions across bilayer membranes (Pickar and Benz, 1978; Gawrisch et al., 1992) and lipid vesicles (Franklin and Cafiso, 1993) indicate that the dipole potential of phosphatidylcholine has a value in the range 100–300 mV, positive in the interior of the membrane.

Let us consider first the interaction of anions with DMPC vesicles. It was found that both membrane-bound RH421 and di-8-ANEPPS experienced an increasing red shift of their fluorescence excitation spectra in the order of anion effectiveness $SO_4^{2-} < F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$. The red shift of the spectra indicates a decrease in the magnitude of the membrane dipole potential (Gross et al., 1994; Clarke and Kane, 1997; Clarke, 1997). Because, as stated above, the dipole potential is believed to have a positive value in the interior of the membrane, this result is, therefore, consistent with the anions binding inside the membrane, with the strength of binding also given by the above order, as proposed by Hodgkin and Horowicz (1960), to explain the effects of anions on the muscle twitch tension. The anions can be considered to screen the positive end of the intrinsic dipole potential, as shown in Fig. 6. The order of anion effectiveness in reducing the dipole potential given above is in good agreement with the order in which their binding influences a number of other experimental effects, e.g., ^2H NMR quadrupole splitting (Rydall and Macdonald, 1992), ^1H NMR chemical shift (Jendrasiak, 1972), phase transition temperature broadening (Jain and Wu, 1977), and electrophoretic mobility (Tatulian, 1987).

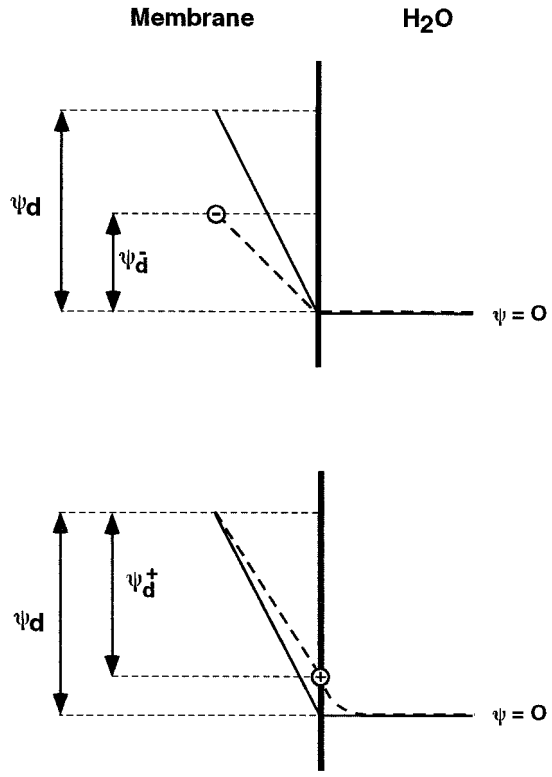

FIGURE 6 Proposed mechanisms for the change in dipole potential, ψ_d, induced by the interaction of the membrane with anions (*top*) and cations (*bottom*). The *solid lines* represent the profile of the electrical potential, ψ, in the absence of bound ions. The *dotted lines* represent the profile of ψ in the presence of anions or cations. The electrical potential is defined to be zero in the aqueous solution far from the membrane surface. ψ_d, as measured by the styrylpyridinium dyes, is defined as the difference in electrical potential between the membrane interior and the membrane surface (not the bulk aqueous phase), i.e., where the dyes are located. ψ_d^- and ψ_d^+ represent the dipole potentials after binding of anions and cations, respectively. The binding plane of the anions is assumed to be located within the membrane, whereas that of the cations is assumed to be located at the membrane/water interface. In principle, both anions and cations will change the electrical potential within the membrane as well as at the membrane–water interface. However, because their effects will dominate at their respective binding planes, for simplicity, the smaller effects of anions at the membrane–water interface and of cations in the membrane interior have been neglected.

To investigate further the hypothesis of anion binding to the membrane, the measured R values of the various anions were compared with their respective values of the Gibbs free energy of hydration. A relatively good correlation was observed (see Fig. 3), with the anions having the lowest free energy of hydration producing the greatest red shifts and hence the greatest decrease in the dipole potential, i.e., the least hydrophilic anions produce the greatest effects. Intuitively, this would be expected, because the least hydrophilic anions would have the greatest tendency to leave the aqueous environment and enter the more hydrophobic environment of the membrane. The behavior shown in Fig. 3 can also be understood in mathematical terms by assuming a model in which there is a competition between aqueous and membrane phases for anions. Introducing the free en-

ergies for the transfer of anions from a vacuum to the aqueous phase, ΔG^0_{hyd}, and from a vacuum to the membrane phase, ΔG^0_{mem}, it can easily be shown that the distribution of anions between the membrane and aqueous phases is given by

$$\frac{[A^-]_m}{[A^-]_{aq}} = \exp(-(\Delta G^0_{mem} - \Delta G^0_{hyd})/RT), \quad (7)$$

where R is, here, the gas constant. Based on Eq. 7 and the assumption that the volume of the aqueous phase is far greater than that of the membrane phase, i.e., $V_{aq} \gg V_m$, it can then be shown that, at any particular concentration of anions, the fraction of the total concentration of anions in the aqueous phase, x, is given by

$$x = \frac{1}{1 + (V_m/V_{aq}) \times \exp(-(\Delta G^0_{mem} - \Delta G^0_{hyd})/RT)}. \quad (8)$$

Because Fig. 3 shows that the strength of interaction of a particular anion is particularly dependent on its free energy of hydration, it will now be assumed in the first instance that the free energy of transfer from a vacuum into the membrane phase, ΔG^0_{mem}, has a constant value for all the anions investigated. Computer simulations (see Fig. 7) based on Eq. 8 show that a reasonably good description of the observed experimental behavior is found if one assumes a value for ΔG^0_{mem} of -235 kJ mol^{-1}. However, it should be noted that the assumption of a constant value for ΔG^0_{mem} is probably an over-simplification. In fact, the various anions are, no doubt, likely to have some specific interactions with the membrane as well, so that ΔG^0_{mem} is likely to vary somewhat. This could account for any divergence of the order of effectiveness in which the ions bind to the membrane from the strict order to be expected based on ΔG^0_{hyd} alone. In a similar fashion, this could explain why the Hofmeister or lyotropic series published for the effects of anions on different proteins are not always identical. The order of the ΔG^0_{prot} values for the transfer of the anions from a vacuum to the protein surface could vary from one protein to another, even though the primary underlying effect of the energy of hydration is constant.

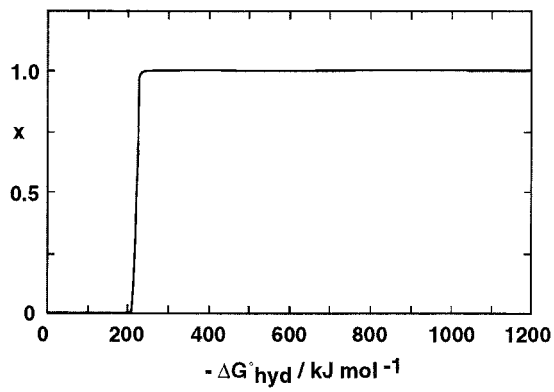

FIGURE 7 Simulation of the dependence of the fraction of the total concentration of anions in the aqueous phase, x, on the Gibbs free energy of hydration, ΔG^0_{hyd}, based on a theoretical model assuming a partition of anions between aqueous and membrane phases. The theoretical curve is defined by Eq. 8, where the Gibbs free energy of interaction of the anions with the membrane phase, ΔG^0_{mem}, was assumed to have a constant value of -235 kJ mol^{-1}. A partial molar volume of DMPC of 0.98 ml g^{-1} (Marsh, 1990) was used to calculate the relative volumes of the membrane and aqueous phases.

Before proceeding, one final point concerning Eqs. 7 and 8 must be mentioned. They assume the membrane to be a phase with an unlimited capacity for accepting anions. In fact, as shown in Fig. 4, a binding model with a limited number of binding sites per vesicle is more appropriate. Nevertheless, at low anion concentrations, before any saturation becomes apparent, Eqs. 7 and 8 could be considered to be adequate approximations.

Now let us consider the interaction of cations with DMPC vesicles. It was found that both membrane-bound RH421 and di-8-ANEPPS experienced an increasing red shift of their fluorescence excitation spectra as the free energy of hydration increased (see Fig. 5). This is in agreement with the cation effectiveness reported by Akutsu and Seelig (1981) and Seelig et al. (1987) for the influence on the ^{2}H NMR quadrupole splitting of DPPC vesicles. It is, however, the complete opposite of the situation with anions. In the case of the cations, it is the most hydrophilic ion, La^{3+}, that causes the greatest effect, whereas, in the case of the anions, it is the least hydrophilic ion, ClO_4^-. Therefore, the most hydrophilic cations cause the greatest reduction in the membrane dipole potential. For the cations, it would seem that a very different mechanism is required to explain their interaction with the membrane than with the anions. If the cations were binding within the lipid membrane, as the results indicate for the anions, one would expect an increase in dipole potential (i.e., a blue shift of the fluorescence excitation spectra) rather than the observed decrease. One can imagine two possible factors to explain the effect of the cations: 1) they are interacting with specific polar sites situated directly on the membrane surface rather than within the membrane, and 2) they are dehydrating the membrane headgroup region. Factor (1) could explain a decrease in dipole potential if the positive dipole potential within the membrane was being counterbalanced by a positive potential from cations on the membrane surface, i.e., the cations are screening the negative end of the intrinsic dipole potential. This mechanism is shown schematically in Fig. 6.

However, factor (2) is also a feasible possibility. The interaction of cations having a high energy of hydration with polar groups of the lipid on the membrane surface could result in the displacement of the water of hydration of the polar groups. Therefore, factor (2) can be seen as a secondary effect occurring as a consequence of cation binding. It has recently been suggested, based on both theoretical calculations (Zheng and Vanderkooi, 1992; Essmann et al., 1995) and experimental data (Gawrisch et al., 1992; Jendrasiak et al., 1997; Moncelli et al., 1998), that water dipoles make a major contribution to the magnitude of the dipole potential. If it is true that the dipole potential arises

from water molecules on the membrane surface, then their displacement by strongly hydrophilic cations might produce a decrease in dipole potential.

In fact, it is possible that both factors are contributing to the decrease in dipole potential. Conclusive evidence has been reported from neutron diffraction (Herbette et al., 1984), luminescence (Herrmann et al., 1986), and NMR investigations (McLaughlin et al., 1978; Petersheim et al., 1989) supporting a specific interaction of metal cations with the phosphate group of phosphatidylcholine. Furthermore, there is a significant amount of evidence from NMR (Hauser et al., 1975), infrared spectroscopy (Dluhy et al., 1983; Casal et al., 1987a,b), and calorimetry (Garidel and Blume, unpublished results) that, at least in the cases of phosphatidylserine and phosphatidylglycerol, the binding of metal cations to the phosphate group causes it to lose its water of hydration. Therefore, it seems likely that the binding of metal cations to the phosphate group of phosphatidylcholine may also involve the loss of water of hydration, and that both cation binding and water release may play a role in the decrease in dipole potential. The relative contributions of the two effects to the dipole potential change is uncertain and requires further investigation.

In fact, it might seem unexpected that the binding of cations to the membrane surface, as shown in Fig. 6, could influence the dipole potential, because it has been shown by Gross et al. (1994) that the incorporation of 20% negatively charged phosphatidylserine into phosphatidylcholine vesicles had no effect on the *R* value of di-8-ANEPPS. In contrast, Ermakov et al. (1992) have reported the results of direct electrical measurements showing that Be^{2+} ions may interact with lipid membranes causing a change in dipole potential. The apparent discrepancy between the results presented here and those of Gross et al. (1994) perhaps could be explained by different locations of the negative charge of phosphatidylserine and the positive charge of the cations relative to the position of the dyes in the membrane. However, it should be noted that the effects of cations on the dipole potential are indeed significantly less than those of lyotropic anions.

The possibility that cations could influence the dipole potential through an effect on lipid packing should still be considered. Verstraeten et al. (1997) have presented evidence from fluorescence polarization studies that Be^{2+} and a number of trivalent metal ions in the submillimolar range increase lipid packing. However, that this effect is responsible for the changes in dipole potential reported here would seem very unlikely, because, based on theoretical considerations and experimental data (Clarke, 1997), an increase in lipid packing would be expected to induce an increase in the dipole potential, i.e., in the opposite direction from that experimentally found.

A final important point to be considered is the effect that anions and cations may have on the lipid conformation. Based on NMR spectroscopic measurements, it has been suggested by Seelig and coworkers (Scherer and Seelig, 1989; Bechinger and Seelig, 1991) that the $^{-}P–N^{+}$ dipole of the phosphatidylcholine headgroup undergoes a change in orientation during interaction with charged molecules and ions. These authors have proposed that positively charged species move the N^{+} end of the dipole toward the aqueous phase, whereas negatively charged species move it toward the hydrocarbon phase. Therefore, this effect would be expected to cause a decrease in the positive dipole potential in the case of cations and an increase in the case of anions. The fact that the lyotropic anions studied here cause a decrease in the dipole potential implies that the direct electrostatic screening effect (see Fig. 6) must, at least in the case of the anions, be the major contributing factor, whereas the headgroup reorientation could only be a minor compensatory effect. However, headgroup reorientation could be an important factor in the smaller decrease in dipole potential observed during binding of cations to the membrane surface.

To conclude, it has been shown that anions bind to phosphatidylcholine vesicles and reduce the membrane dipole potential with an order of effectiveness that corresponds to the Hofmeister or lyotropic series. Therefore, the effect of anions on the dipole potential provides a molecular framework for the interpretation of the Hofmeister effect in the case of ion-translocating membrane proteins. Because such proteins must undergo conformational changes in the process of ion transport, it is reasonable to expect that particular conformations may be stabilized by interaction with the dipole potential. Therefore, any changes in the dipole potential by the addition of lyotropic anions might change the relative stability of the various conformations and influence the kinetics of ion transport. In a subsequent paper, significant effects of lyotropic anion-induced dipole potential changes on the kinetics of partial reactions of the Na^{+},K^{+}-ATPase pump cycle will be presented.

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