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# Adsorption of Monovalent Cations to Bilayer Membranes Containing Negative Phospholipids†

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ABSTRACT: The electrophoretic mobilities of multilamellar phosphatidylserine vesicles were measured in solutions containing monovalent cations, and the $\zeta$ potentials, the electrostatic potentials at the hydrodynamic plane of shear, were calculated from the Helmholtz–Smoluchowski equation. In the presence of 0.1 M lithium, sodium, ammonium, potassium, rubidium, cesium, tetraethylammonium, and tetramethylammonium chloride, the $\zeta$ potentials were −60, −62, −72, −73, −77, −80, −82, and −91 mV, respectively. Similar results were obtained with phosphatidylglycerol vesicles; different results were obtained with cardiolipin, phosphatidylinositol, and phosphatidic acid vesicles. The phosphatidylserine results are interpreted in terms of the Stern equation, a combination of the Gouy equation from the theory of the diffuse double layer, the Boltzmann relation, and the Langmuir adsorption isotherm. Evidence is presented that suggests the hydrodynamic plane of shear is 2 Å from the surface of the membrane in solutions containing the alkali metal cations. With this assumption, the intrinsic association constants of the above monovalent cations with phosphatidylserine are 0.8, 0.6, 0.17, 0.15, 0.08, 0.05, 0.03, and 0 $M^{-1}$, respectively. The validity of this approach was tested in two ways. First, the $\zeta$ potentials of vesicles formed from mixtures of phosphatidylserine and a zwitterionic lipid, phosphatidylcholine, were measured in solutions containing different concentrations of sodium. All the data could be described by the Stern equation if the "relaxation" of the ionic atmosphere, which is predicted by classic electrostatic and hydrodynamic theory to occur at low salt concentrations and high potentials, was circumvented by using only large (diameter >13 $\mu$m) vesicles for these measurements. Second, the fluorescent probe 2-(*p*-toluidinyl)naphthalene-6-sulfonate was used to estimate the potential at the surface of phosphatidylserine and phosphatidylglycerol vesicles sonicated in 0.1 M NaCl. Reasonable agreement with the predicted values of the surface potential was obtained.

This is the third paper in a series of reports on the interaction of inorganic cations with phospholipid bilayer membranes. Our ultimate objective is to understand how divalent cations such as calcium and magnesium interact with negative lipids commonly found in the bilayer component of biological membranes. As biological membranes contain zwitterionic as well as negative lipids, the interaction of cations with these lipids must also be investigated. It has been demonstrated that divalent cations adsorb to the zwitterionic lipid phosphatidylcholine (PC), that this adsorption can be described by the Stern equation (McLaughlin et al., 1978), and that calcium, but none of the other alkaline earth cations, adsorbs more strongly to PC bilayers in the gel than in the liquid-crystalline state (Lau et al., 1979). As biological solutions contain monovalent as well as divalent cations, we also need to know if ions such as sodium and potassium adsorb to a significant degree to negative lipids and if this adsorption can be described by the Stern equation. Phosphatidylserine (PS) is the predominant negative lipid in the membranes of many mammalian cells [e.g., White (1973)]; most of the experiments described below were performed on bilayers containing PS.

There is no completely satisfactory definition of specific adsorption. According to a "quasi-phenomenological" definition of Mohilner (1966) "one says there is specific adsorption if the experimental data cannot be explained by the theory of the diffuse double layer". In the absence of specific adsorption, the theory of the diffuse double layer [e.g., McLaughlin (1977)] predicts that all monovalent cations should exert identical effects on the surface potential of bilayer membranes containing negative lipids.

The first evidence for a specific interaction between the alkali metal cations and PS was obtained by Abramson et al. (1961). These authors noted that Na but not K, at concentrations of 0.4–0.7 M, produced a substantial coagulation of sonicated PS vesicles. Experiments by Hauser et al. (1975) demonstrated that both Na and K but not tetramethylammonium (TMA), at concentrations of 1 M, produced an increase in the optical density of a solution of sonicated PS vesicles. These experiments suggest that the interaction of these cations with PS decreases in the sequence Na > K > TMA. The first quantitative evidence that there was indeed a marked difference in the ability of a variety of monovalent cations to interact with PS was obtained by Puskin (1977). Using electron paramagnetic resonance measurements, he showed that divalent Mn ions were displaced from sonicated PS vesicles by monovalent cations. The relative association constants for Mn in decimolar solutions of Li, Na, K, Rb, Cs, and TMA were 0.31, 1.0, 1.1, 1.3, 1.4, and 7.1, respectively. This marked difference in the ability of Na and TMA to inhibit the binding of Mn to PS led Puskin to suggest that "(a) some specific Na–PS binding or (b) complications arising from penetration of cations into the restricted spaces between the

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0006-2960/79/0418-5213$01.00/0 

head groups of PS" was occurring. These are reasonable suggestions. In an equilibrium dialysis study of the adsorption of calcium to PS vesicles, Nir et al. (1978) concluded that their results could be most easily interpreted if they assumed that the simplest form of the diffuse double layer theory was correct and that some Na binding intrinsic association constant 0.8 $M^{-1}$) was occurring. A recent study by Kurland et al. (1979) utilizing $^{23}Na$ NMR has provided additional evidence that sodium may interact specifically with PS. The NMR results, however, are ambiguous. Similar NMR results obtained in studies of the adsorption of $^{23}Na$ to DNA [e.g., Reuben et al. (1975), Anderson et al. (1978), and Civan & Shporer (1978)] have been interpreted in different ways and have not yet revealed the extent to which sodium adsorbs specifically to this polyanion. Nevertheless, Kurland et al. (1979) were able to conclude, mainly on the basis of competition experiments with tetraethylammonium, that their results were consistent with the intrinsic association constant of Na with PS being about 1 $M^{-1}$.

In summary, if we adopt Mohliner's quasi-phenomenological definition of specific adsorption, there are several lines of evidence suggesting that Na and the other alkali metal cations adsorb specifically to bilayer membranes formed from PS. A more rigorous definition of specific adsorption could be based on a specific structural model of the electrical double layer, for example, that of Grahame (1947). In terms of Grahame's definition, specific adsorption occurs only when the ion interacts so strongly with the surface that it loses its solvation, at least in the direction of the surface. There is good experimental evidence that such "inner sphere complexes", as they are sometimes termed, are formed between cobalt and the phosphate moiety of PC (McLaughlin et al., 1978). At the present time we know of no way to distinguish between the possibility that the alkali metal cations adsorb to PS (in terms of Grahame's definition), the possibility that they interact electrostatically with PS while retaining their water of hydration [Gustavsson & Lindman (1975) suggested that Na interacts with ionic surfactants in this manner, and the interpretation is also consistent with the results Kurland et al. (1979) obtained with PS], and the possibility that one or more of the assumptions inherent in the treatment of all the data are seriously in error. For example, the specificity between the alkali metal cations that is observed experimentally could be due either to a difference in the ability of the cations to penetrate the polar head group region of the membrane or to a difference in the finite size of the cations, an effect that is ignored in the classic Gouy–Chapman theory of the diffuse double layer. This finite-size effect was first considered quantitatively by Stern, who also considered the possibility that ions specifically adsorb to surfaces [for a review see Mohilner (1966)]. Our approach will be to marshal several independent lines of evidence that the electrostatic potential at the surface of a membrane exposed to alkali metal cations can be satisfactorily described by assuming that the simplest form of the diffuse double layer theory is correct and that some specific adsorption of the alkali metal cations also occurs. We relegate to Appendix II a discussion of the possible importance of the finite size of the monovalent cations.

## Theory

(*a*) *Stern Equation.* We assume that the simplest form of the diffuse double layer theory is correct in our interpretation of the experimental data. The major assumptions inherent in this theory are that the charges on the surface, which in our case are located on the negative lipids, are smeared uniformly in a plane located at $x = 0$, that ions do not penetrate beyond this plane to distances $x < 0$, that the ions in solution are point charges, that the dielectric constant of the aqueous phase is equal to its bulk value up to the surface of the membrane, and that image charge effects can be ignored. References to the literature in which these and other assumptions are discussed can be found in reviews by Grahame (1947), Verwey & Overbeek (1948), Haydon (1964), Mohilner (1966), Barlow (1970), Bockris & Reddy (1970), Sparnaay (1972), Aveyard & Haydon (1973), and McLaughlin (1977). By combining the Poisson and Boltzmann equations and applying the appropriate boundary conditions, one obtains the Gouy equation

$$\sinh [e\psi_0/(2kT)] = A\sigma/\sqrt{C} \tag{1}$$

which relates the electrostatic potential in the aqueous phase at the surface of the membrane (relative to the potential in the bulk aqueous phase), $\psi_0$, to the charge density on the surface, $\sigma$, and the concentration of monovalent electrolyte in the bulk aqueous phase, $C$. In eq 1, $e$ is the electronic charge, $k$ is the Boltzmann constant, $T$ is the absolute temperature, and $A$ is a constant equal to $1/(8N\epsilon_r\epsilon_0 kT)^{1/2}$ where $N$ is Avogadro's number, $\epsilon_r$ is the dielectric constant, and $\epsilon_0$ is the permittivity of free space.

The concentration of monovalent cations in the aqueous phase at the membrane solution interface, $C_0$, is given by the Boltzmann relation

$$C_0 = C \exp[-e\psi_0/(kT)] \tag{2}$$

This equation may be derived by noting that the electrochemical potential of the ion must be constant up to the surface of the membrane at equilibrium. Equation 2 follows if it is assumed that the standard chemical potential is independent of distance from the surface and that activity coefficient effects may be ignored.

To describe the adsorption of the monovalent cations to the surface of the membrane, we use a Langmuir adsorption isotherm[1] as shown:

$$\sigma = \sigma^{max}/(1 + KC_0) \tag{3}$$

where $\sigma^{max}$ is the maximum charge density on the membrane (about one electronic charge every 70 Å$^2$ for a PS bilayer) and $K$ is the intrinsic association constant (molarity$^{-1}$).

Note that eq 1–3 may be combined to eliminate $\sigma$ and $C_0$. The resulting equation, which is solved numerically, describes how the surface potential should vary as a function of $\sigma^{max}$, $K$, and $C$.

(*b*) *$\zeta$ Potentials.* We assume that the profile of the electrostatic potential in the aqueous phase, $\psi(x)$, may be described by the Gouy–Chapman theory of the diffuse double layer

$$\psi(x) = \frac{2kT}{e} \ln \left[ \frac{1 + \alpha \exp(-\kappa x)}{1 - \alpha \exp(-\kappa x)} \right] \tag{4}$$

where

$$\alpha = \frac{\exp[e\psi_0/(2kT)] - 1}{\exp[e\psi_0/(2kT)] + 1}$$

and

[1] Note that the use of the Langmuir adsorption isotherm is equivalent to simply assuming that a 1:1 stoichiometric association occurs between a monovalent cation such as Na and a phosphatidylserine molecule [e.g., Gileadi (1967)]. Letting braces denote a surface concentration, the principle of mass action implies that $\{PS\text{–}Na\} = K\{PS\}[Na]_0$ if activity coefficient effects are ignored. Equation 3 follows by noting that $\sigma = -\{PS\}$ and that $\sigma^{max} = -\{PS\} - \{PS\text{–}Na\}$. Aveyard & Haydon (1973) and Bond et al. (1979) may be consulted for a statistical mechanical derivation of the Langmuir and Stern equations.

$$\kappa = \left( \frac{2e^2CNz^2}{\epsilon_r\epsilon_0 kT} \right)^{1/2}$$

The potential profile for a PS vesicle in an aqueous solution containing a 0.1 M monovalent electrolyte that does not adsorb to the membrane is illustrated in Figure 1.

The $\zeta$ potential, $\zeta$, or potential at the hydrodynamic plane of shear, is calculated from the measured value of the electrophoretic mobility, $u$, by the Helmholtz–Smoluchowski equation

$$\zeta = u\epsilon_r\epsilon_0/\eta \qquad (5)$$

where $\eta$ is the viscosity of the aqueous phase. The plane of shear is assumed to be 2 Å from the surface of the membrane in Figure 1. Three major assumptions are inherent in the derivation of the Helmholtz–Smoluchowski equation: (1) the dielectric constant and (2) the viscosity of the aqueous phase are assumed to be independent of position up to the surface of the membrane and (3) the "relaxation" of the ionic atmosphere is ignored [e.g., Overbeek & Wiersema (1967)]. The third assumption is discussed in Appendix I because the experimental results we present there and the theoretical analysis of Wiersema et al. (1966) strongly suggest that the assumption is not valid for phospholipid vesicles under certain conditions.

(*c*) *Fluorescent Probes.* A charged fluorescent molecule must satisfy the following criteria to be considered a good probe of the electrostatic potential at the membrane–solution interface. (1) The molecule must adsorb hydropobically to the membrane, and the intrinsic adsorption coefficient must be independent of the nature of the polar head group of the lipid. (2) To distinguish the molecules adsorbed to the membrane from those in the aqueous phase, both the adsorption coefficient and the ratio of the quantum yield of the probe adsorbed onto the membrane to the quantum yield of the probe in the aqueous phase must be large. (3) The quantum yield of the molecules adsorbed to the membrane must be high because measurements must be made at low concentrations, where the probe molecules do not themselves perturb the surface potential. (4) The fluorescence characteristics of the adsorbed probe (i.e., the excitation and emission spectra, quantum yield, and lifetime) must be independent of the type of lipids present in the membrane and the nature of the electrolyte in the aqueous phase. (5) If the surface potential is to be monitored at only one of the membrane–solution interfaces, the membranes must be essentially impermeable to the probe molecule.

If these criteria are satisfied, the observed fluorescence, $f$, will be proportional to the surface concentration of adsorbed molecules, {F}, which in turn will be proportional to the aqueous concentration of the probe immediately adjacent to the membrane, $[F]_0$. This concentration is related to the electrostatic potential in the aqueous phase adjacent to the membrane, $\psi_0$, and the concentration of the probe in the bulk aqueous phase, [F], by the Boltzmann relation

$$f = \beta\{F\} = \gamma[F]_0 = \gamma[F]\exp[-ze\psi_0/(kT)] \qquad (6)$$

where $\beta$ and $\gamma$ are proportionality constants, $e$ is the electronic charge, $z$ is the valence of the probe, $k$ is the Boltzmann constant, and $T$ is the absolute temperature.

Although we know of no ideal fluorescent probe, these criteria are satisfied sufficiently well by 2-(*p*-toluidinyl)-naphthalene-6-sulfonate (Tns) to consider it an adequate probe. Specifically, (1) Huang & Charlton (1972) concluded from the temperature dependence of the binding of Tns to sonicated lecithin vesicles that the adsorption was essentially governed by hydrophobic forces. In agreement with their conclusion,

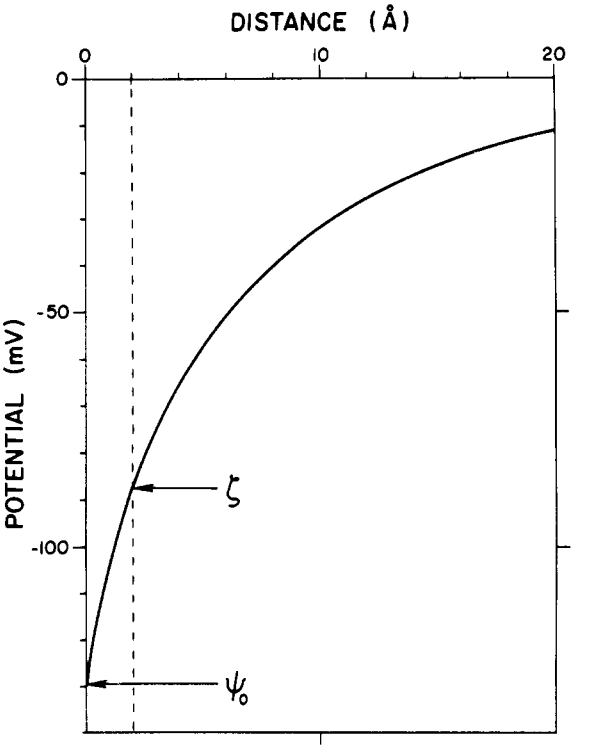


FIGURE 1: Profile of the electrostatic potential in the aqueous phase predicted by the Gouy–Chapman theory of the diffuse double layer (eq 4). The potential is plotted as a function of distance from the surface. The surface has a uniform charge density of one electronic charge per 70 Å$^2$, the concentration of monovalent salt in the bulk aqueous phase is 0.1 M, and the temperature is 25 °C. In this diagram the hydrodynamic plane of shear is assumed to be located 2 Å from the surface of the membrane, as indicated by the dashed line. The $\zeta$ potential, $\zeta$, the potential at the hydrodynamic plane of shear, is −88 mV, and the surface potential, $\psi_0$, the potential in the aqueous phase at the surface of the membrane, is −129 mV.

McLaughlin & Harary (1976) obtained similar binding constants for the adsorption of Tns to lipid bilayers formed from different neutral and zwitterionic lipids. In addition, these results show that the dipole potentials of the different lipids (Hladky, 1974) do not affect the hydrophobic adsorption of Tns to the bilayers. (2) The adsorption coefficient to neutral and zwitterionic bilayers is about $10^4$ M$^{-1}$. Furthermore, when excited at 321 nm and observed at 445 nm, the ratio of the quantum yields of the adsorbed and aqueous Tns is 716 by our measurements. (3) These parameters, together with the fluorescence sensitivity of our equipment, allow us to make measurements in the aqueous concentration range $1 \times 10^{-8} < [\text{Tns}] < 5 \times 10^{-6}$ M. These Tns concentrations are sufficiently low that the effects of the adsorbed Tns ions on the surface potential of the PC bilayers may be ignored (McLaughlin & Harary, 1976). (4) Although the fluorescent properties of the adsorbed Tns molecules are not completely independent of the nature of the lipid, they are very similar for PC, PS, and phosphatidylglycerol (PG) bilayers (see Appendix III). We have measured quantum yields of 0.60, 0.59, and 0.56 for Tns adsorbed to PC, PS, and PG sonicated vesicles in 0.1 M NaCl. (5) The absence of Tns efflux from PC vesicles in 24 h (Huang, personal communication), the inability of Tns to increase the conductance of black lipid membranes (McLaughlin & Harary, 1976), and the lack of any significant increase in the Tns fluorescence 24 h after its addition to multilamellar or sonicated vesicles (our measurements) provide good evidence that the lipid bilayers are essentially impermeable to Tns. In contrast, when multilamellar PC vesicles are exposed to 1-anilinonaphthalene-8-sulfonate (Ans), an increase

of the fluorescence is observed with a time constant of about 20 min (data not shown).

## Materials and Methods

Similar results were obtained with alkali metal chlorides of ultrapure quality from Ventron Corp. (Danvers, MA) or of analytical grade from a variety of sources. Tetramethylammonium chloride was purchased from Eastman (Rochester, NY) and was doubly recrystallized from ethanol–acetone. Mops buffer was obtained from P-L Biochemicals (Milwaukee, WI), Tris buffer was from Schwarz/Mann (Orangeburg, NY), and EDTA was from Fisher (Pittsburgh, PA). Water was purified with a Super-Q system (Millipore Corp., Bedford, MA). The concentrations of all solutions were determined by measuring the conductivity at 20 °C. All lipids (bovine brain phosphatidylserine, PS; egg phosphatidylcholine, PC; egg phosphatidylglycerol, PG; bovine phosphatidylinositol, PI; bovine heart cardiolipin, CL; bovine phosphatidic acid, PA) were obtained from Dr. W. Shaw of Avanti Biochemicals (Birmingham, AL), who prepared the negative lipids by treatment with EDTA to remove divalent ion contaminants. The purity of the lipids was established by one-dimensional TLC in chloroform–methanol–water (65:25:4): 0.5 mg of each lipid chromatographed as a single spot.

For formation of sonicated vesicles, lipids in chloroform (Gold label, spectrophotometric grade from Aldrich, Milwaukee, WI) were dried under nitrogen, resuspended in the appropriate salt solution, and sonicated with a Branson W185 sonifier (Danbury, CT) at maximum allowable power for 30 min under a nitrogen atmosphere in an ice water bath (PC and PG) or at 30 °C (PS). The sonicated material was centrifuged at 120000*g* for 45 min at 4 °C (PC and PG) or at 30 °C (PS). The upper two-thirds of the supernatant was utilized for the fluorescence experiments (Barenholz et al., 1977). The phosphate analysis was performed by the method of Lowry & Tinsley (1974). We observed, however, that this method does not work in the presence of TMA.

Multilamellar vesicles for the electrophoresis experiments were formed by the method of Bangham et al. (1974). Electrokinetic mobilities were measured with a Rank Bros. Mark I microelectrophoresis apparatus (Bottisham, Cambridge, United Kingdom). Care was taken to focus at the stationary layer (Henry, 1938).

Conductance measurements on black lipid membranes in the presence of nonactin were made in a Teflon chamber as previously described (McLaughlin & Harary, 1976).

Steady-state fluorescence was measured with a Spex Fluorocomp fluorometer (Spex Industries, Metuchen, NJ) equipped with a RCA 31034 photon-counting emission photomultiplier. The dark counts were typically 14 ± 2 counts/s. The intensity of the 150-W xenon light source was monitored through a rhodamine B filter by a reference photomultiplier. The excitation bandwidth was set at 2.5 nm, and the emission bandwidth was set at 5.0 nm. Net fluorescence, $f_{net}$, is defined as

$$f_{net} = f(+\text{Tns},+\text{ves}) - f(-\text{Tns},+\text{ves}) - f(+\text{Tns},-\text{ves}) + f(-\text{Tns},-\text{ves}) \quad (7)$$

where each term indicates the observed fluorescence in the presence (+) or in the absence (−) of either Tns or vesicles. Corrected spectra take into account the wavelength dependence of the quantum efficiency of the photomultiplier tube and the wavelength dependence of the exciting light. Above optical densities of 0.08 OD unit, the quenching due to self-absorption was corrected by measuring the optical density in a Zeiss PM1-MQ3 spectrophotometer and applying an empirically determined correction factor.

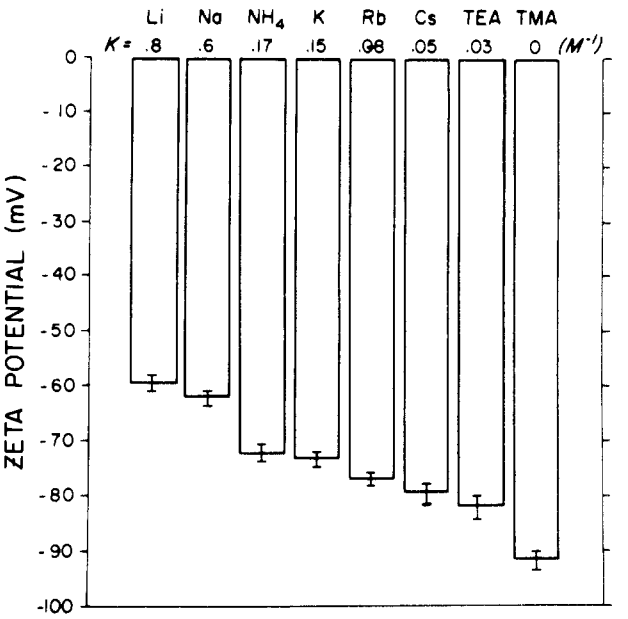


FIGURE 2: ζ potentials potentials of multilamellar PS vesicles formed in decimolar chloride solutions of the indicated cations. The solutions also contain 0.0001 M EDTA and 0.001 M Tris. The pH was 7.5 and the temperature was 25 °C. The error bars indicate the standard deviations of 20 measurements. The values of the intrinsic association constants of the different cations with PS, as calculated from eq 1–4, are also shown.

Fluorescence lifetime measurements were made with an ORTEC 9200 nanosecond fluorometer system (ORTEC, Oak Ridge, TN). The excitation light was a self-flashing spark gap in continuously flowing air. The light was filtered through a Corning UV-Transmitting 7-39 filter. Fluorescence emission was filtered through a Corning 3-73 cutoff filter. For determination of the lamp shape, the scattered light from the vesicle preparation without Tns was collected through a 7-39 filter. Photons received by the photomultiplier tube (RCA 8850) were allowed to accumulate until the number of counts at the peak channel reached at least 10000. Each decay was stored in 255 channels of the multichannel analyzer, at a scale of 0.225 ns/channel.

## Results

*ζ Potential Measurements.* Figure 2 illustrates the ζ potentials of PS vesicles formed in decimolar solutions of the indicated monovalent cation. It is apparent that the magnitude of the ζ potential depends on the nature of the cation. In particular, the ζ potentials in sodium and tetramethylammonium are −62 ± 1 and −90.5 ± 2 mV, respectively.[2] In terms of the Gouy–Chapman theory of the diffuse double layer, all monovalent cations will produce the same surface and ζ potentials if they exert only a "screening" effect. We interpret the selectivity apparent in Figure 2 in terms of the simplest possible model, the Gouy–Chapman–Stern theory discussed above (eq 1–3), which does allow for some specific

[2] Our results differ from those of Hauser et al. (1975), who found that the ζ potentials of PS vesicles in Na and TMA were −50 and −60 mV, respectively. In preliminary experiments with several commercially available samples of PS, we reproduced the values obtained by Hauser et al. (1975). The addition of EDTA to the solutions in which the vesicles were formed, however, produced results identical with those in Figure 2 presumably because these samples contained divalent cationic contaminants. As indicated in Table I, EDTA itself has no significant effect on the ζ potential of vesicles formed from specially purified PS. Thus, it would appear that the results obtained by Hauser et al. (1975) may have been affected by these contaminants.

Table I: $\zeta$ Potentials (Millivolts) of Phospholipid Vesicles[a]

| monovalent cation (0.1 M) as chloride | $\zeta$ potentials: phosphatidylserine, [EDTA] = 0 | phosphatidylserine, [EDTA] = $10^{-4}$ M | phosphatidylserine, [EDTA] = $10^{-3}$ M | phosphatidylglycerol, [EDTA] = $5 \times 10^{-4}$ M | phosphatidylinositol, [EDTA] = $5 \times 10^{-4}$ M | phosphatidic acid, [EDTA] = $5 \times 10^{-4}$ M |
|---|---|---|---|---|---|---|
| Li | −58.5 ± 1.0 | −59.5 ± 1.5 | −58.5 ± 1.0 | −60.0 ± 1.0 | −49.0 ± 1.0 | −65.0 ± 2.5 |
| Na | −61.5 ± 1.0 | −62.0 ± 1.0 | −61.5 ± 1.5 | −63.5 ± 1.0 | −45.0 ± 1.0 | −69.0 ± 3.5 |
| $NH_4$ | −71.5 ± 1.0 | −72.0 ± 1.5 | −71.0 ± 0.5 | −70.0 ± 0.5 | −49.0 ± 1.0 | −81.0 ± 5.0 |
| K | −72.0 ± 1.0 | −73.0 ± 1.5 | −72.0 ± 1.0 | −72.5 ± 0.5 | −47.5 ± 1.0 | −83.5 ± 1.0 |
| Rb | −72.0 ± 2.0 | −76.5 ± 1.0 | −75.5 ± 1.0 | −76.0 ± 1.0 | −50.5 ± 1.0 | −88.5 ± 1.0 |
| Cs | −77.5 ± 1.5 | −79.5 ± 2.0 | −78.0 ± 1.5 | −79.0 ± 1.0 | −51.5 ± 1.5 | −90.5 ± 1.5 |
| TEA | −80.0 ± 2.0 | −81.5 ± 2.5 | −82.0 ± 3.0 | | | |
| TMA | −91.5 ± 2.0 | −90.5 ± 2.0 | −90.0 ± 1.5 | −84.5 ± 1.5 | −70.0 ± 2.5 | |

[a] Measurements were made on at least 20 vesicles in two separate experiments at 25 °C. The results are presented as the average ± SD. The solutions for the PS measurements contained 0.001 M Tris at pH 7.5. The solutions for the other measurements contained 0.001 M Mops at pH 7.5 (PG and PI) or 6.5 (PA). Identical results were obtained, in a few control experiments on PS, in solutions without buffer (pH 6) or with 0.001 M Mops (pH 7.5). It should be noted that Tris does adsorb to PS bilayers but not to a degree sufficient to affect the results reported here: the $\zeta$ potential of PS vesicles in 0.1 M Tris-HCl, pH 7.5, is −5.6 ± 1.5 mV, and the calculated intrinsic association constant is 1.1 $M^{-1}$. As expected theoretically, 0.01 M Tris does lower the magnitude of the $\zeta$ potentials of PS vesicles to values below those reported here. $\zeta$ potentials and standard deviations are reported to the nearest 0.5 mV.

adsorption of the monovalent cations. We present evidence below that the hydrodynamic plane of shear is 2 Å from the surface of a bilayer membrane formed from PS or PG in alkali metal chloride solutions. The application of the Stern equation (eq 1–3), in conjunction with eq 4, which describes the potential profile in the aqueous phase, then allows us to calculate the intrinsic association constants for the different monovalent cations. These constants are indicated in Figure 2.

The data we obtained with PS vesicles are summarized in Table I. Note that EDTA has no significant effect on the results obtained with most cations; the exception is Rb, presumably because our samples of RbCl contained a significant level of divalent contaminants. EDTA at concentrations up to 0.001 M, furthermore, does not affect the $\zeta$ potentials of vesicles formed from PC. These control experiments suggest that EDTA does not adsorb significantly to PC:PS bilayer membranes. In experiments where lower concentrations of monovalent salts were used (Figures 3 and 4), it was necessary to have EDTA present to obtain reproducible results, presumably because a given level of divalent contamination produces a larger effect when the surface potential is high.

The results we obtained with phosphatidylglycerol (PG) vesicles formed in solutions containing the alkali metal cations are essentially identical with those obtained with PS (Table I), and a similar analysis of the data can be made in terms of the Stern equation. With phosphatidylinositol and phosphatidic acid vesicles, however, the $\zeta$ potentials are different (Table I). We do not believe that is is worthwhile to analyze these data in terms of the Stern equation until independent measurements (e.g., surface potential measurements on monolayers, nonactin–$K^+$ probe experiments on planar membranes, and fluorescent and other types of probe measurements on vesicles) are available for comparison with the $\zeta$ potential results.

We now examine whether the Stern equation can predict correctly the $\zeta$ potentials of vesicles formed in different concentrations of salt when the surface concentration of PS is decreased by mixing this lipid with the zwitterionic lipid PC. The results for vesicles formed in TMA solutions are illustrated in Figure 3. The $\zeta$ potential is plotted against the logarithm of the surface concentration of PS. (We assume that the ratio of PS to PC in the outermost monolayer of the vesicle is identical with the ratio of these lipids in the mixture used to form the membrane, that both PS and PC have identical molecular weights, and that they each occupy 70 $Å^2$ in the plane of the membrane.) The theoretical curves are drawn according to the prediction of Gouy–Chapman theory (eq 4), assuming that the plane of shear is 2 Å from the surface of the membrane. Note that the experimental points obtained in the 0.015 and 0.0031 M TMA solutions do not deviate significantly from the theoretical curves. In the 0.105 M TMA solution, however, the points lie 5–10 mV below the theoretical curve.[3] We were originally puzzled by the observation that

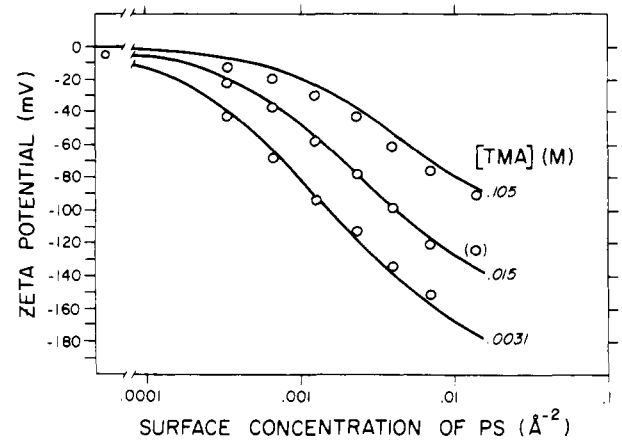


FIGURE 3: $\zeta$ potentials of vesicles formed from PC:PS mixtures of 1:0, 40:1, 20:1, 10:1, 5:1, 2.5:1, 1:1, and 0:1 in the indicated concentrations of tetramethylammonium. The 0.105 and 0.015 (0.0031) M TMA aqueous solutions also contain chloride ions, 0.001 (0.0001) M Mops to buffer the pH to 7.5, and 0.001 (0.0005) M EDTA as the TMA salt to remove traces of multivalent contaminants. The temperature was 25 °C. The standard deviations of the experimental points ($n$ = 20) are about equal to the radii of the circles. The solid lines are the predictions of the Gouy–Chapman theory (eq 4), assuming that the hydrodynamic plane of shear is 2 Å from the surface of the membrane.

[3] If the plane of shear is chosen to be 1.7 Å rather than 2 Å from the surface of the membrane, the theoretical curve passes through the experimental point obtained with PS vesicles, but the fit to the remainder of the points is not substantially improved. It is also possible to fit the point obtained with PS vesicles by assuming that the plane of shear is even closer to the membrane (e.g., 1 and 0 Å) and that some association of TMA occurs (e.g., $K$ = 0.02 and 0.2 $M^{-1}$) but, again, the fit to the remainder of the points is not greatly improved. If we assume that chloride adsorbs to PC, the fit can be improved for low PS:PC ratios, but not for high charge densities. In Appendix II we briefly discuss the possibility that the deviation from the theoretical curve observed in 0.105 M TMA and the discrepancy between $\zeta$ potential measurements and other results are due to the large size of the TMA ion.

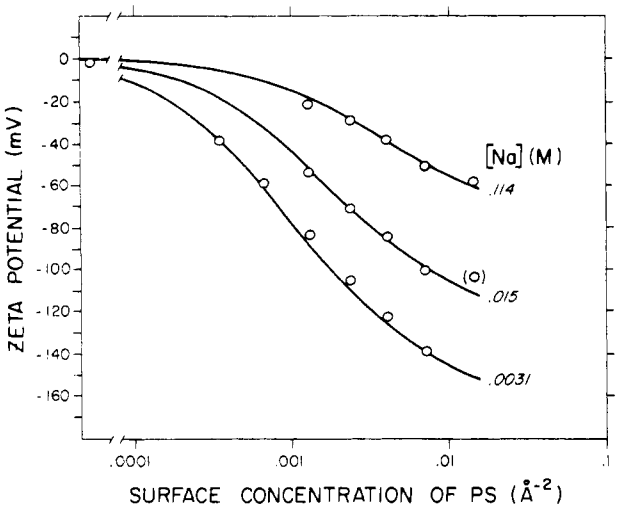


FIGURE 4: $\zeta$ potentials of vesicles formed from PC:PS mixtures in solutions containing the indicated concentrations of sodium, buffered to pH 7.5 at 25 °C. The solid lines are the predictions of the Stern equation (eq 1–3), assuming that the intrinsic PS–Na association constant is 0.6 $M^{-1}$ and that the hydrodynamic plane of shear is 2 Å from the surface of the membrane (eq 4).

the electrokinetic mobility of multilamellar dispersions formed in the low salt solution (0.0031 M) became increasingly dependent on the size of the vesicles as the charge density increased. As discussed in Appendix I, the dependence of mobility on size is predicted quantitatively by the relaxation effect discussed by Wiersema et al. (1976). In Figures 3 and 4 only the mobilities of the largest vesicles (diameter > 13 $\mu$m) were used to estimate the $\zeta$ potentials from eq 5 when a dependence of mobility on size was noted. This procedure circumvents the relaxation effect.

We have also examined whether the Stern equation can describe the $\zeta$ potentials of vesicles formed from PC:PS mixtures in different concentrations of sodium, a cation that appears to adsorb to PS. These results[4] are presented in Figure 4. The theoretical curves are drawn from the Stern equation (eq 1–3), assuming that the plane of shear is 2 Å from the surface of the membrane (eq 4) and that the intrinsic association constant is 0.6 $M^{-1}$. The fit of the Stern equation to the data points is excellent, but we cannot conclude from the good fit that the plane of shear does lie 2 Å from the surface of the membrane. The fit to the data in Figure 4 is not substantially worse, for example, if one assumes that the plane of shear is 1 or 0 Å from the membrane and that the intrinsic association constants are 1.5 and 3 $M^{-1}$, respectively. Thus, independent information about the location of the plane of shear is required. This additional information comes from measurements we have made with the fluorescent probe Tns, which should respond to the electrostatic potential at the membrane–solution interface, and from conductance measurements made with the nonactin–$K^+$ complex on planar black lipid membranes, which yield an estimate of the potential in the center of the membrane.

[4] We note that Barton (1968) obtained curves similar in shape to those presented in Figure 4 for PC:PS vesicles in 0.1 M NaCl. The $\zeta$ potentials he reports, however, are about 30% more negative than the potentials plotted in Figure 4. In another paper, Barton & Jevons (1970) noted that the size of the grid in the microscope of their Rank Bros. Mark I electrophoresis apparatus was 35 $\mu$m. When calibrated in air, the size of the grid in our Rank Bros. Mark I electrophoresis apparatus was also 35 $\mu$m, but, when calibrated under water, the correct procedure, the grid size corresponds to a distance of 27 $\mu$m. This would appear to account for the difference in our experimental results.

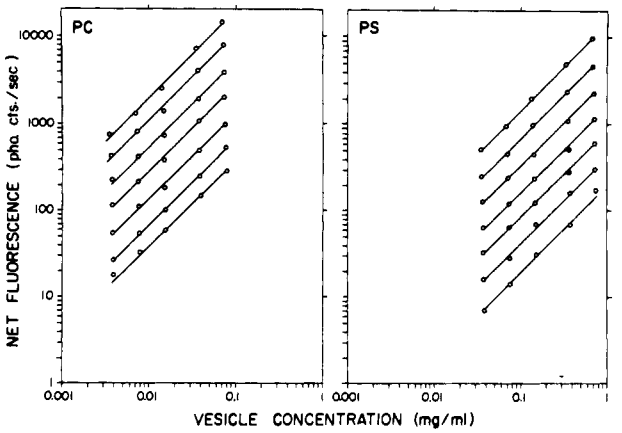


FIGURE 5: Plot of the net fluorescence of Tns adsorbed to PC and PS sonicated vesicles as a function of vesicle concentration. The solutions contain 0.1 M NaCl, 0.001 M EDTA, and 0.001 M Tris, pH 7.5, at 22 °C. The Tns concentrations are 0.11, 0.22, 0.43, 0.82, 1.6, 3.5, and 7.0 $\mu$M. The lines are drawn with a slope of 1.

*Fluorescent Probe Measurements.* Figure 5 shows the net fluorescence of Tns adsorbed to PC and PS vesicles sonicated in buffered 0.1 M NaCl for a range of vesicle and Tns concentrations. As indicated by the lines drawn with a slope of 1, the fluorescence depends linearly on the vesicle concentration, demonstrating that no significant loss of Tns from the aqueous solution occurs because of adsorption onto the vesicles. The data in Figure 5 also show that the fluorescence depends linearly on the Tns concentration, which demonstrates that the adsorbed Tns ions produce a negligible change in the surface potential. The essentially identical fluorescent properties of Tns adsorbed to PC, PS, and PG vesicles (Appendix III) indicate that the probe is in a similar environment in these membranes. The similar dependence of fluorescence on temperature for Tns adsorbed to PC, PS, and PG membranes (Appendix III) suggests that Tns adsorbs hydrophobically to these vesicles. We can, therefore, calculate the electrostatic potential at the surface of the PS vesicles by applying eq 6 and noting that the potential at the surface of a PC vesicle in 0.1 M NaCl should be 0 mV because the $\zeta$ potential is zero (Figure 4).

The results illustrated in Figure 5 indicate that the potential at the surface of the PS vesicle is −70 mV. The results from four different experiments yield an average value (±SD) of −71 ± 2 mV. The fluorescence produced by Tns adsorbed to PC and PG vesicles sonicated in 0.1 M NaCl (plus 0.001 M EDTA and 0.001 M Mops, pH 7.3) was measured in three different experiments. The potential at the surface of the PG bilayer was calculated (eq 6) to be −81 ± 2 mV. The results obtained with PS and PG vesicles agree reasonably well with the surface potentials of −83 and −85 mV predicted by eq 4 from the measured $\zeta$ potentials of PS and PG vesicles in 0.1 M NaCl (Table I), assuming the plane of shear is 2 Å from the surface of the membrane.

When NaCl was replaced by TMACl in analogous experiments, the results were indeterminate because TMA ions affected the phosphorus analysis (Lowry & Tinsley, 1974) for reasons that are not clear to us. In an attempt to circumvent this problem, we sonicated PS in 0.001 M EDTA and 0.001 M Mops, pH 7.3, and split the vesicle preparation in two. Buffered 1 M salt solutions were added to the vesicles to bring the final concentration to 0.1 M NaCl or 0.1 M TMACl, and the net fluorescence of adsorbed Tns was measured in both preparations. The results of four such experiments indicated that the surface potential of PS vesicles in TMA is 19 ± 1 mV more negative than the surface potential of the vesicles in Na.

Similar results were obtained with Tns in experiments on multilamellar vesicles.[5] The $\zeta$ potential of PS vesicles in TMA is about 30 mV more negative than the $\zeta$ potential of PS vesicles in Na (Table I). From the results of Puskin (1977), one calculates that the potential at the manganese binding site of PS vesicles in 0.1 M TMACl is 25 mV more negative than the potential of PS vesicles in 0.1 M NaCl. The difference between these three independent measurements suggests that no simple quantitative interpretation of the results obtained with TMA is possible at the present time. The difficulties are possibly related to the large size of this cation (Appendix II).

*Conductance Measurements.* The conductance, $G$, produced on a planar black lipid membrane by the nonactin–$K^+$ complex may be used to estimate changes in the electrostatic potential within the membrane because

$$G \propto \exp[-e\psi_{0^-}/(kT)] \quad (8)$$

where $\psi_{0^-}$ is the potential within the membrane (McLaughlin, 1977). We used conductance measurements in two different ways to test the working hypothesis developed above.

First, if the plane of shear is 2 Å from the surface of the membrane, the observed 20-mV difference in the $\zeta$ potential of PS vesicles formed in Li and Cs (Table I) should correspond to a difference of about 30 mV in the surface potential (eq 4). We tested this prediction experimentally by forming black lipid membranes from PS in either 0.1 M LiCl or CsCl. The solutions also contained 0.01 M KCl to provide the charged permeant species (Eisenman et al., 1973), 0.001 M Mops to buffer the pH to 7.5, and 0.0005 M EDTA to remove any traces of multivalent contaminants. By use of the values of the binding constants for Li, K, and Cs illustrated in Figure 2, the obvious extension of eq 1–3 to the case where more than one cation adsorbs predicts that the surface potentials in the Li and the Cs solutions should be −76.9 and −106.9 mV, respectively. When $6 \times 10^{-7}$ M nonactin was added to the aqueous solutions, the values of the specific conductances in Li and Cs solutions ($G^{Li}$ and $G^{Cs}$ in mho/cm$^2$) were as follows: $\log G^{Li} = -4.28 \pm 0.06$ and $\log G^{Cs} = -3.79 \pm 0.03$ (±SD; $n = 5$). From eq 8 the difference in potential within the membrane is $58.5(\log G^{Li} - \log G^{Cs}) = -29$ mV. This difference in potential agrees, within experimental error, with the predicted value of −30 mV, and the measurements support the suggestion that the plane of shear is 2 Å from the surface of PS vesicles in solutions containing the alkali metal cations.

Second, conductance measurements made in the presence of the nonactin–$K^+$ complex can be combined with mobility measurements in another way to test our working hypothesis. This comparison is based on the observation that, when calcium is added to a solution containing either PS or PG vesicles, the $\zeta$ potentials become less negative and, at high concentrations of calcium, reverse sign and become positive. In terms of the Gouy–Chapman–Stern theory (eq 1–4), the surface potential, $\psi_0$, will be zero when the $\zeta$ potential is zero, irrespective of the distance of the plane of shear from the membrane. Conductance measurements made at calcium concentrations that reduce the $\zeta$ potential to zero thus provide a reference point for the nonactin–$K^+$ conductance measurements, which by themselves can only be used to determine changes in the potential within the membrane. The calcium concentrations at which charge reversal occurs for PS and PG vesicles were measured in 0.1 M KCl. These concentrations were 0.085 and 0.120 M, respectively (data not shown). The experimentally determined value of the $\zeta$ potential of PS and PG vesicles in 0.1 M KCl (−73 mV; Table I) was then used to predict, by using eq 4 and the assumption that the plane of shear is 2 Å from the membrane, the change in surface potential that should have occurred. This predicted value, $\Delta\psi_0 = 101$ mV, was then compared with the experimentally determined values of $\Delta\psi_{0^-}$, the changes in potential within planar black lipid membranes that occurred at these calcium concentrations. From the data of McLaughlin et al. (1971), the change in potential within a PS bilayer estimated from nonactin–$K^+$ conductance measurements is 98 mV at a calcium concentration of 0.085 M, and the change in potential within a PG bilayer is 93 mV at a calcium concentration of 0.120 M. These numbers are in reasonable agreement with the predicted value of 101 mV and provide additional support for our assumption that the plane of shear is about 2 Å from the surface of the membrane in a solution containing the alkali metal cations.

*Comparison of Mobility and Surface Potential Measurements.* Mobility measurements can be used in a similar manner to "calibrate" surface potential measurements made on monolayers formed from PS in decimolar sodium chloride solutions. In 0.1 M NaCl, the measured $\zeta$ potential of −62 mV for PS vesicles (Table I) should correspond to a surface potential of −83 mV (eq 4). It was determined experimentally that the addition of 0.095 M $CaCl_2$ to this solution reduced the mobility of the PS vesicles to zero (data not shown). Unfortunately, we know of no surface potential data on PS monolayers where the calcium concentration has been increased to this level. Perhaps the best data available are those of Ohki & Sauve (1978), who measured the change in surface potential, $\Delta\Delta V$, of a PS monolayer (65 Å$^2$/molecule) formed over a 0.09 M NaCl and 0.01 M Tris-HCl solution as the calcium concentration was increased to 0.02 M. As expected theoretically, $\Delta\Delta V$ changed linearly with the log of the calcium concentration for $0.001 \leq [CaCl_2] \leq 0.02$ M. Extrapolating their results to a calcium concentration of 0.095 M, we find that the value of $\Delta\Delta V$ is about 80 mV, in good agreement with the predicted value of 83 mV.

In conclusion, fluorescence measurements with the probe Tns on sonicated vesicles, conductance measurements with the nonactin–$K^+$ complex on planar black lipid membranes, and surface potential measurements on monolayers (Ohki & Sauve, 1978) all provide evidence that the plane of shear may be considered to be about 2 Å from the surface of multilamellar vesicles formed from either PS or PG in a solution of alkali metal cations.

## Discussion

Our main conclusion is that the simple Stern equation (eq 1–3) can describe the effects of alkali metal cations on the surface and $\zeta$ potentials of lipid bilayer membranes containing PS, the major negatively charged lipid in many biological membranes. The intrinsic association constants deduced for the adsorption of the alkali metal cations to PS decrease in the lyotropic sequence Li > Na > K > Rb > Cs, in agreement with the results of Puskin (1977), who measured the ability of these cations to depress the adsorption of manganese ions to PS bilayers. The fact that identical sequences are observed with independent techniques strongly argues that the results are not due to an artifact inherent in any one of the methods.

[5] Multilamellar PS vesicles were formed in 0.1 M buffered NaCl (or TMACl). Several 5-mL aliquots were loaded into dialysis tubing and dialyzed against 0.1 M buffered TMACl (or NaCl). Half of these samples was subsequently dialyzed against the initial NaCl (or TMACl) buffer. In addition, some original samples were dialyzed against the same salt in which they were prepared. At each of the above steps, Tns was added and the net fluorescence was measured. The results indicate that the adsorption of Tns to PS is larger by a factor of 2 (32% error) in Na than in TMA and that it is reversible. The dialysis protocol was necessary because there is no accurate procedure to determine the total area of the outermost monolayers in multilamellar vesicle preparations.

It is unlikely, for example, that the selectivity observed with mobility measurements (Figure 2) is due to the hydrodynamic plane of shear having different locations for the different alkali metal cations or that the Li/Cs selectivity observed with nonactin–$K^+$ conductance measurements (see above) is due to the dipole potential of the PS bilayer having different values for these two cations. We know of no measurements of the adsorption of monovalent cations to bilayers formed from phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid other than those presented in Table I, and we prefer not to discuss the selectivity apparent in these mobility measurements until our independent fluorescence, NMR, and conductance measurements are completed. We also measured the mobilities of cardiolipin vesicles in decimolar salt solutions; they increased in the sequence Li $\sim$ Na $<$ $NH_4$ $\sim$ K $\sim$ Rb $\sim$ Cs $\sim$ TMA, in qualitative agreement with the Mn competition results of Puskin (1977). The standard deviations of the mobilities, however, were 1 order of magnitude higher than those recorded in Table I for vesicles formed from other lipids, and we prefer not to present numerical data until the source of this variability is understood.

It is perhaps relevant that the intrinsic binding constants of the alkali metal cations for the phosphate groups of polyphosphates are of the same order of magnitude as those calculated here for PS and also decrease in the lyotropic sequence [e.g., Strauss & Ross (1959)]. The binding of the alkali metal cations to adenosine phosphates (Smith & Alberty, 1956) and DNA (Ross & Scruggs, 1964) also follows the lyotropic sequence, and ab initio calculations indicate that this should indeed be the binding sequence for the adsorption of the alkali metal cations to phosphate groups [e.g., Marynick & Schaefer (1975) and Pullman & Pullman (1977)].

While the phosphate moieties of phospholipids are presumably involved in the adsorption of the alkali metal cations, it should be stressed that the nature of this adsorption is unclear at the present time. There is some controversy in the NMR literature about this point with respect to the adsorption of $^{23}Na$ to DNA (Reuben et al., 1975; Reuben, 1977; Andersen et al., 1978; Civan & Shporer, 1978) and to amphipathic molecules [e.g., Persson & Johansson (1971), Lindblom & Lindman (1973), and Gustavsson & Lindman (1975)], and the same arguments could be applied to the very similar results obtained in a recent NMR study of the adsorption of $^{23}Na$ to PS bilayers (Kurland et al., 1979).

The agreement of our $\zeta$ potential data (e.g., Figure 4) with the simple Stern equation (assuming the plane of shear is 2 Å from the surface of the membrane in solutions containing alkali metal cations) is surprising in view of the many simplifying assumptions inherent in the theory. Indeed, poor agreement with any simple theory was noted in an extensive study of the $\zeta$ potentials of decane droplets made charged by the adsorption of cationic surfactants (Carroll & Haydon, 1975). For whatever reason, $\zeta$ potential data obtained on vesicles formed from PC:PS mixtures (Figure 4) and other lipids in NaCl (McLaughlin, 1977; McLaughlin et al., 1978) are much easier to interpret than data obtained on charged oil droplets in NaCl. The association constant for the PS–Na complex deduced from $\zeta$ potential measurements agrees well with that deduced from independent techniques (Puskin, 1977; Nir et al., 1978; Kurland et al., 1979). We conclude, therefore, that $\zeta$ potential measurements should be valuable in studying the adsorption of other charged molecules to lipid bilayer membranes.

The utility of Tns as a fluorescent probe in this study suggests that it may also prove useful as a probe of electrostatic potentials on other membrane systems. More calibration studies are, of course, required on model membranes before measurements on biological membranes can be interpreted with confidence. Charged amphipathic spin-labeled molecules have been recently used for this purpose (Castle & Hubbell, 1976; Gaffney & Mich, 1976; Quintanilha & Packer, 1977), but a fluorescent probe could be more sensitive and have advantages with respect to spatial and temporal resolution.

In summary, the observations on artificial bilayers containing PS and PG presented here and in the papers cited above all suggest that the alkali metal cations adsorb to these negative lipids. This observation should be useful to both biochemists and biologists. Cowley et al. (1978), for example, measured the repulsive forces between bilayer membranes containing PG. Their results "suggest clearly that not all the lipid is dissociated from protons or perhaps from the Na counterions". The results presented in Table I and Figure 4 support this suggestion. As an example of the biological relevance of the adsorption of sodium to negative lipids, we note that there is now good evidence that negative charges exist on the outer surface of nerve and muscle membranes in the vicinity of the excitable channels [e.g., Hille et al. (1975)]. If these charges arise from PS, the major negatively charged lipid in nerve membranes, one should be able to increase the magnitude of the negative surface potential by replacing Na with Cs or TMA. (The concentration of divalent cations in the bathing solution must be low in these experiments.) If an increase in the magnitude of the surface potential is produced by this maneuver, it will manifest itself as a shift in the conductance–voltage curves along the voltage axis.

## Acknowledgments

We thank Drs. J. Cohen, R. Kurland, A. McLaughlin, S. Nir, D. Papahadjopoulos, V. A. Parsegian, J. Puskin, and P. Rand for stimulating discussions.

## Appendix

(I) As discussed in detail by Overbeek & Wiersema (1967) in their comprehensive review, there are four forces acting on a vesicle when it achieves its terminal velocity in an electric field. The force in the direction of motion is the product of the electric field and the total charge on the vesicle within the plane of shear. This force is opposed by the Stokes friction, which is proportional to the electrophoretic mobility. The applied field also exerts a force on the counterions in the electrical double layer. The movement of the counterions produces a movement of the solvent relative to the vesicle and, therefore, a retarding force on the vesicle (electrophoretic retardation). These three forces are accounted for in the derivation of the Helmholtz–Smoluchowski equation (eq 5). The analysis predicts that the mobility of a particle should be independent of its size and shape for all charge densities; this is approximately the case for phospholipid vesicles in decimolar salt solutions (unpublished observations). It is well-known from the Debye–Hückel theory of the conductivity of electrolyte solutions, however, that there exists a fourth force on a charged particle undergoing electrophoretic movement. The force arises because the center of the ionic atmosphere lags slightly behind the center of the particle when it is in uniform motion. This "relaxation" effect produces an electrical force that retards the motion of the particle. The relaxation effect has been incorporated into an analysis of the electrophoretic mobility by Wiersema et al. (1966). Their analysis indicates that for all experimentally accessible charge densities the mobility of a phospholipid vesicle in a 0.114 M monovalent

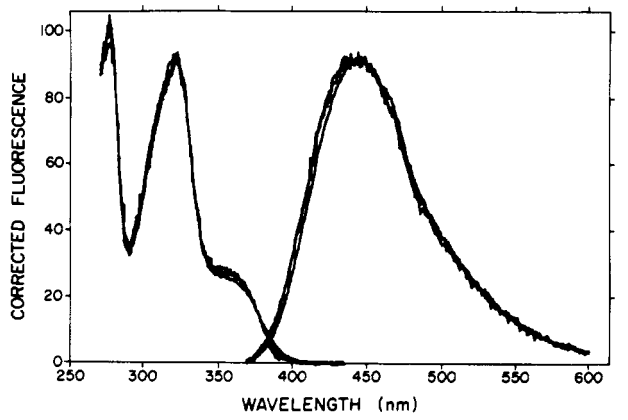


FIGURE 6: Peak normalized, corrected excitation and emission spectra of Tns adsorbed to PC, PS, and PG vesicles. The aqueous solutions contain 0.1 mg/mL vesicles, 0.1 M NaCl, 0.001 M EDTA, 0.001 M Mops, pH 7.3, and 2.0 μM Tns, at 22 °C. The slightly blue shifted emission spectra are those of PS and PG.

salt solution should be essentially independent of size, in agreement with our experimental observations (Table II) on vesicles formed from 1:1 PC:PS mixtures. Their analysis also indicates that the $\zeta$ potentials of these vesicles calculated from the Helmholtz–Smoluchowski equation (eq 5) will deviate from the correct value as the salt concentration is decreased and that the deviation will be largest for the smallest vesicles. This is precisely what is observed experimentally, as illustrated in Table II. In the 0.015 M solution, the mobility of small vesicles is 5% less than the mobility of large vesicles; the predicted deviation is about half this value. In the 0.0031 M sodium solution, the mobility of small vesicles is 12% less than the mobility of large vesicles; the predicted deviation is 9% (Table II). In a 0.0001 M $Na_4EDTA$ solution, the experimentally observed and theoretically expected differences between the mobilities of small and large vesicles are both 25%.

We observed that the percentage difference between the mobilities of large and small vesicles decreased, at a given salt concentration, as the $\zeta$ potential (or charge density or fraction of PS) decreased. This result is also in accord with the predictions of Wiersema et al. (1966). Results similar to those presented in Table II were also obtained in TMA solutions (data not shown). The points obtained with PS vesicles in 0.015 M solutions are in parentheses in Figures 3 and 4 be-

Table II: Dependence of Mobility on Size for Vesicles Formed from 1:1 PS:PC Mixtures

| sodium concn (M) | size of vesicle[a] | $\zeta$ potential from eq 5 (mV) | % difference in $\zeta$ potential (exptl) | % difference in $\zeta$ potential (theor)[b] |
|---|---|---|---|---|
| 0.114 | small | −51.5 ± 1.0 | 0.5 | 0.5 |
| | large | −51.5 ± 1.0 | | |
| 0.015 | small | −96.5 ± 1.0 | 5.5 | 2.5 |
| | large | −102.0 ± 3.0 | | |
| 0.0031 | small | −121.0 ± 2.5 | 12 | 8.5 |
| | large | −136.5 ± 6.0 | | |

[a] The diameters of the small vesicles were between 1 and 5 μm; the diameters of the large vesicles were between 13 and 20 μm. [b] These numbers were calculated from Figure 2 of Wiersema et al. (1966).

Table III: Lifetimes ($\tau_1$ and $\tau_2$ in Nanoseconds), Corresponding Amplitudes ($\alpha_1$ and $\alpha_2$), and Average Lifetimes ($\langle\tau\rangle$ in Nanoseconds) for Tns Adsorbed to Sonicated PC, PS, and PG Vesicles in 0.1 M Buffered NaCl at 22 °C

| | $\tau_1$ | $\alpha_1$ | $\tau_2$ | $\alpha_2$ | $\langle\tau\rangle = (\Sigma\alpha_i\tau_i^2)/(\Sigma\alpha_i\tau_i)$ |
|---|---|---|---|---|---|
| PC | 4.7 | 0.38 | 9.8 | 0.036 | 5.5 |
| PS | 4.7 | 0.38 | 10.0 | 0.026 | 5.4 |
| PG | 4.5 | 0.39 | 9.5 | 0.025 | 5.1 |

cause we could not form large vesicles with PS, irrespective of how gently we agitated the lipid–water mixture. All other data presented in these figures were obtained on large vesicles when the mobility depended on size.

In conclusion, the data presented in this section provide evidence that the dependence of mobility on size is due to the relaxation effect discussed by Wiersema et al. (1966). Our conclusion agrees with that of Shaw & Ottewill (1965), who studied the mobility of polystyrene latex particles. The conclusion is of some theoretical significance in understanding the interaction of ions with membranes. For example, with small PC:PS vesicles in 0.003 M NaCl solutions, the mobility first increases with charge density and then passes through a maximum and decreases (data not shown). It has been pointed out that such maxima could arise from discrete charge effects

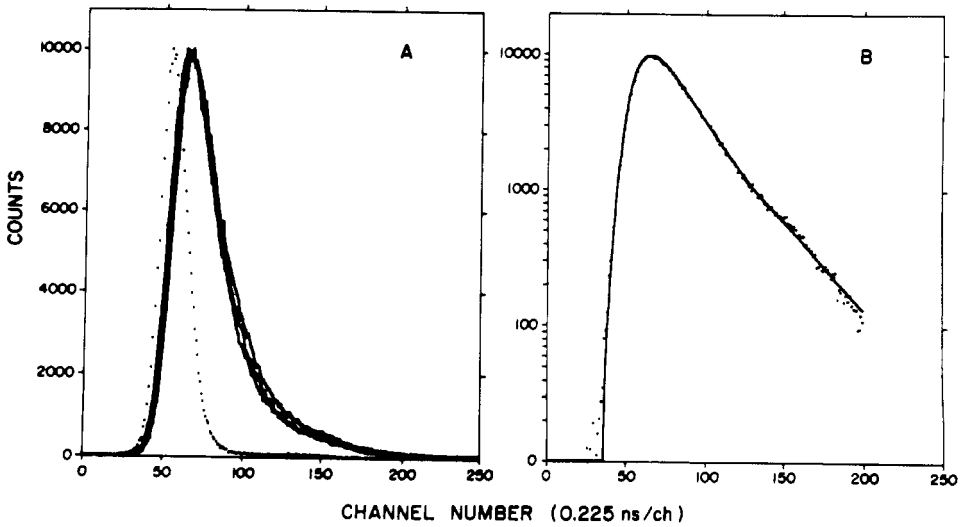


FIGURE 7: (A) Peak normalized, nanosecond decays of Tns adsorbed to PC, PS, and PG vesicles (same conditions as described in the legend of Figure 6). The dotted curve represents the lamp shape. (B) The dotted curve is the observed decay for Tns adsorbed to PS vesicles. The solid line is the reconstructed decay from the lamp shape and the amplitudes and lifetimes determined for this decay by the two-exponential deconvolution (Table III), shown to illustrate the degree of fit.

(Levine & Bell, 1963); our results on large vesicles and the analysis of Wiersema et al. (1966) suggest that the relaxation effect plays a major role in producing the maximum we observed.

(II) The factors responsible for the deviations of the experimentally observed $\zeta$ potentials from the theoretically predicted values in 0.105 M TMA (Figure 3) are not well understood at the present time. An obvious explanation is that the finite size of the ions is important [e.g., Stern (1924)]. Stern assumed that the plane of closest approach for a hydrated ion, the outer Helmholtz plane (OHP), was the same for both cations and anions. If, however, the OHP for TMA, which has a large radius [3.47 Å; Robinson & Stokes (1959)], is further from the surface than the OHP for chloride, a negative space charge will be formed in the region between the two planes. This space charge will produce a negative $\zeta$ potential. Mohilner (1966) may be consulted for a further discussion of this phenomenon, which could explain why the $\zeta$ potentials of PC vesicles in 0.1 M NaCl are close to zero ($-1.1 \pm 0.3$ mV) whereas the $\zeta$ potentials of vesicles formed from the same batch of lipid in 0.1 M TMACl are negative ($-5.1 \pm 0.6$ mV). If the argument has any validity, it should be possible to produce PC vesicles with positive $\zeta$ potentials by replacing chloride with an anion for which the OHP is further from the surface than the OHP for sodium. In accordance with this prediction, the $\zeta$ potentials of PC vesicles in 0.1 M $NaH_2PO_4$ solutions (pH 5.4) are positive, $+2.2 \pm 0.4$ mV, but other effects must be important as well. For example, raising the pH of the solution to pH 7.7, where most of the phosphate exists in the divalent monohydrogen form, increases the $\zeta$ potential to $+5.7 \pm 0.4$ mV. This increase in the $\zeta$ potential probably occurs because the repulsive "image charge" forces are stronger for the divalent than for the monovalent phosphate anions and these forces will act to keep the divalent species further from the surface. The $\zeta$ potentials of PC vesicles were also significantly positive, $+5.5 \pm 1.1$ mV, in 0.1 M $Na_2SO_4$ solutions. The $\zeta$ potentials of PC vesicles in decimolar solutions of the alkali metal cations range from $+4.3 \pm 0.7$ mV for LiCl to $-2.9 \pm 0.5$ mV for CsCl (data not shown). To reconcile these data with the finite-size effect, one would have to postulate that it is the size of the unhydrated cation that is important. Other factors, such as a specific adsorption of chloride anions and alkali metal cations to PC are probably important. Finally, as the polar head group region of a bilayer membrane bears little resemblance to the idealized membrane–solution interface of our model, it does not seem worthwhile to attempt a quantitative application of the finite-size effect to membranes.

(III) Since the use $\tau/q$, a fluorescence probe to measure surface potentials at the membrane–solution interface is based on the assumption that the fluorescence is proportional to the number of adsorbed probe molecules, it is essential to know if the quantum yield of the adsorbed probe is independent of the lipid composition of the bilayer. This assumption can be tested by measuring the lifetimes and corrected spectra of Tns adsorbed to bilayers formed from different lipids.

By definition, $\tau_0 = \tau/q$, where $\tau_0$ and $\tau$ are the intrinsic and measured lifetimes and $q$ is the quantum yield. If the intrinsic lifetime of Tns is the same when it is adsorbed to PC, PS, and PG vesicles, then the relative quantum yields will be proportional to the relative measured lifetimes. The Strickler–Berg equation (Strickler & Berg, 1962) allows one to calculate the intrinsic lifetime from the emission and absorption spectra if the geometry of the molecule does not undergo large changes upon excitation. We assume that the geometry of Tns adsorbed to phospholipid bilayers does not change upon excitation.

The corrected spectra for Tns adsorbed to PC, PS, and PG bilayers are shown, peak normalized, in Figure 6. Note that they are virtually superimposable. Our calculations using the Strickler–Berg equation indicate that the relative intrinsic lifetimes are within 1.5% of each other.

Easter et al. (1976) have shown that the fluorescence lifetime of Tns adsorbed to PC vesicles is dependent on the wavelength of the emission light and can be described by three-exponential components. To obtain approximate quantum yield ratios in our three phospholipid vesicle systems, we made fluorescence lifetime measurements as described under Materials and Methods and used a double-exponential deconvolution fit. Our results are shown in Figure 7 and are summarized in Table III. Our mean lifetime for Tns adsorbed to PC vesicles at 22 °C agrees well with that measured by Easter et al. (1978) from a three-exponential average. We have, therefore, corrected our fluorescence data by the ratios of the lifetimes (i.e., we have multiplied the fluorescence from PC, PS, and PG by 1.00, 1.02, and 1.08, respectively).

Since corrected emission spectra of Tns adsorbed to PC vesicles and Tns in methanol are identical when normalized (results not shown), we can obtain the value of the quantum yield of the adsorbed Tns from the measured lifetimes and the quantum yield of Tns in methanol. Our measurements give a lifetime of 5.7 ns for Tns in methanol. We have calculated the quantum yield of Tns in methanol by comparing the integrated corrected emission spectra of 5.0 $\mu$M solutions of Tns in methanol and Ans in methanol, using Yguerabide's (1972) value of 0.216 for the quantum yield of Ans in methanol. We obtain a value of 0.632 for the quantum yield of Tns in methanol (and, incidentally, a value of 0.0027 for Tns in 0.1 M NaCl). The quantum yield for Tns adsorbed to PC vesicles is, therefore, 0.60 (and, similarly, 0.59 and 0.56 for Tns adsorbed to PS and to PG vesicles, respectively). A critical test of this procedure is possible because Huang & Charlton (1972) have measured directly the adsorption of Tns to sonicated PC vesicles. A dissociation constant of $1.7 \times 10^{-4}$ M at 22 °C may be calculated by fitting their data at 20 and 25 °C to the Stern equation [e.g., McLaughlin & Harary (1976)] and interpolating to 22 °C. From our fluorescence data we calculate a dissociation constant of $1.1 \times 10^{-4}$ M at 22 °C, assuming a maximum surface density of one adsorbed Tns molecule per 70 Å$^2$. The agreement of these two dissociation constants is quite satisfactory.

We also studied the temperature dependence of the corrected emission of Tns adsorbed to the three phospholipid bilayers in the range from 15 to 40 °C. A plot of the natural logarithm of the fluorescence vs. the inverse of the absolute temperature is well described by a straight line. The slopes ($\times 10^{-3}$ in kelvin) and correlation coefficients are as follows: 4.79 and 0.999, 4.06 and 0.999, and 5.49 and 0.999 for PC, PS, and PG, respectively (data not shown). A linear fit of the analogous plot from Huang & Charlton's (1972) data gives a slope of $4.31 \times 10^3$ K and a correlation coefficient of 0.995 for the number of Tns molecules adsorbed per lipid molecule in PC bilayers at 5.00 $\mu$M free Tns concentration. Our main point here is that the temperature dependence of the fluorescence is similar in the three lipid systems. This indicates that Tns adsorbs to bilayers formed from these lipids by essentially "hydrophobic" forces.

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