THE INTRINSIC pK_a VALUES FOR PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE IN PHOSPHATIDYLCHOLINE HOST BILAYERS

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ABSTRACT Potentiometric titrations and surface potential measurements have been used to determine the intrinsic pK_a values of both the carboxyl and amino groups of phosphatidylserine (PS) in mixed vesicles of PS and phosphatidylcholine (PC), and also of the amino group of phosphatidylethanolamine (PE) in mixed PE-PC vesicles. The pK_a of the carboxyl group of PS in liposomes with different PS/PC lipid ratios measured by the two different methods is 3.6 ± 0.1, and the pK_a of its amino group is 9.8 ± 0.1. The pK_a of the amino group of PE in PE-PC vesicles, determined solely by surface potential measurements, is 9.6 ± 0.1. These pK_a values are independent of the aqueous phase ionic strength and of the effect of the liposome's surface potential due to the presence of these partially charged lipids.

INTRODUCTION

Biological membranes generally have on their surfaces negatively charged groups that give rise to a surface electrostatic potential. This potential is an important determinant of many physical properties of the system. For example, in systems having closely apposed membranes, such as photoreceptor disks (Corless, 1972; Chabre, 1975) and chloroplast thylakoids (Sculley et al., 1980), the free energy of interaction between membranes has important contributions from the surface potential. Fixed charges on the membrane surface give rise to concentration gradients of ions at the surface, and this phenomenon has important functional consequences for enzymes and transport systems that operate on charged substrates.

A substantial fraction of the charge at membrane surfaces is due to charged phospholipids, such as phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL), and, to a lesser extent at neutral pH, phosphatidylethanolamine (PE). Due to its relatively high abundance, PS is an important contributor to membrane charge. Although PE bears little net charge at neutral pH, its high abundance makes it a significant contributor at mildly alkaline pH values. It is with these latter two lipids that the present study is concerned.

In general, acidic lipids bind both protons and metal ions (Papahadjopoulos, 1968; Eisenberg et al., 1979; Hauser and Shipley, 1984). Thus in order to determine the charge contribution from a specific lipid, the intrinsic pK_a, surface pH and metal ion binding constants must all be known. The distinction between the so-called apparent pK_a and the intrinsic pK_a is important to appreciate. The apparent pK_a for a single site group is determined as the bulk pH at which 50% of the group population is charged. The intrinsic pK_a is determined as the surface pH at which 50% of the group population is charged. The relationship between these quantities is simply

$$\text{pH(surface)} = \text{pH(bulk)} + F\psi/2.303\,RT, \qquad (1)$$

where F is the Faraday constant, R is the gas constant and ψ is the surface potential. Eq. 1 can be rewritten to give the equivalent expression for pK_a's,

$$pK_a(\text{intrinsic}) = pK_a(\text{apparent}) + F\psi/2.303\,RT. \qquad (2)$$

The apparent pK_a is a directly measurable quantity, but depends on experimental conditions such as the ionic strength of the medium and the amount of charged lipid in the membrane. The intrinsic pK_a, on the other hand, is a fundamental property of the system and is the most useful quantity.

Various investigators have already reported measurements of the apparent pK_a values of the carboxyl group on PS (MacDonald et al., 1976; Seddon et al., 1983) and the amino group on PE in bilayers (Szabo et al., 1972). To obtain the intrinsic pK_a from these values, both the surface potential and ion bindng constants must be known. Unfortunately, literature measurements of the apparent pK_a values for PS have been carried out on vesicles, dispersions, or monolayers of the pure lipid. Under these conditions the

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surface potentials are very high, ion binding is quite significant, and the area per headgroup of phospholipid (which is needed to compute charge density) is a function of pH and ionic strength. Thus the corrections to the apparent pK_a required to obtain the intrinsic pK_a are very large and not known with accuracy.

In the present paper, we investigate both proton binding (by potentiometric titration) and surface electrostatic potential (by the spin labeling method) in a host PC bilayer containing relatively small amounts of PS, PE, or mixtures thereof at a variety of ionic strengths and pH values. The surface potentials are measured directly and the binding constants for alkali cations to PS are known. Analysis of the data taking into account ion binding allows determination of intrinsic pK_a values that are found to be independent of ionic strength in the range studied.

Since the determinations are done in dilute systems with a relatively low charge density, the corrections for ion binding and surface pH needed to obtain the intrinsic pK_a are relatively small and believed to be accurate. The PS and PE mole fractions studied here are similar to those found in native membranes, and the pK_a's found should be applicable to those systems. Indeed, the primary incentive for this work is to provide a basis for modeling the electrostatic structure of native membranes.

MATERIALS AND METHODS

PC was purified from locally purchased fresh hen eggs according to the procedure of Singleton et al. (1965), except that aluminum oxide (neutral, activity grade 3 from Woelm) was substituted for aluminum oxide (Merck) for column chromatography of the crude phosphatides. PS and PE, both from egg, were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The spin-labeled amphiphile *N,N*-dimethyl-*N*-nonyl-*N*-tempoylammonium bromide (C_9Q^+) was synthesized as described previously (Hubbell et al., 1970).

Depending on the pH desired, the following buffer solutions were used: 10.0 mM glycine/HCl, pH 3.10; 20.0 mM succinate, 10.0 mM arginine (arg.), pH 4.20; 90.0 mM 2-*N* Morpholino Ethane Sulfonic Acid (MES), 10.0 mM arg., pH 5.10; 40.0 mM MES, 10.0 mM arg., pH 5.70; 20.0 mM MES, 10.0 mM arg., pH 6.30; 20.0 mM *N*-2-Hydroxyethyl Piperazine-N-2-Ethanesulfonic Acid (HEPES), 8.0 mM arg., pH 7.10; 20.0 mM *N*-2-Hydroxyethyl Piperazine Propane Sulfonic Acid (HEPPS), 10.0 mM arg., pH 7.85; 16.0 mM *N*-Tris-Hydroxymethyl Methyl-Aminopropane Sulfonic Acid (TAPS), 10.0 mM arg., pH 8.45; 8.0 mM TAPS, 10.0 mM arg., pH 8.85; 6.0 mM TAPS, 10.0 mM arg., pH 9.00; 2.0 mM TAPS, 10.0 mM arg., pH 9.50; 25.0 mM $NaHCO_3$, 12.2 mM NaOH, pH 10.1; 25.0 mM $NaHCO_3$, 19.1 mM NaOH, pH 10.6; 25.0 mM $NaHCO_3$, 23.7 mM NaOH, pH 11.1.

Potentiometric Titrations

Aliquots of stock lipids, stored under nitrogen or argon at −20°C, were removed in quantity to give 25 mg/ml of the appropriate combination of PC and PS in the final vesicle preparation. The solvent was removed under a flow of argon, and the lipid mixture was dried in a vacuum desiccator for 16 h. Sodium chloride aqueous solution (2 mM) was added (1 ml/25 mg of dried lipid) and the samples were cooled in an ice bath and subjected to sonication (Sonifier, Heat Systems-Ultrasonics, Inc., Plainview, NY) under argon for 10 min at 30 W. The sonicates were centrifuged for 20 min at 31,000 *g*, yielding pellets of titanium dust from the sonifier tip and tiny amounts of poorly sonicated lipid. The final concentration of the stock vesicle suspension was determined according to the inorganic phosphate assay of Bartlett (1959). The stock vesicle suspension in 2 mM NaCl was stored under argon prior to its dilution for potentiometric titration measurements.

Potentiometric titration of the vesicle suspension was carried out with a pH meter model 701A/digital ionalyzer; Orion Research Inc. Cambridge, MA) calibrated with reference buffer solutions under an argon atmosphere at pH values of 4.0, 7.0, and 10.0. All the samples were prepared by diluting the stock lipid solution with 10 or 100 mM NaCl to a final concentration of 6.0 mg of lipid in 24 ml. Samples were maintained under an argon atmosphere throughout the measurements. Gramicidin, 2–3 molecules per vesicle, was added to prevent the buildup of transmembrane proton or sodium ion gradients. Concentrated HCl in 10 or 100 mM NaCl solution was used to bring the initial pH value to 2.5, and 20 mM NaOH was used to titrate the vesicle suspension. Bulk pH values were taken 1.5 to 2 min after the addition of the base. All experiments were carried out in plastic containers to prevent proton adsoprtion to the glass surface. The titration curve of the vesicle suspension after subtraction of a blank titration curve was used to deduce what fraction of the carboxyl groups in the PS molecules is deprotonated at different bulk pH values.

Surface Potential Measurements at Different Bulk pH Values

For the EPR determination of surface potentials, dried lipids (50 mg/ml) were suspended in 2 mM NaCl solution, and this phospholipid solution was added to buffers with different pH values ranging from 3.1 to 11.1 for mixed PS-PC and PS-PE-PC liposomes, and pH values ranging from 7.5 to 11.1 for mixed PE-PC liposomes. The suspensions were sonicated separately under argon for 15 to 30 min at 30 W, depending on the lipid composition, and then centrifuged to remove titanium dust and poorly sonicated lipid. The final lipid concentration was determined according to the phosphate assay of Bartlett (1959). The final ionic strength of the phospholipid supernatants was carefully adjusted to 50 mM with concentrated NaCl solution prepared in appropriate buffers. The stock vesicle suspensions in buffers with different pH values were stored overnight under argon prior to their dilution for EPR experiments.

Samples were prepared from stock phospholipid vesicles, 250 μM stock C_9Q^+ spin label, and concentrated NaCl prepared in appropriate buffers. The final lipid and spin label concentrations were 0.8 % wt/vol and 20 μM, respectively. The samples were mixed 15 min before conducting EPR measurements.

Samples of 200 μl were contained in a Varian quartz flat cell (Varian Instrument Group, Palo Alto, CA). The Varian Model E-109 spectrometer interfaced to a Nicolet 1280 data acquisition system (Nicolet Instrument Corporation, Madison, WI) was operated at X-band with a klystron power of 10 mW. Spectra were signal averaged where necessary. The principle of the spin label surface potential determination and the analysis of the EPR spectra have been previously discussed at great length by Castle and Hubbell (1976) and Cafiso and Hubbell (1981).

A difficulty that one might confront in this study is the possibility of oxidation and hydrolysis of PE at a high pH range. However, thin layer chromatography studies in our laboratory revealed that no significant hydrolysis of PE had occurred.

Surface Potential Determination at a Fixed pH with Different Ionic Concentrations

Sample preparation was the same as described in previous section except that the dried lipids were suspended directly in 20 mM succinate, 10 mM arginine at pH 4.1 for mixed PS-PC.

Samples were prepared from stock phospholipid vesicles (4.8% wt/vol lipid), 250 μM stock C_9Q^+ spin label, and concentrated NaCl prepared in buffer as described above. The final spin-label concentration was 20 μM and the NaCl concentration ranged from 0 to 100 mM. EPR spectra were recorded under the conditions described above.

Vesicle Size Determination by Electron Microscopy

Samples of the vesicle preparations were diluted with water to ~1 mg/ml, and 0.1 ml of the diluted suspension was mixed with 0.6 ml of water and 0.1 ml of 4% OsO_4. Fixation was carried out for 30 min at 0°C. Drops of fixed vesicles were applied to formvar- and carbon-coated 200-mesh copper grids and, after 3 min, were negatively stained for 15 s with 2% uranyl acetate (Castle and Hubbell, 1976). Examination in a JEOL 100C electron microscope rendered an average vesicle radius of 140–150 Å. For vesicles composed of a high concentration of PS or PE, the average radius is approximately 110–120 Å.

THEORY AND RESULTS

Surface Potentials of PS-PC Vesicles

In different aqueous NaCl concentrations, the surface potentials of 4, 8, and 14 mol % PS-PC vesicles at bulk pH values ranging from 3.1 to 7.5 were determined by the spin label method (Castle and Hubbell, 1976; Cafiso and Hubbell, 1981). Measurements at higher pH values will be discussed separately below.

The alkyl quarternary ammonium nitroxide spin labels used in this method are almost impermeable in phospholipid vesicle systems because of the positive charge on the quaternary nitrogen atom (Castle and Hubbell, 1976), so that the phase distribution is restricted to the external aqueous medium and the external half of the bilayer. The partitioning of this spin label between the membrane and aqueous phases can then be used to deduce the outer surface potential, which arises from the existence of immobilized surface charges. This potential is related to the ion distribution according to the Boltzmann equation

$$\psi_{so} = (-ZF/RT) \ln [\lambda(0)/\lambda'(0)], \tag{3}$$

where ψ_{so} is the external surface potential and $\lambda(0)$ and $\lambda'(0)$ are the relative partition coefficients in the presence and absence, respectively, of a surface potential. For PS-PC systems sufficiently dilute in PS (≤8 mol %) one can take $\lambda'(0)$ to be the partition coefficient of the spin label in a pure egg PC vesicle (Castle and Hubbell, 1976). For higher PS to PC ratios (over 8 mol %), the intrinsic binding constant of spin-label to vesicles has been measured by suspending the vesicles in a buffer containing 200 mM NaCl at a pH of 2.5. Under these conditions, PS has a very low net charge and the moderately high salt effectively screens the small potential that does exist. Even at the highest PS concentrations studied here (14 mol %), the intrinsic binding constant of C_9Q^+ differs by only ~10% from that for pure PC.

The surface potentials can be related to a smear charge density of spherical vesicles according to

$$2 \sinh\left(\frac{ZF\psi}{2RT}\right) + \frac{4}{A}\tanh\left(\frac{ZF\psi}{4RT}\right) - \frac{\sigma F}{RTK\epsilon_0\epsilon_r} = 0. \tag{4}$$

In Eq. 4, $A = K \cdot r$, where r is the radius of the vesicle (140–150 Å) and K is the Debye constant, σ is the charge density, ψ is the surface potential, and the other symbols have their usual physical meanings. Eq. 4 is an accurate approximation to the exact solution of the nonlinearized spherical Poisson-Boltzmann equation derived by Ohshima et al. (1982).[1]

For PS-PC membranes the charge density in Eq. 4 is due entirely to the carboxyl group on PS in bulk pH's ranging from 3.1 to 7.5.[2] Taking into account the binding of cations to the PS carboxyl group, which reduces the effective charge density, σ has the form

$$\sigma = \frac{N}{1 + \left(\frac{[H^+]}{K_a} + K_m[M^+]\right)\exp\left(-\frac{ZF\psi}{RT}\right)}, \tag{5}$$

where N is the density of $-COOH$ groups per μm^2 with dissociation constant K_a, H^+ and M^+ are the bulk proton and cation concentrations, K_m is the association constant for 1:1 binding of the monovalent cation to PS, and ψ is the surface potential. Knowing the mol % of PS in PS-PC vesicles and the cation concentration and its association constant to PS molecules, one can solve Eqs. 4 and 5 simultaneously to obtain the calculated surface potential as a function of bulk pH for any given pK_a value. In these studies, the predominant binding cation is Na^+, for which K_m has been determined to be in the range 0.6–1 M^{-1} (Eisenberg et al., 1979). In the analysis presented here it was found that $K_m = 0.7\ M^{-1}$ gave the best results, although varying this value within 0.1 unit did not change the final result by more than experimental error. For the sake of comparison, tetramethylammonium (TMA) was also used in some experiments, since this ion is reported to have no affinity for binding to PS, i.e. $K_m = 0$ (Eisenberg et al., 1979). Calculation of N in Eq. 5 is based on a constant area per phospholipid of 70 $Å^2$ (Johnson et al., 1973; Castle and Hubbell, 1976).

In Fig. 1 *a*, *b*, and *c*, the measured and calculated surface potentials are shown as a function of bulk pH. The

[1]In earlier work described by Castle and Hubbell (1976), the Gouy-Chapman theory was employed to relate surface charge densities and surface potentials since this provided a mathematically simple formulation. Although the Gouy-Chapman equation is derived on the basis of planar geometry, it gives values for the potential at the surface very close to those obtained from the spherical Poisson-Boltzmann equation as long as $K \cdot r \gg 1$, a situation satisfied for sonicated vesicles at moderate salt concentrations, low surface potentials. At lower salt concentrations, however, the differences become appreciable. For example, for 8 mol % PS-PC vesicles in 10 mM monovalent salt, the Gouy-Chapman equation predicts a surface potential that is 7.5% higher than predicted by the spherical Poisson-Boltzmann equation; in 500 mM salt, the difference is 0.4%. Since the relatively simple and highly accurate expression given by Eq. 4 has recently become available for the spherical geometry, we here choose to employ it in lieu of the less precise Gouy-Chapman equation.

[2]Under the same salt concentration, the partition coefficients of C_9Q^+ to pure PC vesicles in buffers with pH values of 3.1 and 7.3 were virtually identical, suggesting that the phosphate group in PC or PS will be fully charged at the pH range that we investigated.

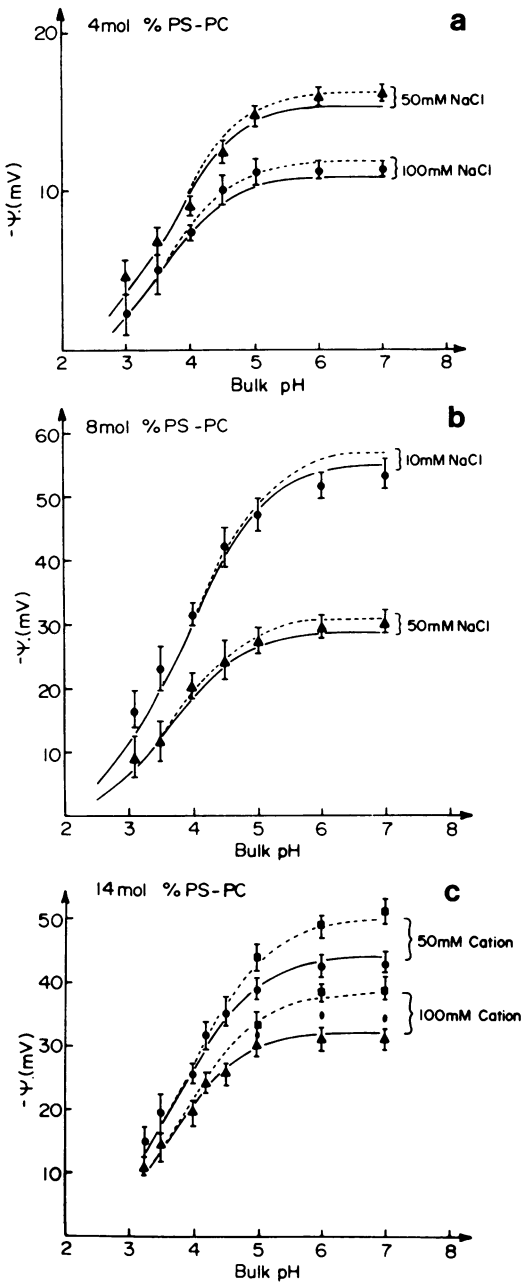

FIGURE 1 Surface potential profiles of mixed PS-PC vesicles as a function of bulk pH. Vesicles were suspended in the indicated concentration of NaCl and the appropriate buffer for the pH range (see Materials and Methods). (*a*) 4 mol % PS-PC in 50 and 100 mM NaCl. ▲ and ● represent experimental values, solid lines are calculated by Eqs. 4 and 5 with a pK_a of 3.60 and a K_m for Na^+ to PS of 0.7 M^{-1}. Dashed lines were calculated the same way as the solid lines, except that $K_m = 0$ for Na^+ binding. (*b*) 8 mol % PS-PC in 10 and 50 mM NaCl. ● and ▲ represent experimental values; solid lines are calculated with Eqs. 4 and 5 with a pK_a of 3.50 and $K_m = 0.7$ M^{-1} for Na^+ binding to PS. Dashed lines are calculated on the basis of $K_m = 0$ M^{-1}. (*c*) 14 mol % PS-PC in 50 and 100 mM cation solutions. ■ represent vesicles in tetramethylammonium chloride (TMA^+Cl^-), ● and ▲ are vesicles in NaCl, and ● are vesicles in 50 mM NaCl and 50 mM TMA^+Cl^-. Solid lines were calculated on the basis of a pK_a of 3.55 and $K_m = 0.7$ M^{-1} for Na^+ binding. Dashed lines were calculated values using $K_m = 0$. Error bars on the experimental points are the standard deviations obtained from the average of three independent measurements.

best fit[3] for 4 mol % PS-PC is obtained by choosing an intrinsic pK_a value of 3.6 ± 0.1, for 8 mol % PS-PC the best choice is 3.5 ± 0.1, and for 14 mol % PS-PC, 3.6 ± 0.1. In all these calculations, it was assumed that the size and shape of the vesicles are independent of the bulk pH and salt concentration. The broken lines in these figures were calculated on the assumption that there is no ion binding to PS in order to emphasize the need for including this effect as the PS:PC ratio or ion concentration increases. In Fig. 1 *c*, the surface potential values obtained using TMA as the screening cation agree well with the lines calculated by assuming no ion binding to PS. Thus we confirm earlier observations indicating no binding of this ion to PS.

An alternative graphical approach can also be used to estimate the intrinsic pK_a of the PS carboxyl group when ion binding is insignificant. Measured surface potentials can be used to obtain charge densities according to Eq. 4. The bulk pH at which half the maximal charge density is obtained is the apparent pK_a. The intrinsic pK_a is then obtained by means of Eq. 2.

Fig. 2 *a* and *b* show the computed charge density for 4 and 8 mol % PS assuming an area of 70 $Å^2$ per phospholipid. For the 8 mol % PS-PC vesicle, the maximum charge is -1.14×10^{-3} charges/$Å^2$ and the apparent pK_a values in 10 mM and 50 mM NaCl are read as ~4.0 and 3.7, respectively, corresponding to a charge density of -5.7×10^{-4} charges/$Å^2$. This is the charge density at which half of the $-COOH$ groups are deprotonated. When the apparent pK_a's are known, the intrinsic pK_a's can then be calculated via Eq. 2 on the basis of the measured surface potential (Fig. 1 *b*). The intrinsic pK_a for the $-COOH$ groups in PS at both bulk salt concentrations is thus found to be ~3.4 when calculated by Eq. 2. Similar calculations were applied to 4 mol % PS-PC vesicles and the intrinsic pK_a was found to be ~3.5. In these calculations, it was assumed that the ion binding effect is negligible due to the low PS concentration (<10 mol %) and low surface potential. The fact that the pK_a value determined in this fashion is very similar to the one determined by fitting the measured values to the calculated surface potentials supported this assumption.

We also measured the binding of the quaternary ammonium spin label to 4 and 14 mol % PS-PC vesicle suspensions at a bulk pH of 4.1, varying the NaCl concentration from 10 to 100 mM. This pH was chosen because it lies close to the value for the intrinsic pK_a of the PS carboxyl that was determined from previous experiments. At this pH, addition of salt will decrease the surface potential. Consequently, the surface pH will increase, causing a change in the degree of ionization of the carboxyl groups.

[3]Surface potentials as a function of bulk pH were calculated by means of Eqs. 4 and 5, for a range of pK_a values. The curves that enclosed the dispersion of experimental data points gave the maximum and minimum values for the pK_a. The "best fit" was taken as the central value of these extremes with the probable error given by the range of the extremes.

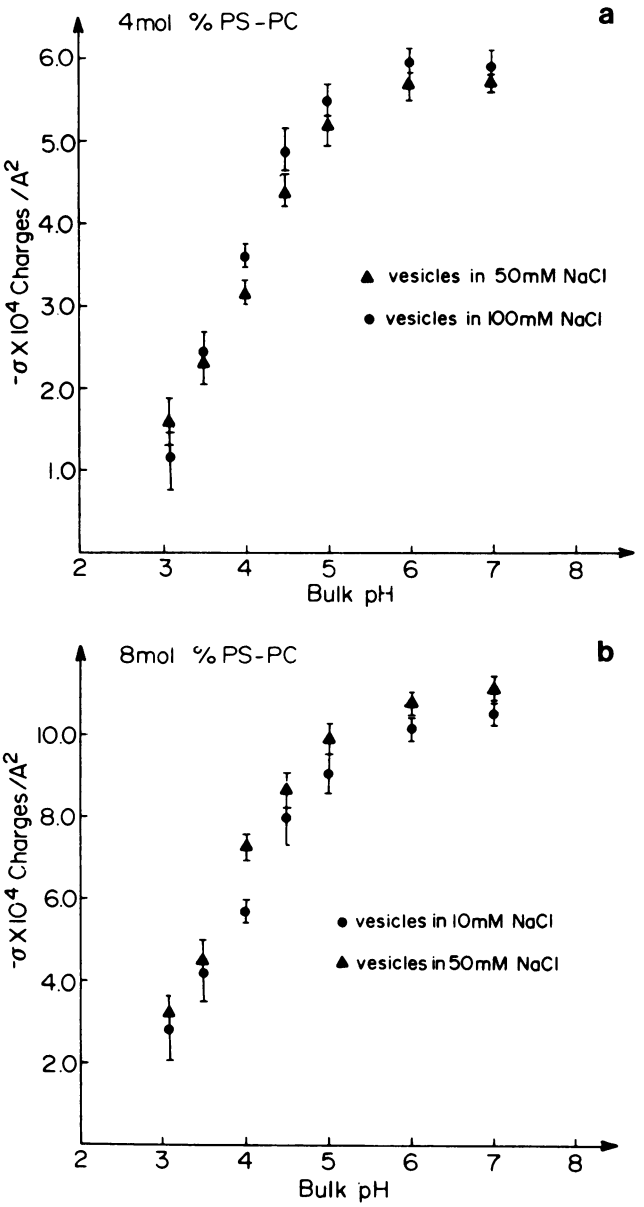

FIGURE 2 Surface charge density profiles of mixed PS-PC vesicles as a function of bulk pH. (*a*) 4 mol % PS-PC in 50 mM (▲) and 100 mM (●) NaCl. The apparent pK_a for vesicles in 50 mM NaCl is ~3.7, as read from the midpoint, corresponding to an intrinsic pK_a of ~3.4. The apparent pK_a = ~3.6 for vesicles in 100 mM NaCl, corresponding to an intrinsic pK_a of ~3.5. (*b*) 8 mol % PS-PC in 10 mM (●) and 50 mM (▲) NaCl. The apparent pK_a = ~4.0 for vesicles in 10 mM NaCl, as read from the midpoint, corresponding to an intrinsic pK_a of ~3.5. The apparent pK_a = ~3.7 for vesicles in 50 mM NaCl, corresponding to an intrinsic pK_a of ~3.4. Error bars on the experimental points are the standard deviations obtained from the average of three independent measurements.

Thus the charge density should be salt dependent in this pH range; this complex situation will commonly be encountered in native membrane systems. Experimental surface potentials were determined by the spin label approach described above. Theoretical surface potentials at various NaCl concentrations were calculated according to Eqs. 4 and 5 with K_m = 0.7 M^{-1} for Na^+, and a value of pK_a was chosen to best fit the experimental data. Figs. 3 *a* and *b* show the experimental salt-dependent surface potentials of 4 and 14 mol % PS-PC vesicles; the solid lines were calculated on the basis of different pK_a values. At a bulk pH of 4.1, the best values for pK_a, obtained by fitting the surface potentials calculated at different salt concentra-

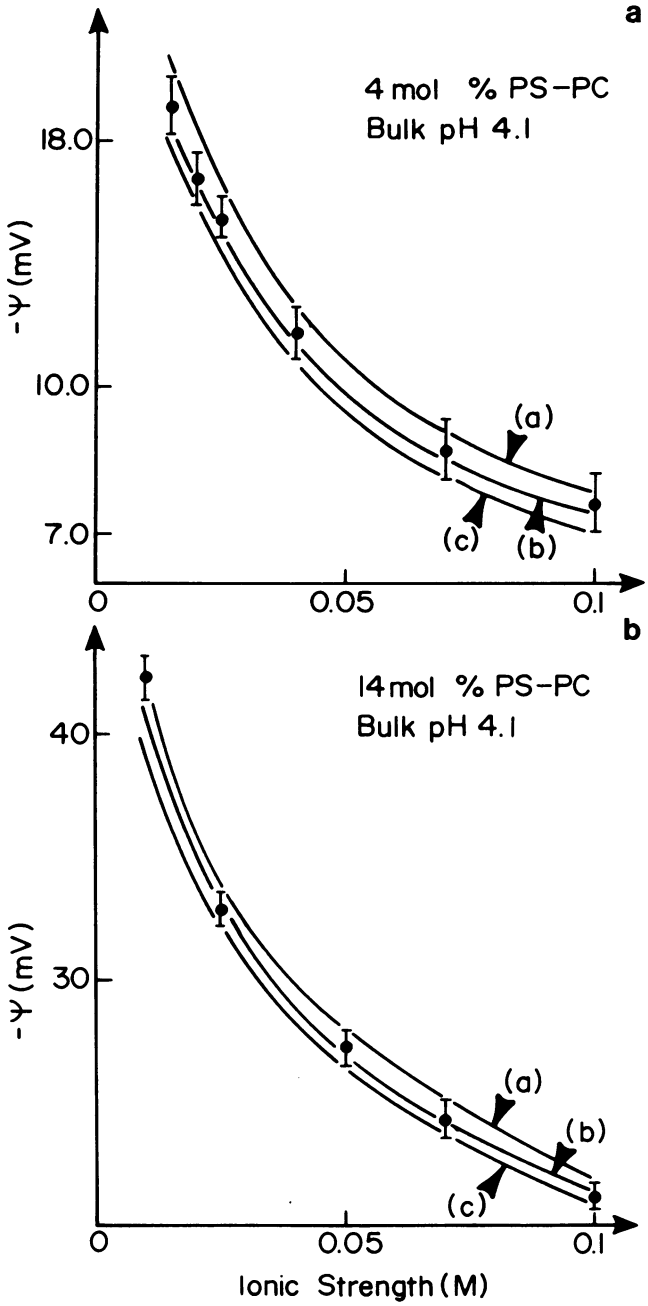

FIGURE 3 Salt-dependent surface potential profiles of mixed PS-PC vesicles at a bulk pH of 4.1. (*a*) Measured values (●) for 4 mol % PS-PC. Solid lines *a*, *b*, and *c* were calculated with pK_a's of 3.65, 3.70 and 3.75, respectively, and a K_m of 0.7 M^{-1} for Na^+. (*b*) Measured values (●) for 14 mol % PS-PC. Solid lines *a*, *b*, and *c* were calculated with pK_a's of 3.55, 3.60, and 3.65, respectively, and a K_m for Na^+ of 0.7 M^{-1}. Error bars on the experimental points are the standard deviations obtained from the average of three independent measurements.

tions by Eqs. 4 and 5, are 3.7 ± 0.1 for 4 mol % PS-PC and 3.6 ± 0.1 for 14 mol % PS-PC. Thus, whether the bulk pH or salt concentration was varied, the preferred value of the pK_a for the carboxyl group obtained by the data fitting procedure is found to be 3.6 ± 0.1.

As shown above, at pH values below 7.5, PS carries one negative charge, suggesting that the amino group is protonated. As the pH is increased, however, the $-NH_3^+$ groups deprotonate, so that PS carries two negative charges, one from the carboxyl group and the other from the phosphate. By measuring the surface potential of PS-PC vesicles as a function of pH at a fixed salt concentration, the pK_a^2 (the amino group pK_a) for PS can be determined. Applying the data-fitting approach described above to the experimental points in Fig. 4 *a*, it was found that pK_a^2 is 9.8 ± 0.1.

From the measured surface potentials at different pH's, surface charge densities were calculated by means of Eq. 4 and plotted in Fig. 4 *b* for 8 mol % PS-PC vesicles. As mentioned above, an estimate of the apparent pK_a for the amino group can be readily obtained from the midpoint of the second titration branch. From Fig. 4 *b*, this is found to be ~10.6. Correcting for the effect of the surface potential with Eq. 2, the intrinsic pK_a becomes ~9.9, in close agreement with the value obtained from the fitting procedure. These values compare well with the electrophoretic mobility results of S. McLaughlin and his co-workers (personal communication).

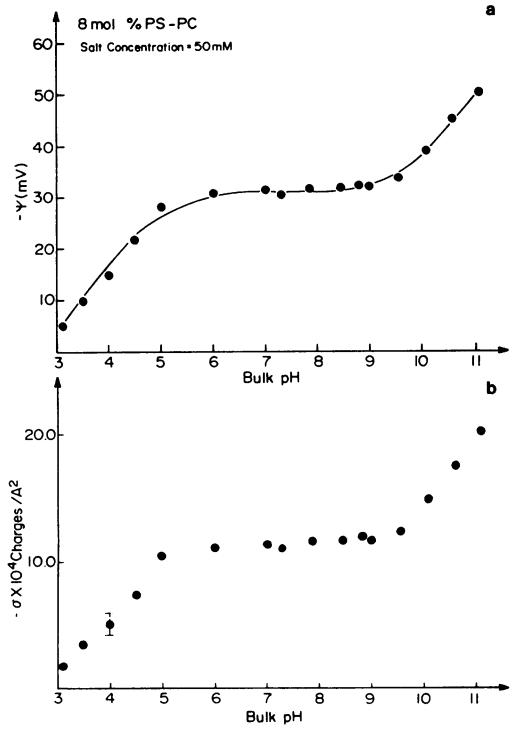

FIGURE 4 Surface potential (*a*) and surface charge density (*b*) profiles of 8 mol % PS-PC in 50 mM NaCl as a function of bulk pH. Measured values are shown as ●; solid line in *a* was calculated using $pK_a = 3.50$ for the carboxyl group, $pK_a = 9.80$ for the amino group, and a $K_m = 0.7\ M^{-1}$ for Na^+ binding to the carboxyl group and $K_m = 0\ M^{-1}$ for Na^+ binding to the amino group. The error bars represent error limits for measurements of EPR signal amplitudes.

Potentiometric Titrations of PS-PC Vesicles

To complement the spin-label binding studies, we performed potentiometric titrations of the mixed PS-PC vesicles; results are shown in Fig. 5. No vesicle compositions other than 14 mol % PS were investigated by potentiometric titration because at lower percentages the number of protons released by the carboxyl groups is too small to be reliably measured. In addition, 14% is a typical mole percentage of PS in native membrane systems. The starting concentration of the 14 mol % PS-PC vesicles in both 10 and 100 mM NaCl was 0.25 mg/ml. The titration was started by lowering the bulk pH to 2.5 with concentrated HCl. (No pH values below 2.5 were investigated because of the possibility that the phosphate groups of the phospholipids might be protonated, thus complicating the data analysis.) At pH values between 2.5 and 3.0, the blank and the vesicle suspension titration curves are very similar, suggesting that only a few protons are released from the carboxyl groups of the PS molecules or from the phosphate groups of the PC molecules in this pH range. By subtracting the blank titration curve from the vesicle titration, one can determine the titration valency per PS molecule as a function of pH. The results under different NaCl concentrations are displayed in Fig. 6. The ionic strength effect on the titration valency profile shown in Fig. 6 is similar to that shown in Fig. 1, indicating that the surface potential has an effect in binding protons. The apparent pK_a values can be read from the midpoints of the titration valency curves. The apparent pK_a values of 14 mol % PS-PC in 10 and 100 mM NaCl were read as 4.7 ± 0.1 and 3.9 ± 0.1 respectively. The intrinsic pK_a value was calculated to be 3.6 ± 0.2 by Eq. 2. This value compares well with those found by the spin-label binding studies. The number of protons released from the vesicles at a pH higher than 7.5 was more than the total number of PS molecules, which may be due to the release of protons from the amino groups.

Surface Potential Measurements of PE-PC Vesicles

Larger mol percentages of PE were studied because of its higher pK_a, which caused the surface charge density to be lower at reasonable pH values. For example, at a pH near the pK_a of PE (9.6) the surface charge density of 14 mol % PE is about the same as for 8 mol % PS vesicles at neutral pH. A concentration of 30 mol % PE was also studied because of its resemblance to the PE composition of native membranes.

At pH values below 6.0, changing the NaCl concentration has no effect on the binding of the C_9Q^+ spin label to 14 and 30 mol % PE-PC vesicles, which suggests that the

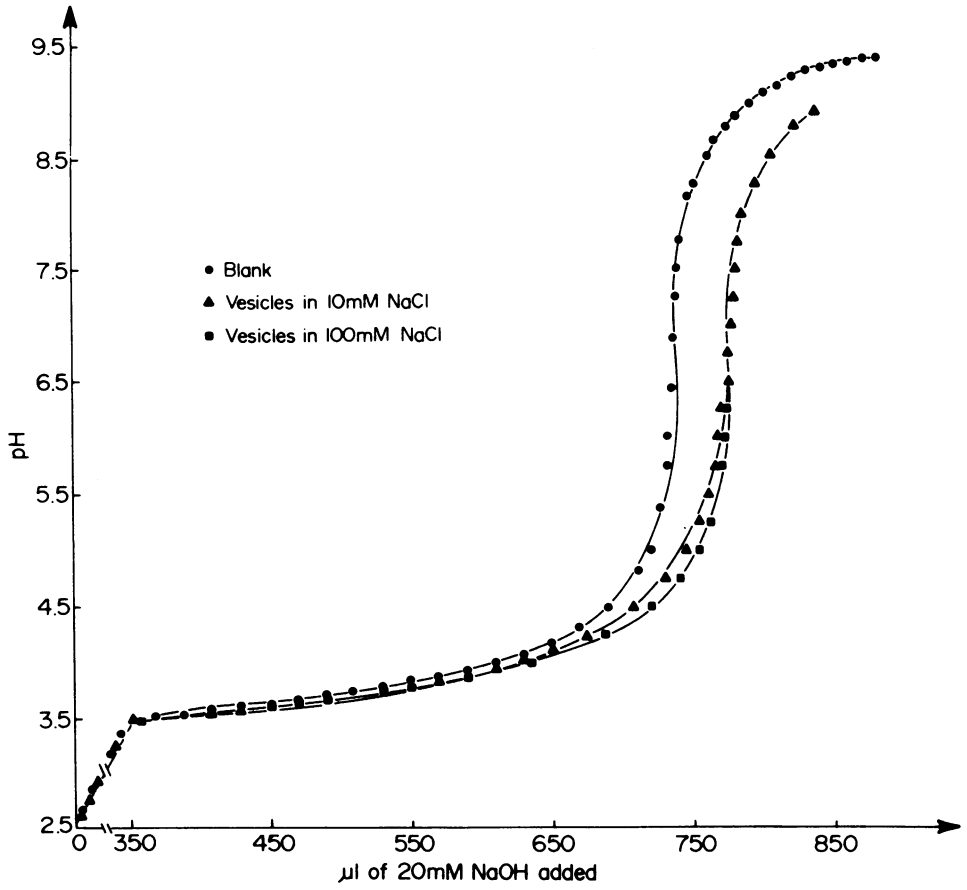

FIGURE 5 14 mol % PS-PC acid-base titration curves.

PE molecule carries no net charge. At higher pH values, the $-NH_3^+$ groups deprotonate so that the PE molecule carries a single negative charge owing to the presence of the negatively charged phosphate group. Therefore, the number of negative charges at different pH values should be equivalent to the number of $-NH_3^+$ groups deprotonated at that pH. This assumption was used to deduce the apparent and the intrinsic pK_a values of PE molecules in 14 and 30 mol % PE-PC vesicles. The intrinsic binding constant was determined directly by fitting the measured surface potentials to a set of calculated values obtained by varying the intrinsic pK_a value as described in the previous section. Figs. 7 *a* and *b* show the measured and predicted surface potentials as a function of bulk pH. For 14 mol % PE-PC, the best fit and probable error for the pK_a is 9.6 ± 0.1, and for 30 mol % PE-PC, 9.7 ± 0.1; or an average of 9.6 ± 0.1. This value compares well with those found by other researchers (see the Discussion for details). The results obtained from using Na^+ or TMA as screening cations are virtually identical, suggesting that PE does not bind Na^+, i.e. $K_m = 0$. Hence, ion binding was not considered in these calculations.

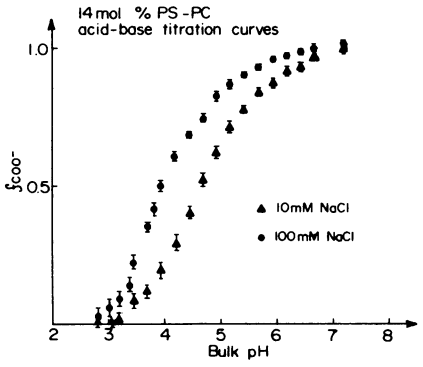

FIGURE 6 Fraction of deprotonated PS carboxyl groups ($f_{COO^-} = N_{COO^-}/[N_{COO^-} + N_{COOH}]$) as a function of bulk pH. The apparent pK_a's for vesicles in 10 and 100 mM NaCl are ~4.7 and ~3.9, respectively, as read from the midpoints. The intrinsic pK_a value, calculated with Eq. 2, is ~3.6. Error bars on the experimental points are the standard deviation obtained from the average of three independent measurements.

Again, the graphical method presented above can be used to obtain an approximate pK_a. If all the $-NH_3^+$ groups are deprotonated, 14 mol % PE-PC vesicles will have a maximum surface charge density of -2.0×10^{-3} charges/A^2. Fig. 7 *c* shows the derived charge density of PE in 14 mol % PE-PC vesicles at pH values ranging from 7.0 to 11.1. The apparent pK_a in 50 mM Na^+ was ~10, as read from the charge density at which half of the $-NH_3^+$ groups are deprotonated. From the measured surface

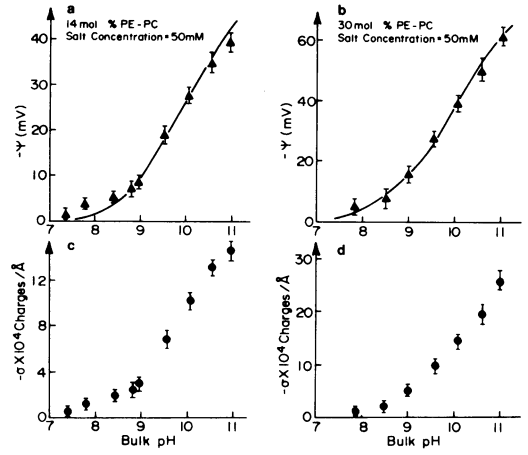

FIGURE 7 *a* and *b* represent surface potential profiles of 14 and 30 mol % PE-PC vesicles as a function of bulk pH. Measured values are shown as ▲; solid line in *a* is calculated with a pK_a of 9.60 and in *b* with a pK_a of 9.72. *c* and *d* are surface charge density profiles of 14 and 30 mol % PE-PC vesicles as a function of bulk pH. Measured values are shown as ●; the apparent pK_a's for 14 and 30 mol % PE-PC vesicles in 50 mM NaCl as read from the midpoints are ~10.0 and ~10.6, respectively, corresponding to an average intrinsic pK_a of ~9.6, obtained via Eq. 2. The surface charge density for 14 and 30 mol % PE-PC at the apparent pK_a is equal to -1.0×10^{-3} and -2.1×10^{-3} charges/A^2, respectively. Error bars on the experimental points are the standard deviations obtained from the average of three independent measurements.

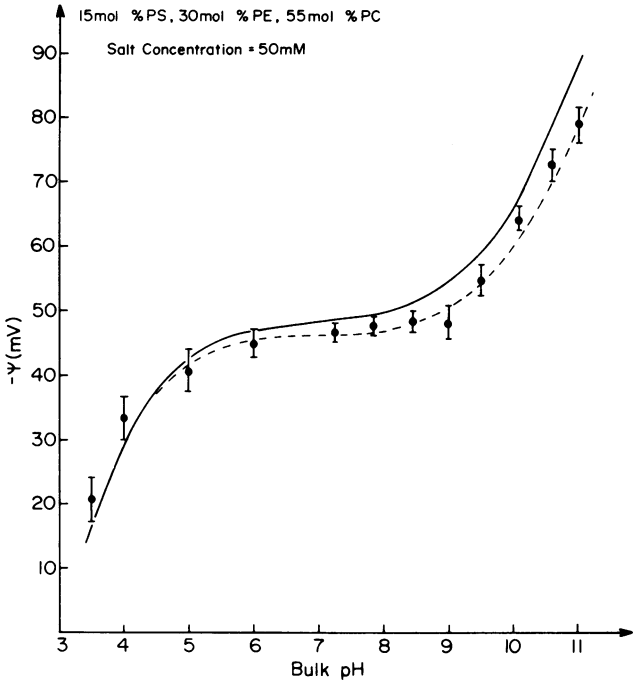

FIGURE 8 Surface potential profiles of mixed 15 mol % PS, 30 mol % PE, and 55 mol % PC vesicles as a function of bulk pH. Solid line is calculated with 3.50 and 9.80 for the carboxyl and amino pK_a's of PS, and 9.60 for the amino pK_a of PE. The broken line is calculated with the same pK_a's for PS and choosing an amino pK_a for PE of 10.1. Error bars on the experimental points are the standard deviations obtained from the average of three independent measurements.

potential at this pH and the apparent pK_a value, the intrinsic pK_a of the $-NH_3^+$ groups in PE is found to be ~9.6 (Eq. 2). Similar calculations for 30 mol % PE-PC vesicles gave apparent and intrinsic pK_a values in 50 mM Na^+ of ~10.6 and ~9.7, respectively (Fig. 7 *d*).

Surface Titrations of Mixed PS-PE-PC Vesicles

The primary incentive of this work was to provide pK_a values for PS and PE that would allow calculation of expected charge densities in native membranes, particularly the disk membrane of photoreceptor cells. The values provided above should allow this, provided that mixtures of the lipids can be described with independent pK_a values. To test this, mixtures of PS/PE/PC were prepared in approximately the same ratio as they occur in the photoreceptor disk membrane, i.e. 15:30:55. The surface potentials were measured as a function of pH at a fixed ionic strength and the results shown in Fig. 8. The solid line is calculated by choosing 3.6 and 9.8 for the carboxyl and amino pK_a's of PS, and 9.6 for the amino pK_a of PE. The broken line is obtained by using the same pK_a's for PS but changing the amino pK_a of PE to 10.1. The broken line fits the measured values better, suggesting that the PE amino group has a slightly higher pK_a value in mixed vesicles containing PS.

DISCUSSION

To create an electrical structure model of the biological membrane surface, one has to know the pK_a values of the ionizable groups on the proteins and phospholipids present on the surface. At a physiological pH value of 7.4, the major functional groups contributing surface charges to the membrane are the carboxyl groups of phosphatidylserine, aspartic acid and glutamic acid, and the amino groups of arginine and lysine. The pK_a values for amino acid side-chains in aqueous solution or in protein hydrophobic domains have been widely reported in the literature (Cantor and Schimmel, 1980). However, many of the studies reporting pK_a values for phospholipids have involved rather indirect methods such as phase separation measurements (MacDonald et al., 1976; Seddon et al., 1983), and in most cases did not distinguish between apparent and intrinsic pK_a values (Garvin and Karnovsky, 1956; Van Dijck et al., 1978; Abramson et al., 1964). A drawback of these and other studies (Hendrickson and Fullington, 1965) is the use of pure PS bilayers, whose surface area per phospholipid can change as a function of pH or ionic strength because of the high surface potential, and large corrections are required to account for ion binding. Eisenberg et al. (1979) have shown that sodium binds to PS with an association constant of 0.6–1.0 M^{-1}, and that for pure PS liposomes in a 100 mM NaCl solution at a pH of 7.5 this effect decreases the surface potential by almost 30%. As a consequence, although values for the apparent pK_a of the carboxyl group in pure PS liposomes are available, it is

difficult to calculate the corresponding intrinsic pK_a or to reconcile the broad range of apparent pK_a's, from 2.2 to 5.5, that have been reported (Cevc et al., 1981).

In this manuscript, we describe a direct spin label approach to determine the pK_a values of the ionizable groups of charged phospholipids dilute in PC vesicles. The vesicles were prepared with rather low PS-PC mole ratios to minimize the effect of ion binding. Low mol percent PS-PC vesicles also exhibit a low surface potential, a major correction factor that must be applied to convert the measured apparent pK_a to intrinsic pK_a (Eq. 2). The advantages of using the cationic quaternary ammonium nitroxide C_9Q^+ as surface potential probe were discussed in detail by Castle and Hubbell (1976). One of the attractive features of this probe is its high sensitivity, which allows its use at low concentrations (2×10^{-5} M) that will not substantially affect the intrinsic charge of the electrostatic surface being tested. As an ammonium ion, this particular probe also does not show specific association with individual anionic groups which might result in lowering the effective charge on the surface.

The intrinsic pK_a values reported here are independent (within experimental error) of the vesicle surface potential and the effect of cation binding. In the experiments presented above, binding of Na^+ to PS molecules significantly reduced the surface potential at mol percentages >8. We carried out spin-labeling experiments using Na^+ or tetramethylammonium (TMA), a cation that does not bind to PS (Eisenberg et al., 1979), as screening cations for studies using 14 mol % PS-PC vesicles, a PS concentration high enough for ion binding to be significant. The measured surface potentials obtained while using Na^+ as screening cation were always lower than those found by using TMA. Using a mixture of Na^+ and TMA as screening cations gave intermediate results (Fig. 1 *c*). All these suggest that the binding of Na^+ to PS lowers the effective surface charge densities and hence the surface potentials. To account for the data, a binding constant of Na^+ to PS of 0.7 M^{-1} was used, which is in the range of values reported by Eisenberg et al. (1979). The binding constant of Na^+ to the amino on PE is essentially zero.

The binding of the spin-label probe to phospholipid vesicles was measured as a function of bulk pH or salt concentrations. From the binding parameter, the surface potentials can be determined as described in Results. The intrinsic pK_a's of the titratable groups on PS and PE were obtained by fitting the measured data to a set of lines calculated by means of Eqs. 4 and 5 for for different pK_a values. The intrinsic pK_a of the carboxyl group on PS was found to be 3.6 ± 0.1. The results obtained by both spin label binding and potentiometric titration are comparable, giving us confidence that EPR can be used as an alternative physical method to study the properties of charged phospholipid vesicle surfaces in different pH environments.

The pK_a of the amino group of PE in PE-PC vesicles was determined solely by surface potential measurements to be 9.6 ± 0.1. The pK_a of the amino group on PS is 9.8 ± 0.1, which is slightly higher than for PE. This might be due to a higher negative potential at the site of the PS amino group due to the nearby carboxylate anion.

MacDonald et al. (1976) reported an intrinsic pK_a value of 2.7 for the PS carboxyl group in pure PS liposomes. In their report, the very significant effect of sodium ion binding, which lowers the effective surface charge density and hence the surface potential, was not taken into account. For pure PS vesicles in a 0.1 M NaCl solution, MacDonald et al. (1976) reported an apparent pK_a of 4.6. If the effect of ion binding is taken into account, the intrinsic pK_a becomes 3.1, which compares well with our measured value.

Dielectric constants in the range 10–30 have been reported for the phospholipid vesicle surface (Fernandez and Fromherz, 1977; Lelkes and Miller, 1980; Cevc et al., 1981), as compared with 78 for the aqueous phase. Fernandez and Fromherz (1977) showed that the low dielectric environment results in an increase of the pK_a value of the acidic titratable group by approximately one unit. Thus PS in bilayers should have a pK_a value at least one unit higher than found in phosphoserine, which is 2.7 as determined by acid-base titration (Neuhaus and Korkes, 1958). With this assumption, PS should have a pK_a of ~3.7, which is close to the value reported here. The carboxyl group of glutamic acid and the carboxyl group of aspartic acid in globular proteins have a pK_a value of 4.5 to 5.0 (Cantor and Schimmel, 1980), which is also substantially higher than found in phosphoserine. By the same token, the low dielectric constant, by favoring the existence of the neutral form, should decrease the intrinsic pK_a of basic titratable groups by one unit. Ethylamine, which can exist as a monomer in aqueous solution, has a pK_a of 10.8. If the dielectric effect is taken into account, the amino group of PE in phospholipid vesicles should have an intrinsic pK_a of $10.8 - 1.0 = 9.8$, which is very close to the value reported here.

Szabo et al. (1972) measured the conductance of black lipid bilayers formed from PE and containing nonactin and 100 mM KCl. In these experiments, nonactin, an ionophore that forms a charged complex with K^+, is used to measure the conductance of the bilayer; from the conductance the surface potential is directly calculated. Their conductance vs. pH curve is almost exactly superimposable onto the surface potential vs. pH curve reported by Papahadjopoulos (1968) for the titration of PE monolayers. From their curve one could assert that the intrinsic pK_a of PE is ~10. Szabo et al. (1972) contend that the agreement between their conductance measurements and the surface potential results on monolayers supports the notion that the conductance changes are due to changes in the membrane surface potential.

In biological membranes, the predominant phospholipids are PC, PE, and PS. Using the pK_a values determined

above, one could model a titration curve for phospholipid vesicles with compositions similar to those found in native systems by choosing the PC/PE/PS ratio of a typical membrane, such as 55:30:15, which is the disk membrane composition. We found that the predicted curve for such a mixture agrees tolerably well with the experimental measurement, with some deviation at high pH. Even here, however, the expected and measured values do not differ by more than ~10 to 15%. The agreement can be improved, however, by setting the amino pK_a for PE in the PC-PE-PS vesicles at 10.1, almost half a pH unit higher than found in PE-PC vesicles. An increae in pK_a might be due to the formation of hydrogen bonds between the proton of the amino group on PE and the oxygen of the carboxyl group on PS, which would favor the existence of the protonated species. Likewise, the pK_a shift could be due to the larger repulsion between phospholipid headgroups in this mixture at basic pH, which would cause the charge density to decrease and consequently the pK_a to increase.

We would like to thank Dr. S. McLaughlin for helpful suggestions and comments and Drs. J. Trudell and C. Altenbach for careful readings of the manuscript.

This work was supported by National Institutes of Health grants EY-05216 to W. L. Hubbell, Postdoctoral Training Fellowship EY-07026 to F. C. Tsui, Vision Research Center grant EY-00331 to the Jules Stein Eye Institute, and the Jules Stein Professorship Endowment to W. L. Hubbell.

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