The Intrinsic pK_a Values for Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylserine in Monolayers Deposited on Mercury Electrodes

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ABSTRACT The intrinsic pK_a values of the phosphate groups of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and of the phosphate and carboxyl groups of phosphatidylserine (PS) in self-organized monolayers deposited on a hanging mercury drop electrode were determined by a novel procedure based on measurements of the differential capacity C of this lipid-coated electrode. In view of the Gouy-Chapman theory, plots of $1/C$ at constant bulk pH and variable KCl concentration against the reciprocal of the calculated diffuse-layer capacity $C_{d,0}$ at zero charge exhibit slopes that decrease from an almost unit value to vanishingly low values as the absolute value of the charge density on the lipid increases from zero to ≈ 2 μC cm^{-2}. The intrinsic pK_a values so determined are 0.5 for PE and 0.8 for PC. The plots of $1/C$ against $1/C_{d,0}$ for pure PS exhibit slopes that pass from zero to a maximum value and then back to zero as pH is varied from 7.5 to 3, indicating that the charge density of the lipid film passes from slight negative to slight positive values over this pH range. An explanation for this anomalous behavior, which is ascribed to the phosphate group of PS, is provided. Interdispersion of PS and PC molecules in the film decreases the "formal" pK_a value of the latter group by about three orders of magnitude.

INTRODUCTION

Bilayer lipid membranes (BLMs) are extensively employed as models of biological membranes, because their structural characteristics and electrical properties are similar to those of biomembranes. This is particularly true for "reconstituted" BLMs, namely BLMs incorporating some basic structural units of natural membranes. A more recent model of biomembranes is represented by a phospholipid-coated mercury electrode (Miller, 1981; Nelson and Benton, 1986). The phospholipid coating is provided by spreading a solution of the phospholipid in a suitable solvent (e.g., pentane) on the surface of an aqueous electrolyte, allowing the solvent to evaporate, and immersing a hanging mercury drop electrode in the electrolyte. This procedure gives rise to half a bilayer.

Self-assembled phospholipid monolayers on mercury electrodes offer some advantages over BLMs, when used as models of biomembranes. Thus, the electric potential and the flux of electroreducible metal ions across these monolayers can be controlled more accurately and more directly than across BLMs. Moreover, these half-membranes provide an inherent mechanical stability and a resistance to high electric fields that is is not shared by BLMs. Thus, application of a double potential step of short duration to a dioleoylphosphatidylcholine (PC)-coated mercury electrode, from the potential region of minimum capacity (-0.2 V $< E <$ -0.8 V/*SCE*) to -1.8 V and backwards, leaves the film completely unaltered. Over the region of minimum capacity, the film is impermeable to inorganic metal ions, whereas it becomes permeable outside this region. Incorporation of the channel-forming gramicidin (Nelson, 1991a) and of the ionophore Antibiotic 23187 (Nelson, 1991b) into phospholipid monolayers on mercury has been recently carried out by Nelson, who has investigated the effect of these ion carriers on the permeability of the monolayer to electroreducible metal ions.

As expected, the differential capacity C_m of a lipid monolayer on mercury over the flat region of minimum capacity is ≈ 1.7–1.9 μF cm^{-2}, namely twice the value for a BLM. If we consider that the diffuse layer capacity $C_{d,0}$ at zero charge for a 10^{-3}M solution of a 1,1-valent electrolyte amounts to ≈ 8 μF cm^{-2}, we may conclude that at this low electrolyte concentration $C_{d,0}$ contributes to the overall capacity $C = C_m C_{d,0}/(C_m + C_{d,0})$ of the lipid-coated electrode by about 20%. This may permit an investigation of the double layer structure at the half-membrane/water interface on the basis of differential capacity measurements, provided the reproducibility and accuracy of these measurements is sufficiently high. In a previous note (Moncelli and Guidelli, 1992), this procedure was adopted to test the validity of the Gouy-Chapman (GC) theory for an uncharged monolayer of PC deposited on mercury by plotting experimental values of $1/C$ at different concentrations c of a 1,1-valent electrolyte against $1/C_{d,0}$ values calculated from the GC theory: in agreement with the theory, these plots exhibit an almost unit slope.

Under conditions in which a neutral lipid monolayer starts to become progressively charged, the GC theory predicts a rapid increase in its diffuse-layer capacity C_d and, hence, a rapid decrease in its contribution to the overall capacity $C = C_m C_d/(C_m + C_d)$. According to this theory a plot of $1/C$ vs. $1/C_{d,0}$ is expected to exhibit an average slope that becomes

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vanishingly small within the limits of experimental accuracy for absolute values of the charge density σ_1 on the lipid monolayer greater than $\approx 2\ \mu C\ cm^{-2}$. Differential capacity measurements on lipid-coated mercury electrodes may permit us, therefore, to determine the experimental conditions under which the lipid monolayer is only slightly charged and to estimate the magnitude of its charge density. In the present note, this novel procedure is applied to the investigation of pH effects on self-organized monolayers of PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and mixtures of PS and PC. Results obtained with phosphatidic acid (PA) monolayers deposited on mercury will also be reported.

Both PC and PE are known to be isoelectric over a wide pH range, as indicated by the pH dependence of a number of physicochemical properties such as surface potential (Shah and Schulman, 1967), electrophoretic mobility (Bangham and Dawson, 1959), and ^{1}H-chemical shifts (Hauser et al., 1975). PE differs from PC insofar as the primary ammonium group becomes deprotonated at a pH of about 8 (Rojas and Tobias, 1965; Seimiya and Ohki, 1972). PS is the predominant negative lipid in the membranes of many mammalian cells. Thanks to its anionic character at physiological pH, it plays an important role in processes involving the interactions between the membrane and the intra- and extracellular electrolytes by regulating the concentration of protons, cations, and any charged metabolites at the membrane surface. Its charge contributes to the establishment of transmembrane potentials that are involved in energy-linked transmembrane transport processes and that are postulated to regulate the activity of certain membrane proteins. To determine the contribution to the membrane charge from PS, one must know its intrinsic pK_a, namely the surface pH at which one-half of the PS molecules in the membrane is charged. It is now well established that the intrinsic pK_a of negatively charged groups in self-organized films is significantly lower than the apparent value, namely the value determined with reference to the bulk pH, because the surface pH is less than the bulk pH. A Boltzmann distribution of hydrogen ions is usually assumed at the lipid/solution interphase, and the surface ionic potential ψ is expressed by the Gouy-Chapman (GC) theory. Literature measurements of the intrinsic pK_a values for PS have been carried out on vesicles, dispersions, monolayers, or BLMs of PS (Tsui et al., 1986; Matinyan et al., 1985; Mac Donald et al., 1976; Ohki and Kurland, 1981). Application of our procedure to mercury electrodes coated with PC and with PE yields pK_a values for the phosphate groups of these lipids that are in fairly good agreement with the literature. Conversely, the results on PS monolayers, albeit quite reproducible, are at variance with those in the literature. A plausible explanation for these discrepancies is provided.

MATERIALS AND METHODS

The adsorbed monolayers of PC, PE, and PS on mercury were prepared as described by Nelson and Auffret (1988a, b). The water used was obtained from light mineral water by distilling it once, and by then distilling the water so obtained from alkaline permanganate, while constantly discarding the heads. Tap water from our waterworks was found to be unsuitable because neither the above distillation from permanganate nor distillation and subsequent deionization by the Millipore system succeeded in eliminating traces of low boiling organic impurities. Merck Suprapur KCl was baked at 500°C before use to remove any organic impurities. Dioleoyl PC, dioleoyl PE, and dioleoyl PS were obtained from Lipid Products (South Nutfield, Surrey, England), whereas dioleoyl PA was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The desired pH values were realized with Merck Suprapur HCl over the pH range 2–5, with a 1×10^{-3} M $H(PO_4)^{2-}/H_2(PO_4)^-$ buffer over the pH range 6.5–7.5 and with a 1×10^{-3} M H_3BO_3/NaOH buffer over the pH range 8.5–9.8.

A homemade hanging mercury drop electrode (HMDE) was used, with a grinded cylindrical piston of stainless steel, 1 mm in diameter. The HMDE was made particularly mercury-tight by using two O-rings. The piston was driven by a micrometric head (no. 350–541 Mitutoyo, Japan) with a digital millesimal position sensor, which permitted us to estimate a 2-μm shift of the piston. The whole cell was contained in a water-jacketed box thermostated at 25 ± 0.1°C. The HMDE was housed in a water-jacketed sleeve on the top of the box, with the only exclusion of the micrometric head, to permit an effective thermostatization of the mercury reservoir. This contrivance produced an appreciable improvement in the time constancy of the drop area and, hence, of the differential capacity of the film. Before spreading the lipid solution in pentane on the surface of the electrolytic solution, the latter was deaerated by purging with high purity argon for no less than 30 min; the absence of detectable traces of organic impurities was then constantly checked by verifying the constancy of the corresponding differential capacity versus potential curve for a period of no less than 20 min. The amount of lipid spread on the argon/solution interface corresponded to 2.5 monolayers if the cross sectional area of the lipid is estimated at 65 Å^2. After spreading the lipid film, the HMDE was lowered into the solution through the film by means of an oleopneumatic piston; this ensured the complete absence of vibrations while permitting an appreciable range of velocities. The reproducibility of the transfer of a monomolecular film of the lipid from the argon/solution interface to the mercury drop was found to depend upon the rate of lowering of the HMDE; a pressure gauze connected to the oleopneumatic system permitted us to reproduce the optimum rate as determined in preliminary experiments.

Measurements of the differential capacity, C, were carried out using a Metrohm Polarecord E506 (Herisau, Switzerland). In view of the low capacity of the lipid-coated electrode ($<2\ \mu F\ cm^{-2}$), C was directly measured by the quadrature component of the AC current, other than at the lowest salt concentrations; in the latter case, both quadrature and in-phase components of the AC current were measured, to correct for the cell resistance. The AC signal had a 10-mV amplitude and a 75-Hz frequency. The system was calibrated using a precision capacitor (Decade Capacitor type 1412-BC, General Radio, Concord, MA). All potentials were measured versus a saturated calomel electrode (SCE). The reproducibility of the differential capacity in passing from one mercury drop to another was better than $0.05\ \mu F\ cm^{-2}$. At any rate each set of differential capacity measurements at variable KCl concentration and constant pH was carried out on the same lipid-coated mercury drop, to eliminate practically the effect of any slight irreproducibilities in the drop surface area or in the lipid transfer. This permitted us to estimate the changes in differential capacity after an increase in electrolyte concentration with an accuracy better than $0.02\ \mu F\ cm^{-2}$. The electrolyte concentration in the cell was progressively increased by adding a deaerated solution of the concentrated electrolyte from a microsyringe (Hamilton, Reno, NV). The plunger of the syringe was fastened tightly to the rod of a digital-display micrometer screw with a 0.005-mm pitch (n.297-101-01, Mitutoyo, Japan). The micrometer screw was held by a movable stand that permitted the syringe needle to be lowered into the solution during the addition and raised above the solution just after the addition. After each addition, the solution was stirred mildly for about 30 s with a magnetic stirrer on the bottom of the cell. The stability of the differential capacity was tested by recording it over the whole potential region of minimum capacity 2 or 3 times consecutively, interposing a mild stirring between each measurement; whenever detectable differences between these recordings were observed, the whole series of measurements was discarded.

To prepare films of PS-PC mixtures, aliquots of stock solutions of these lipids in 50% (v/v) $CHCl_3/CH_3OH$, stored at −20°C, were dissolved in pentane in quantity to give the appropriate combination of PS and PC in the lipid film on the solution surface. As distinct from BLMs (Matinyan et al., 1985), mixed lipid monolayers deposited on mercury cannot have a composition different from that of the membrane-forming mixture. In fact, the time τ during which the mercury drop remains in contact with the lipid film on the solution surface is of the order of 2 s. Let us assume as a limiting case that PS tends to be completely expelled from the PS-PC mixture in contact with mercury: the quantity of PS that may actually escape from the drop surface must be necessarily less than that diffusing across the maximum contact line among mercury, solution and argon (namely $2\pi r$, where $r \approx 0.035$ cm is the drop radius) during τ under limiting conditions, namely for a zero surface concentration of PS along this line. This quantity is given by $(2\pi r)2xN(D\tau/\pi)^{1/2}$, where x is the mole fraction of PS in the PS-PC mixture, N is the overall density of the phospholipids in the monolayer, D is the diffusion coefficient of PS, and $(\pi Dt)^{1/2}$ is the diffusion layer thickness at a given time $t \leq \tau$. For the typical values $r = 0.035$ cm, $D = 2 \times 10^{-8}$ cm^2 s^{-1}, $N = 2 \times 10^{-10}$ mol cm^{-2}, and $x = 0.15$, this quantity equals 1.49×10^{-15} mol and, hence, is only 0.32% of the quantity of PS dragged into the solution by the mercury drop.

RESULTS AND DISCUSSION

Each series of differential capacity measurements was carried out on a single mercury drop coated with PC, PE, PS, or with a PS-PC mixture in a solution of constant pH by increasing gradually the KCl concentration from 5×10^{-3} to 0.2 M. Several series at different pH were performed. Although C varies only very slightly over the potential region of minimum capacity, namely from −0.2 to −0.8 V, differential capacity measurements were constantly carried out at −0.5 V. Plots of $1/C$ at constant pH against the reciprocal of the GC diffuse-layer capacity at zero charge,

$$C_{d,0} = F\sqrt{\frac{\epsilon c}{2\pi RT}},$$

show only slight deviations from linear behavior at all pH values investigated, where $\epsilon = 78$ is the dielectric constant of the solvent and c is the overall concentration of the 1,1-valent electrolytes present in the solution; in all cases, the slight curvature of these $1/C$ vs. $1/C_{d,0}$ plots turns the concavity towards the horizontal axis, as shown in Fig. 1 for PC films. Although plots of $1/C$ vs. $1/c^{1/2}$ exhibit the same behavior, $1/C$ vs. $1/C_{d,0}$ plots were preferred because their average slope is dimensionless and is close to unity when the lipid monolayer is uncharged.

We shall assume that the experimental differential capacity C is given by

$$\frac{1}{C} = \frac{1}{C_m} + \frac{1}{C_d} \quad (1)$$

and that the differential capacity C_m of the lipid monolayer is not affected by changes in pH or in the electrolyte concentration c. The latter assumption is supported by the rigorous constancy of the area per lipid molecule during a single series of measurements at constant pH and variable ionic strength. In fact each series, which provides each of the experimental points to be reported in the following, was carried out on a single lipid-coated mercury drop completely immersed in the solution. Therefore, the lipid monolayer is practically separated from any lipid "reservoir" in a condition of accepting lipid molecules from or releasing them to the lipid monolayer. That this is the case is demonstrated by the following experiment: expanding the drop within the solution causes the differential capacity to increase proportionally to the surface area S of the drop and, hence, proportionally to the reciprocal of the monolayer thickness d as estimated upon assuming that the volume Sd of lipid material on the drop surface remains constant during the expansion (Nelson and Benton, 1986); we have also ascertained that a contraction of the surface area S up to 20% causes C to decrease proportionally to S, thus excluding accumulation of lipid material around the drop neck. In this respect, the situation is different from that encountered with lipid-coated mercury drops maintaining a contact with the solution surface (Lecompte and Miller, 1980), where a change in experimental conditions may cause an appreciable change in the area per lipid molecule. Such a change may also take place with BLMs, whose Plateau-Gibbs boundary extending along the circumference of the flat bilayer may act as an effective lipid reservoir, or with vesicles, whose radius may freely change with a change in experimental conditions.

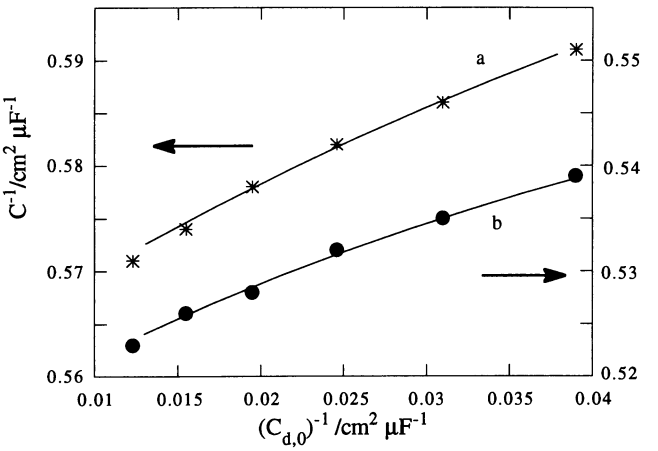

FIGURE 1 Points are experimental values of $1/C$ against $1/C_{d,0}$ at $E = -0.5$ V for PC-coated mercury electrodes immersed in KCl solutions of pH 3 (*a*) and 2.6 (*b*). The solid curves are $1/C_d$ vs. $1/C_{d,0}$ plots calculated as described in Appendix I for $K = 6\ M^{-1}$, $C_{ht} = 1.7\ \mu F\ cm^{-2}$, $\sigma_{max} = 25\ \mu C\ cm^{-2}$, and $\Delta\phi - \chi = -0.3$ V; these curves were shifted along the vertical axis to attain the best overlapping with the corresponding $1/C$ vs. $1/C_{d,0}$ plots.

With the above assumptions the experimental plots of $1/C$ vs. $1/C_{d,0}$ at constant pH and variable c will have the same slope as those of $1/C_d$ vs. $1/C_{d,0}$ in view of Eq. 1. The diffuse layer capacity C_d was calculated, therefore, from the GC theory as a function of c at different bulk pH values upon assuming that the protons bind to the phosphate or carboxyl groups according to a Langmuir isotherm and taking into account the changes in surface pH after the changes in c at constant bulk pH and applied potential (see appendices). In practice, the estimate of C_d as a function of c and bulk pH requires the knowledge of the protonation constants of the ionizable groups of the lipid. Hence, fitting the slope of calculated $1/C_d$ vs. $1/C_{d,0}$ plots to that of the experimental $1/C$

vs. $1/C_{d,0}$ plots allows an estimate of the protonation constants, provided the fitting is satisfactory over the whole range of c and bulk pH values investigated.

PC and PE monolayers

In Fig. 1, the experimental $1/C$ vs. $1/C_{d,0}$ plots for PC at two different bulk pH values are compared with $1/C_d$ vs. $1/C_{d,0}$ plots calculated for the same pH values by setting the protonation constant K of the phosphate group equal to 6 M^{-1}; the area per phospholipid molecule was estimated at 65 $Å^2$. The calculated curves were shifted along the vertical axis to attain the best overlapping with the corresponding experimental plots. It is apparent that the calculated curves reproduce satisfactorily both the average slope and the slight curvature of the experimental plots. To compare the slopes of the experimental $1/C$ vs. $1/C_{d,0}$ plots with those of the calculated $1/C_d$ vs. $1/C_{d,0}$ plots over the whole pH range investigated, the average slopes of both plots, as estimated by least-squares fitting to a straight line over the c range 0.01–0.1 M, were plotted against pH. In what follows, these slopes will be briefly referred to as "experimental slopes" and "calculated slopes" and will be denoted by S_{exp} and S_{cal}. In Figs. 2 and 3, the experimental slopes for PC and PE are plotted against pH together with a series of slopes calculated for different K values. It should be noted that the maximum limiting value of the calculated slopes in Figs. 2 and 3 is somewhat less than unity because of the presence of a slight negative charge σ_M of about -0.5 $\mu C\ cm^{-2}$ on the mercury surface at the applied potential $E = -0.5$ V at which C measurements were carried out. Under these conditions, the GC diffuse-layer capacity C^{GC} is given by $d(\sigma_M + \sigma_1)/d\psi$, where $\sigma_M + \sigma_1$ is the overall charge density "seen" by the diffuse-layer ions, whereas the diffuse-layer contribution C_d to the overall differential capacity C is given by $d\sigma_M/d\psi$, where $d\sigma_M/dt$ is the capacitive current density flowing across the external circuit as a consequence of the AC signal. The S_{exp} values for PE in Fig. 3 are in good agreement with the S_{cal} vs. pH curve calculated for $K = 3$ M^{-1}. Conversely, whereas the higher experimental slopes for PC in Fig. 2 lie on the S_{cal} vs. pH curve calculated for $K = 6$ M^{-1}, the lower slopes undergo a rather abrupt fall, thus deviating from this curve.

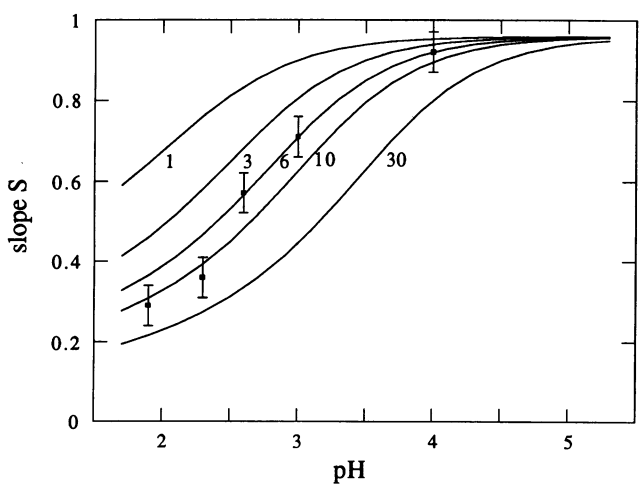

FIGURE 2 S_{exp} values at $E = -0.5$ V against pH for PC monolayers deposited on mercury. The solid curves are S_{cal} vs. pH plots calculated as described in Appendix I for $C_{ht} = 1.7$ $\mu F\ cm^{-2}$, $\sigma_{max} = 25$ $\mu C\ cm^{-2}$, and $\Delta\phi - \chi = -0.3$ V upon assuming that the protonation of the phosphate group takes place according to a Langmuir isotherm; numbers on each curve denote K values in mol^{-1}.

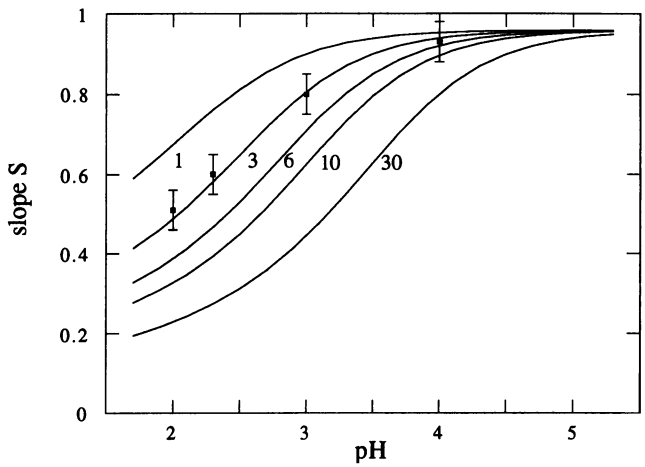

FIGURE 3 S_{exp} values at $E = -0.5$ V against pH for PE monolayers deposited on mercury. The solid curves are S_{cal} vs. pH plots calculated as in Fig. 2.

The intrinsic log K value of ≈0.5 for PE as estimated from Fig. 3 is less than that for free $H_2(PO_4)^-$ ion (2.12) or for free dimethyl phosphate (1.22) (Osterberg, 1962). The reason for this behavior is to be found in the conformation of the zwitterionic polar head of PE, which is generally regarded as approximately parallel to the plane of the lipid layer (Hauser and Phillips, 1979). This arrangement leads to intermolecular electrostatic as well as H-bonding interactions between adjacent ammonium and phosphate groups, with a resulting stabilization of the unprotonated form of the latter groups. Our intrinsic log K value for PE is also less than the apparent log K value for films of 1,2-didodecyl PE (1.7) (Seddon et al., 1983), although an apparent log K value <1 was reported for films of 1,2-dilauroyl PE (London and Feigenson, 1979). At any rate, our results cannot be regarded as contrasting with the above apparent log K values, because apparent values are constantly somewhat greater than the intrinsic ones.

The intrinsic log K value of ≈0.8 for PC as estimated from the upper portion of the S_{exp} vs. pH plot in Fig. 2 is only slightly greater than that for PE, suggesting that the zwitterionic polar head of PC is arranged approximately parallel to the plane of the monolayer just as in PE. The slightly higher log K value for PC may possibly be justified by the fact that adjacent P-N dipoles can only interact electrostatically, whereas in PE monolayers H-bonding interactions are also operative. A coplanar arrangement of the choline segment, with the P-N dipole approximately parallel to the plane

of the PC layer, was actually proposed on the basis of neutron diffraction profiles of PC bilayers (Worcester, 1976; Buldt et al., 1978).

An attempt to explain the deviations of the S_{exp} vs. pH plots for PC in Fig. 2 from the S_{cal} vs. pH plots was made by accounting for discreteness-of-charge effects, which are disregarded in the Langmuir isotherm. To this end, Levine's isotherm (Levine et al., 1962, 1965) was employed (see Eqs. A8–A14 in Appendix I). In practice, this amounts to multiplying the hydrogen ion concentration on the surface of the lipid monolayer by a factor $\exp(-a\sigma_1)$, where a is a positive parameter that takes values of the order of 1 cm^2 μC^{-1}. Fig. 4 shows a series of S_{cal} vs. pH curves calculated on the basis of Levine's isotherm for $K' = 6$ M^{-1} and for different positive values of the parameter a. It can be seen that a gradual increase in a merely shifts the S_{cal} vs. pH curve for $a = 0$, corresponding to Langmuirian behavior, towards increasing pH values. In view of the behavior of the S_{exp} vs. pH curve for PC, we must conclude that discreteness-of-charge effects cannot account for such a behavior.

We are lead, therefore, to conclude that the rather abrupt decrease in the slope S_{exp} of the $1/C$ vs. $1/C_{d,0}$ plots for PC as pH is decreased below 2.5 is due to some slight conformational change in the PC polar head. In this connection, it should be noted that the average conformation of the polar head of PC is reported to be sensitive to the environmental conditions (Hauser and Phillips, 1979), contrary to that of PE, which is constantly characterized by the P-N dipole parallel to the lipid layer. Thus, the ^{1}H, ^{31}P, and ^{13}C NMR spectra of PC vesicles in the presence of the transition metal ions of the lanthanides seem to point to a conformation of the polar group of PC in the presence of these polyvalent cations with the the P-N dipole oriented at about 45° to the bilayer plane (Hauser et al., 1976). That some conformational changes of the PC polar head involve only slight changes in Gibbs energy is suggested by deuteron magnetic resonance spectroscopic measurements on PC deuterated on the polar head (Gally et al., 1975). These measurements indicate that the conformation of the choline group is not fixed in space, but rather undergoes rapid angular oscillations of various degrees of restrictions; in particular, a rotation about the OC-CN bond is sufficient to produce a passage from an orientation of the P-N dipole parallel to the bilayer plane to an orientation at about 45° to this plane. Now, a rotation of the P-N dipole from a parallel to a tilted orientation with the negative (P) end of the dipole towards the hydrocarbon tail region creates a negative potential difference that attracts positive ions towards the innermost portion of the polar head region the more the higher their positive charge is. In this respect, a conformation with tilted P-N dipoles may be energetically favored over that with parallel P-N dipoles when in the presence of polyvalent cations that may penetrate the polar head region.

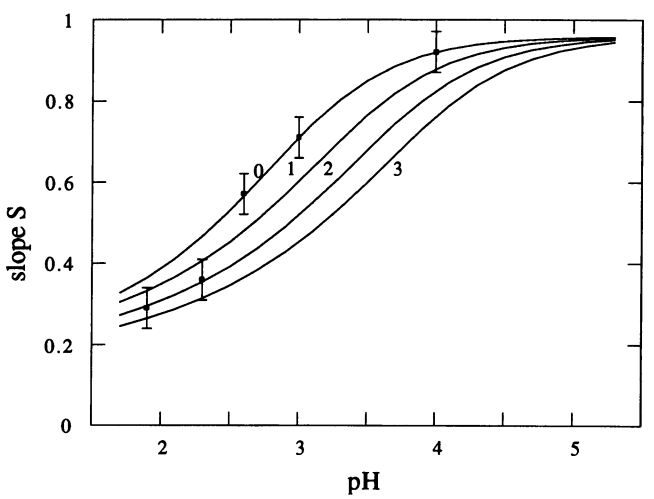

FIGURE 4 The solid curves are S_{cal} vs. pH plots calculated as described in Appendix I for $K = 6$ M^{-1}, $C_{ht} = 1.7$ μF cm^{-2}, $\sigma_{max} = 25$ μC cm^{-2}, and $\Delta\phi - \chi = -0.3$ V upon assuming that the protonation of the phosphate group takes place according to a Levine isotherm; numbers on each curve denote a values in cm^2 μC^{-1}. For comparison, the same S_{exp} values for PC as in Fig. 2 are also reported against pH.

It is well known that a two-dimensional lattice of point dipoles in the absence of external electric fields attains a minimum internal energy when the dipoles are oriented parallel to the lattice plane in a (positive pole)-to-(negative pole) configuration (Guidelli, 1990); if an increasing external electric field normal to the lattice plane is applied, the network of dipoles will ultimately collapse due to the relaxation of the attractive dipole-dipole electrostatic interactions as soon as a sufficiently high number of dipoles is forced to align along the direction of the external field. A similar effect, albeit less dramatic, may be expected for the parallel P-N dipoles of the PC layer: as an incipient protonation of the phosphate group begins to convert a number of P-N zwitterions into $-N(CH_3)_3^+$ cations, the network of parallel P-N dipoles starts to be undermined. As a result, the residual P-N dipoles will tend to assume a tilted orientation which, by creating a favorable potential difference across the polar head region, will cause the protons to be attracted towards the innermost portion of this region and to protonate the phosphate groups there. This may explain the relatively rapid decrease in the slope S_{exp} of the $1/C$ vs. $1/C_{d,0}$ plots for PC with a decrease in pH below 2.5, as shown in Fig. 2. It should be noted that the charge density σ_1 on the PC monolayer at pH 2.5 as estimated from Eq. A3 for $K = 10$ M^{-1} and $c = 0.1$ M equals 0.66 μC cm^{-2} and, hence, corresponds to the protonation of only 2.6% of the PC molecules.

PS and PS-PC monolayers

The points in Figs. 5–7 are the average slopes S_{exp} of the experimental $1/C$ vs. $1/C_{d,0}$ plots for pure PS and for 15 and 7 mol% PS-PC mixtures, as obtained by least-squares fitting of these plots to a straight line for 0.01 M $\leq c \leq$ 0.1 M; these points are plotted against pH. In examining these curves, we must consider that the GC theory predicts a very rapid increase in C_d with an increase both in c and in the absolute value $|\sigma_1|$ of the charge density on the lipid monolayer. In practice, $1/C_d$ becomes entirely negligible with respect to

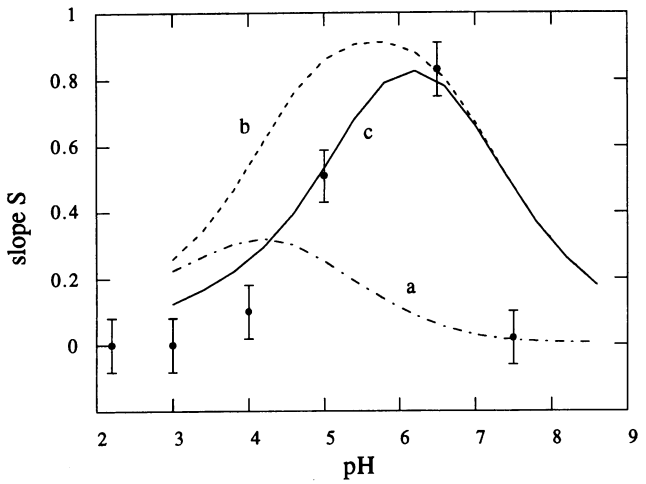

FIGURE 5 S_{exp} values against pH for pure PS monolayers deposited on mercury. The S_{cal} vs. pH curves *a*, *b*, and *c* were calculated as described in Appendix II for $K = 6\ M^{-1}$, $C_{ht} = 1.7\ \mu F\ cm^{-2}$, $\sigma_{max} = 20\ \mu C\ cm^{-2}$, $\Delta\phi - \chi = -0.2$ V and for: $K_1 = 5 \times 10^5\ M^{-1}$, $K_2 = 2 \times 10^2\ M^{-1}$, $K_M = 5\ M^{-1}$ (*a*); $K_1 = 5 \times 10^8\ M^{-1}$, $K_2 = 2 \times 10^2\ M^{-1}$, $K_M = 5\ M^{-1}$ (*b*); $K_1 = 5 \times 10^8\ M^{-1}$, $K_2 = 2 \times 10^3\ M^{-1}$, $K_M = 5\ M^{-1}$ (*c*).

$1/C_m$ for $|\sigma_1| \geq 2\ \mu C\ cm^{-2}$ within the limits of experimental error; when this is the case, the experimental plots of $1/C$ vs. $1/C_{d,0}$ will exhibit a slope $S_{exp} \approx 0$. The passage of S_{exp} for pure PS in Fig. 5 from zero to a maximum value of 0.85 and then back to zero in passing from pH 7.5 to pH 3 must be interpreted, therefore, as a passage of the charge density σ_1 of the PS monolayer from a slight negative to a slight positive value over this pH range. By analogous considerations, the fact that S_{exp} for the two PS-PC mixtures is practically pH-independent and greater than zero over the pH range 7–10 (see Figs. 6 and 7) indicates that under these conditions the ionizible groups of PS are not affected by pH and impart to the mixed monolayer a charge density that is less negative than $-2\ \mu C\ cm^{-2}$. Taking into account that the charge density of the 15 and 7 mol% PS-PC mixtures would be equal to -3 and $-1.4\ \mu C\ cm^{-2}$ for $N = 2 \times 10^{-10}$ mol cm^{-2} if all PS molecules bore a single negative charge, this implies that the K^+ ions bind strongly to the PS polar heads. As distinct from PS, phosphatidic acid (PA) yields slopes S_{exp} of $1/C$ vs. $1/C_{d,0}$ plots that are vanishingly small over the pH range 4–9.

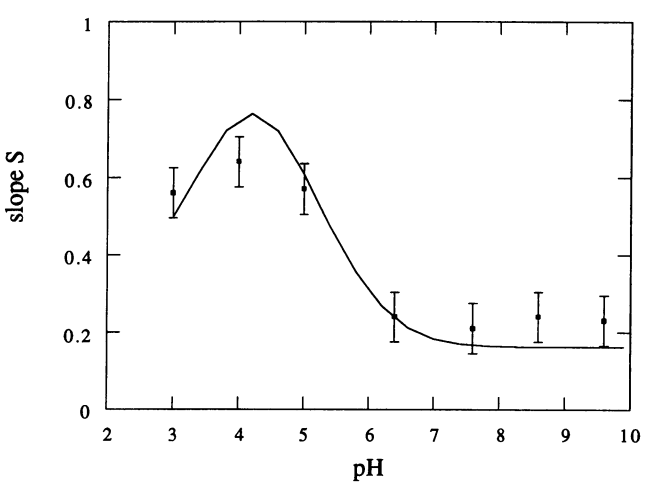

FIGURE 6 S_{exp} values against pH for 15 mol% PS-PC monolayers deposited on mercury. The S_{cal} vs. pH solid curve was calculated as described in Appendix II for $K = 6\ M^{-1}$, $K_1 = 5 \times 10^5\ M^{-1}$, $K_2 = 2 \times 10^2\ M^{-1}$, $K_M = 5\ M^{-1}$ and for the same C_{ht}, σ_{max}, and $\Delta\phi - \chi$ values as in Fig. 5.

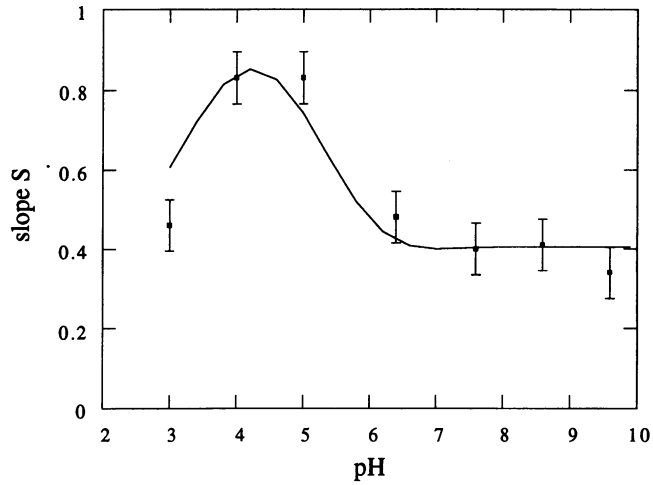

FIGURE 7 S_{exp} values against pH for 7 mol% PS-PC monolayers deposited on mercury. The S_{cal} vs. pH solid curve was calculated for the same parameters as in Fig. 6.

To draw quantitative conclusions from the plots in Figs 5–7, the diffuse-layer capacity C_d was calculated from the GC theory as a function of *c* and bulk pH upon assuming that protons bind to the phosphate groups of both PS and PC and to the carboxyl group of PS according to Langmuir isotherms (see Appendix II); the two consecutive protonation constants for PS are herein denoted by K_1 and K_2, whereas the single protonation constant for PC is still denoted by K. In view of the tendency of alkali metal ions to bind to PS (Tsui et al., 1986; Eisenberg et al., 1979), potassium ions were assumed to bind to PS according to a Langmuir isotherm, with a binding constant K_M. Changes in surface pH with varying *c* at constant bulk pH and applied potential were adequately taken into account in the calculations.

The average slopes S_{cal} of $1/C_d$ vs. $1/C_{d,0}$ plots at variable *c* and constant pH, calculated as described in Appendix II at different pH values for pure PS and for the two PS-PC mixtures, were fitted to the average slopes S_{exp} of the corresponding experimental $1/C$ vs. $1/C_{d,0}$ plots over the pH range 3–10. Such a fitting allows an estimate of the various equilibrium constants K_1, K_2, and K_M. The protonation constant K for PC was set equal to the value previously determined from pure PC monolayers, namely $6\ M^{-1}$. The fitting of the S_{cal} vs. pH curves to the S_{exp} vs. pH plots for the 15 and 7 mol% PS-PC mixtures was carried out for $0.01\ M \leq c \leq 0.1$ M using identical values of the equilibrium constants for both mixtures. The best fit, albeit not entirely satisfactory, was achieved for $K_1 \approx 5 \times 10^5\ M^{-1}$, $K_2 \approx 2 \times 10^2\ M^{-1}$ and $K_M \approx 5\ M^{-1}$, yielding the solid curves in Figs. 6 and 7. At pH > 7, the only constant that influences the fitting is K_M,

because the two protonation constants K_1 and K_2 are only required to be small enough to exclude the protonation of the carboxyl and phosphate groups. In particular, with this K_2 value the nonbonded negatively charged fraction of PS in 0.11 M KCl is estimated at 44 and 51% in the 15 and 7 mol% PS-PC mixtures, respectively. For an acceptable fitting of the S_{cal} vs. pH curve to the S_{exp} vs. pH plot of pure PS in Fig. 5, the K_1 and K_2 values used for the two PS-PC mixtures cannot be retained. The best possible fit is indeed obtained for $K_1 \approx 5 \times 10^8$ M^{-1} and $K_2 \approx 2 \times 10^3$ M^{-1}; with these values of the two protonation constants, the effect of the K^+ binding to the PC polar heads at pH < 8 is minor, such that almost identical S_{cal} vs. pH curves are obtained for $K_M = 0$ and for $K_M = 5$ M^{-1}. The logarithms of the binding constants that provide the best fitting between S_{exp} vs. pH and S_{calc} vs. pH curves for the phospholipids examined in this work are summarized in Table 1.

The intrinsic K_1 and K_M values herein obtained under the present experimental conditions are at variance with those reported in the literature. Literature measurements of the intrinsic $pK_a \equiv \log K_1$ values for PS have been carried out on vesicles (Tsui et al., 1986), dispersions (Mac Donald et al., 1976), monolayers (Ohki and Kurland, 1981), or BLMs (Matinyan et al., 1985) of PS either alone (Mac Donald et al., 1976; Ohki and Kurland, 1981) or in mixtures with PC (Tsui et al., 1986; Matinyan et al., 1985). The measurements span from the rather indirect calorimetric or turbidity measurements (Mac Donald et al., 1976) to surface potential measurements by means of a radioactive electrode (Ohki and Kurland, 1981), transmembrane potential measurements by the potentiodynamic technique (Matinyan et al., 1985), ζ potential measurements by electrophoresis (Mac Donald et al., 1976), surface ionic potential measurements by spin labels (Tsui et al., 1986), and proton binding measurements by acid-base titration (Tsui et al., 1986). Intrinsic pK_a values of 2.1 (Matinyan et al., 1985), 2.7 (Mac Donald et al., 1976), 3.6 (Tsui et al., 1986) and 4 (Ohki and Kurland, 1981) have been reported for the carboxyl group, whereas the protonation constant for the phosphate group was found to be too low to be estimated over the accessible pH range.

Our anomalously high values of K_M and, even more so, of K_1 can be explained if the negative group responsible for these values is located deep inside the polar head region with respect to the bathing solution. This generates an additional negative potential difference between the position of this group and the bathing solution, which will move additional protons from the solution across the polar head region; an equilibrium situation will then be rapidly attained corresponding to a high degree of protonation of the negative group at hand and, hence, to a low charge density σ_1 on the PS monolayer. A model that accounts qualitatively for the above behavior by combining the diffuse layer effects expressed by the GC theory with the electrostatic effects produced by a different location of the three ionizable groups of PS within the polar head region will be the subject of a further note.

In view of the above considerations, the anomalous K_1 and K_M values must be regarded as "formal," in that they depend on the particular conformation of the polar head. Nonetheless, they are not "apparent" but rather "intrinsic" values, because diffuse-layer effects upon the surface concentrations of H^+ and K^+ ions are accounted for in their calculation. These values are quite likely to be ascribed to the phosphate group, in view of its closer vicinity to the hydrocarbon tails. Moreover, our value 5 M^{-1} for the binding constant K_M being higher than the literature values of ≈0.6–1 M^{-1} (Tsui et al., 1986; Eisenberg et al., 1979) can be more reasonably justified for the phosphate group than for the carboxyl group. In fact, although the question of whether the phosphate group, or the carboxyl group, or both, are involved in the binding of metal ions is still an argument of debate, there is some indirect evidence in favor of the hypothesis that the phosphate group in PS is the main binding site for cations. This evidence relies on the notable similarities between the binding of Ca^{2+} ion to PS monolayers and that to phosphatidylinositol monolayers at different surface pressures (Dawson and Hauser, 1970). Once our K_1 and K_M values are ascribed to the phosphate group, our log K_2 value (≈3.3 in pure PS and ≈2.3 in PS-PC mixtures) is to be ascribed to the carboxyl group, and is indeed in fairly good agreement with the literature values (Tsui et al., 1986; Matinyan et al., 1985; Mac Donald et al., 1976; Ohki and Kurland, 1981) for this group.

The discrepancies between our results and those in the literature indicate that the conformation of the polar head of PS and the intrinsic protonation constants of the ionizable groups for such a conformation are strongly sensitive to the experimental conditions. This also implies that some conformations of PS are characterized by quite similar Gibbs energies. The difference in behavior between PS on one hand and PC and PE on the other can be explained by considering that with the latter two lipids a conformation with the P-N dipoles parallel to the lipid layer and aligned in a (negative pole)-to-(positive pole) configuration is strongly favored from an electrostatic point of view. Conversely, it is difficult to envisage a conformation of the PS polar heads with two negative and one positive charge on the same plane parallel to the lipid layer, which may be as electrostatically favored as the conformation assumed by zwitterionic lipids: a conformation with the phosphate group deep inside the polar head region and a C-N dipole roughly parallel to the lipid plane and in direct contact with the aqueous phase may well

TABLE 1 Intrinsic binding constants of PC, PE, and PS with protons and of PS with K^+ ions

			PS		
			mol% PS-PC		
	PC	PE	100	15	7
pK_a	0.8	0.5			
pK_{a1}			8.7	5.7	5.7
pK_{a2}			3.3	2.3	2.3
pK_M				0.7	0.7

have a comparable Gibbs energy. That the anomalous behavior of PS under our experimental conditions is related to the presence of three ionizable groups is also suggested by the observation that PA, whose polar head has only one phosphate group, is characterized by $S_{exp} \approx 0$ and, hence, by a charge density σ_1 that is certainly more negative than -2 μC cm^{-2} at pH > 4.

The intrinsic pK_a of an ionizable group in a lipid is generally believed to be a fundamental property of the lipid (Tsui et al., 1986) that should not depend on experimental conditions such as the pH and the ionic strength of the medium, the amount of charged lipids, or the area per head group of the lipids in the membrane. Nonetheless, experimental results that contradict this prediction have been sometimes reported when using the GC theory to estimate the surface ionic potential ψ and, hence, the surface pH (Matinyan et al., 1985); these discrepancies have been ascribed to secondary pH effects such as a pH-dependent reorientation of the polar heads of the lipid, resulting in a drastic change of the surface dipole potential χ (Matinyan et al., 1985). A pH-dependent change in χ may indeed affect measurements of changes ΔV in the transmembrane potential as carried out, say, by the vibrating electrode or by potentiodynamic techniques. However, a point that has not been sufficiently emphasized is that a reorientation of the polar heads may also cause a change in the intrinsic pK_a values of their ionizable groups, independent of whether this reorientation originates from a bulk pH change or by some other factor. When this is the case, discrepancies in the intrinsic pK_a values obtained under different experimental conditions are to be expected even by using techniques that are exclusively sensitive to changes in the surface ionic potential ψ. In fact, the forces acting among the lipid molecules of a self-organized film are generally cooperative in nature. Hence, any external factors causing a drastic change in the area per lipid polar head (e.g., a mechanical compression or expansion of the film) or in the composition of the nearest neighbors of a given lipid molecule (e.g., a change in composition of a mixture of different lipids) may induce a collective reorientation of the polar heads and a resulting change in the intrinsic pK_a values of their ionizable groups. Analogous effects may be produced by a decrease in pH causing the breaking of intermolecular bonds (dipole-dipole interactions or H-bonds) between neighboring polar heads as a consequence of the partial protonation of some of their ionizable groups. Thus, the decrease in our K_1 value for PS by about three orders of magnitude in passing from pure PS to PS-PC mixtures with a low PS content (see Figs. 5–7) must be ascribed to some rearrangement of the PS polar heads induced by the neighboring PC molecules. Analogously, the decrease in reactivity of the amino group of PE against 2,4,6-trinitrobenzenesulphonic acid or formaldehyde when passing from the pure PE to PE-PC mixtures has been explained by a resulting decrease in intermolecular ammonium-phosphate interactions between contiguous PE molecules due to the interdispersion of PE with PC molecules (Papahadjopoulos and Weiss, 1969); such a breaking of intermolecular bonds may well affect the pK_a values of the ionizable groups involved. By the same token, the surface potential versus pH plots for PC show a progressive shift in the onset of phosphate protonation towards lower pH values in passing from dipalmitoyl PC to egg PC and from this to dioleoyl PC (Shah and Schulman, 1967), which is the reverse of the order of their intermolecular spacing in monolayers; this suggests that the pK_a of the phosphate group is related to intermolecular spacing and, hence, to unsaturation of the fatty acyl chains of PC molecules.

In view of the sensitivity of the conformation of PS and of the resulting acidity of its ionizable groups to the experimental conditions, it is interesting to examine the different conditions under which the intrinsic and apparent pK_a values of this lipid have been determined.

In phase separation methods (Cevc et al., 1981), the shifts in the phase transition temperature of PS dispersions with varying pH are caused by some changes in molecular packing brought about by the protonation of the acid groups; hence, these phase transitions may well be accompanied by a drastic conformational change in the polar headgroups of the lipids and by a resulting change in their pK_a values.

From ζ potential measurements of PS dispersions in 0.1 M NaCl by electrophoresis an isoelectric point was reported at pH 1.2 (Abramson et al., 1964). Although this result points unambiguously to a negative charge on the PS polar head in neutral media, PS dispersions consist of micelles with an average radius of 200 Å (Abramson et al., 1964), single-layered (Mac Donald et al., 1976), or multilamellar vesicles (Eisenberg et al., 1979) in which the spacing between neighboring polar heads and the state of compression may differ appreciably from those in flat bilayers or in monolayers. Analogous considerations apply to ψ measurements carried out on PS dispersions using fluorescent probes (Eisenberg et al., 1979) or spin labels (Tsui et al., 1986), apart from a major difference: although electrophoretic measurements are sensitive to the average electrostatic potential at the shear plane, ionic probes are sensitive to the local electrostatic potential that their adsorption in the polar head region may contribute to alter to a notable extent.

Surface potential measurements (Bangham and Papahadjopoulos, 1966; Papahadjopoulos, 1968; Standish and Pethica, 1968; Shah and Schulman, 1965; Lakhdar-Ghazal et al., 1983) are often performed at the collapse pressure, which imposes on the lipid monolayer a state of compression (e.g., 50 $Å^2$ per PS molecule (Papahadjopoulos, 1968)) that is higher than that commonly realized with BLMs. Moreover, the changes $\Delta\Delta V$ in the transmembrane potential ΔV estimated either from surface potential measurements on monomolecular films or from potentiodynamic measurements on BLMs (Matinyan et al., 1985) consist of two contributions, namely a contribution from the change $\Delta\psi$ in the surface ionic potential and a contribution from the change $\Delta\chi$ in the surface dipole potential; in examining $\Delta\Delta V$ vs. pH curves, the latter contribution is usually disregarded (Matinyan et al., 1985; Ohki and Kurland, 1981; Standish and Pethica, 1968) or else is invoked and justified on the basis of qualitative considerations (Lakhdar-Ghazal et al., 1983; Betts and

Pethica, 1956). Even assuming that $\Delta\chi$ is independent of the pH of the solution, experimental $\Delta\Delta V$ vs. pH plots differ from $\Delta\psi$ vs. pH plots by a constant; hence, a comparison of these experimental plots with $\Delta\psi$ vs. pH plots calculated on the basis of the GC theory is not completely unambiguous. Thus, the plateau region interposed between an upswing at low pH and a downswing at high pH in the $\Delta\Delta V$ vs. pH plots for PE (Papahadjopoulos, 1968; Standish and Pethica, 1968) and PC (Matinyan et al., 1985) is associated with a very small charge density σ_1 on the lipid, whereas an entirely analogous plateau for PS (Matinyan et al., 1985; Papahadjopoulos, 1968; Standish and Pethica, 1968) is associated to a constant charge density of one electron per PS molecule. Fig. 8 shows that the qualitative features of these plots for PS can also be simulated using our K_1, K_2, and K_M values, which predict a small charge density over the plateau region.

As concerns the technique adopted herein, it is directly sensitive to the absolute value of the charge density σ_1 on the lipid monolayer deposited on the Hg electrode, provided $|\sigma_1|$ does not exceed 1.5–2 μC cm^{-2}. In view of the low differential capacity of the lipid film, at constant applied potential the charge density σ_M on mercury varies by only a few tenths of a μC cm^{-2} with varying the electrolyte concentration over the accessible range. Moreover, the small AC signal employed in differential capacity measurements (10 mV peak-to-peak) causes σ_M to change by less than 0.02 μC cm^{-2}; this permits us to exclude an appreciable change in the surface dipole potential χ during these measurements. Thus, if the dipole moment μ of the dipole consisting of the charged carboxyl group and of the charged ammonium group is estimated at 6 Debye, the change in χ involved in its passage from an orientation parallel to the monolayer to a vertical orientation will be equal to $4\pi N N_A \mu/\epsilon_1$, where $N \approx 2 \times 10^{-10}$ mol cm^{-2} is the density of PS in the monolayer, N_A is Avogadro's number and ϵ_1 is the dielectric constant of the polar head region. If we set $\epsilon_1 = 2$, the change in χ will be equal to 1.36 V and will involve a charge flow of about (2 μF cm^{-2}) $\times$ 1.36 V = 2.7 μC cm^{-2}; if this charge flows over a potential range of 0.1 V, it will give rise to a pseudocapacitance peak of about 27 μF cm^{-2}. Incidentally, this is the order of magnitude of the differential capacity peak that delimitates the potential region of the capacity minimum over which the lipid monolayer is self-organized and impermeable to ions. Hence, over this region no appreciable reorientation of the polar groups takes place as a consequence of the AC signal.

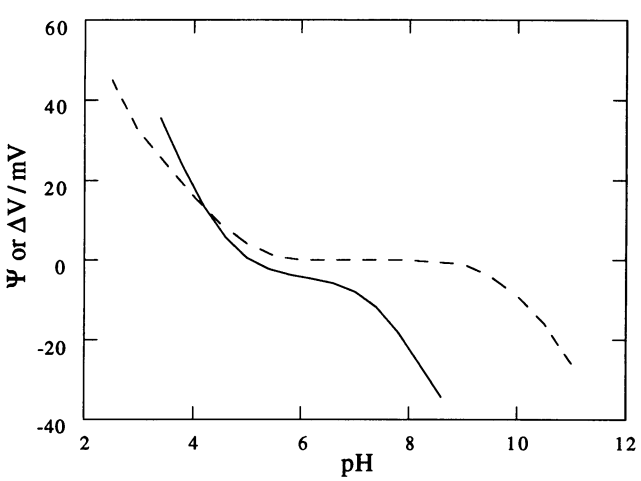

FIGURE 8 The solid curve is a ψ vs. pH plot calculated as described in Appendix II for the same parameters as in Fig. 5, curve *c*. The dashed curve is the ΔV vs. pH plot for a PS monolayer at the air/solution interphase as reported in Bangham and Papahadjopoulos (1966).

Our results for PS and PS-PC monolayers deposited on mercury may or may not be entirely consistent with those obtained on vesicles, BLMs or monolayers at the air/water interface, where the electric field acting on the hydrocarbon tails and the paramaters related to intermolecular spacing and state of compression may be somewhat different. In particular our experimental results were obtained under conditions in which the area per lipid molecule remains practically constant during each series of measurements, whereas this requirement is not generally satisfied with BLMs or vesicles. Nonetheless, the differences between our experimental conditions and those realized with other biomimetical membranes cannot be too large because our results with PC, PE, and PA monolayers deposited on mercury are in substantial agreement with those reported in the literature for different films of these zwitterionic and acidic phospholipids. We must conclude, therefore, that self-organized films of PS may assume at least two different conformations of the polar head with similar Gibbs energies but quite different acidities of the ionizable groups. The strong dependence of the acidic properties of the polar groups of PS (and possibly of other components of biomembranes such as proteins) upon conformation may have some relevance to the molecular mechanisms of nerve excitation.

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APPENDIX I

The potential difference $\Delta\phi$ across the whole interface is given by

$$\Delta\phi = \frac{\sigma_M}{C_{ht}} + \chi + \psi, \qquad \text{(A1)}$$

where σ_M is the charge density on the metal surface, χ is the surface dipole potential, ψ is the surface ionic potential, and $C_{ht} \approx 1.7$ μC cm^{-2} is the differential capacity of the hydrocarbon tail region, which can be regarded as almost coincident with that, C_m, of the whole lipid monolayer. The $\Delta\phi$ value is not experimentally accessible, but it differs from the applied potential E by a constant that depends exclusively upon the choice of the reference electrode; hence $\Delta\phi$ is constant at constant E. If we assume that χ remains approximately constant at constant E with varying the composition of the solution and we use the GC theory to estimate ψ, independent

measurements of σ_M (in preparation) permit us to ascribe a value of about -0.3 V to the quantity $(\Delta\phi - \chi)$ at $E = -0.5$ V by using Eq. A1.

Let us assume that protons bind to the phosphate moiety of PC according to a Langmuir isotherm, with a protonation constant K that is independent of the electric field within the hydrocarbon tail region. The charge density σ_1 on the polar heads is then given by

$$\sigma_1 = \sigma_{max}\frac{Kc_{H^+}y^2}{1 + Kc_{H^+}y^2}, \qquad \text{with} \quad y \equiv \exp\left(-\frac{F\psi}{2RT}\right). \tag{A2}$$

Here c_{H+} and $c_{H+}y^2$ are the hydrogen ion concentrations in the bulk solution and on the lipid surface respectively, whereas σ_{max} is the maximum value of σ_1, which corresponds to a proton charge per cross sectional area of the phospholipid. Setting this area equal to 65 Å^2, σ_{max} equals 20 μC cm^{-2}. Application of the GC theory yields

$$\begin{aligned}\sigma_M + \sigma_1 &= \left(\Delta\phi - \chi + \frac{2RT}{F}\ln y\right)C_{ht} + \sigma_{max}\frac{Kc_{H^+}y^2}{1 + Kc_{H^+}y^2} \\ &= A\left(\frac{1}{y} - y\right),\end{aligned} \tag{A3}$$

with

$$A \equiv \sqrt{\frac{RT\epsilon(c_{M+} + c_{H+})}{2\pi}}.$$

Here c_{M+} is the bulk concentration of the 1,1-valent electrolyte used to vary the ionic strength and $\epsilon = 78$ is the dielectric constant of the solvent. This relationship expresses y implicitly as a function of K, C_{ht}, σ_{max}, $(\Delta\phi - \chi)$, c_{H+}, and c_{M+}, and is readily solved using the Newton-Raphson iterative procedure.

The actual diffuse-layer capacity C_d is expressed by the equation

$$\frac{1}{C_d} \equiv \frac{d\psi}{d\sigma_M} = \frac{d\psi}{d(\sigma_M + \sigma_1)}\left(1 + \frac{d\sigma_1}{d\sigma_M}\right), \tag{A4}$$

where $d\psi/d(\sigma_M + \sigma_1)$ is directly provided by the GC theory

$$\frac{d\psi}{d(\sigma_M + \sigma_1)} \equiv \frac{1}{C^{GC}} = \left[\frac{FA}{2RT}\left(y + \frac{1}{y}\right)\right]^{-1}. \tag{A5}$$

Having assumed that K is independent of σ_M, σ_1 depends upon σ_M only through the ψ potential. Hence, taking Eq. A2 into account, we have

$$\frac{d\sigma_1}{d\sigma_M} = \frac{d\sigma_1}{d\psi}\frac{d\psi}{d\sigma_M} = -\frac{F}{RT}\frac{\sigma_{max}}{C_d}\frac{Kc_{H^+}y^2}{(1 + Kc_{H^+}y^2)^2}. \tag{A6}$$

Substituting $d\sigma_1/d\sigma_M$ from Eq. A6 into Eq. A4 and rearranging terms, we get

$$\frac{1}{C_d} = \frac{1/C^{GC}}{1 + \frac{F}{RT}\frac{\sigma_{max}}{C^{GC}}\frac{Kc_{H^+}y^2}{(1 + Kc_{H^+}y^2)^2}}, \tag{A7}$$

where C^{GC} is expressed by Eq. A5.

The Langmuir isotherm combined with the GC correction for the hydrogen ion concentration in its position of closest approach to the lipid monolayer (the so called outer Helmholtz plane $x = d$, where the distance x is measured from the electrode surface plane) does not account for the local electrostatic potential ψ_{loc} at the binding site of the proton within the polar head region. This effect can be accounted for by using an isotherm derived by Levine et al. (1962, 1965) for the case of ionic specific adsorption. This isotherm can be applied to the present situation by considering that each neutral polar head constitutes an "adsorption site" that can only be occupied by a single "adsorbing" hydrogen ion. In this case Levine's isotherm takes the form

$$\frac{\sigma_1}{\sigma_{max} - \sigma_1} = Kc_{H^+}\exp\left(-\frac{F}{RT}\psi_{loc}\right), \tag{A8}$$

with

$$\psi_{loc} = \psi_d + \frac{4\pi}{\epsilon_1}\gamma(\sigma_M + \sigma_1) - \frac{4\pi}{\epsilon_1}\frac{\beta\gamma}{d}g\sigma_1. \tag{A9}$$

The first two terms on the right-hand side of Eq. A9 express the mean electrostatic potential at the adsorption site, whereas the last term is the "perturbation" potential due to the "self-atmosphere" of the protonated polar head and of its induced images in the electrode surface plane $x = 0$ and in the outer Helmholtz plane $x = d = \beta + \gamma$; β and γ are the distances of the center of the positive charge within the polar head from the electrode surface plane and from the outer Helmholtz plane, respectively; ϵ_1 is the dielectric constant of the phospholipid monolayer; g is a dimensionless factor, which is a function of β, γ, and the radius of the cross sectional area of the polar head and takes values very close to unity. Setting $g = 1$ in Eq. A9 as a good approximation and expressing the differential capacity C_{ht} by the equation for a parallel plate capacitor ($C_{ht} \approx \epsilon/4\pi d$), Eqs. A8 and A9 yield

$$\frac{\sigma_1}{\sigma_{max} - \sigma_1} = Kc_{H^+}y^2\exp(-a\sigma_1 - b\sigma_M), \tag{A10}$$

where

$$a \equiv \frac{F}{RTC_{ht}}\left(\frac{\gamma}{d}\right)^2; \qquad b \equiv \frac{F}{RTC_{ht}}\frac{\gamma}{d}. \tag{A11}$$

As an example, if γ and d are given the reasonable values 3 and 25 Å respectively, a and b are equal to 0.33 and 2.75 cm^2 μC^{-1}.

In view of Eq. A1 and of the first and third member of Eq. A3, Eq. A10 takes the form

$$\sigma_1 = \sigma_{max}\frac{z}{1 + z}, \tag{A12}$$

with

$$\begin{aligned}z &\equiv K'c_{H^+}y^{2[1+(a-b)RTC_{ht}/F]}\exp\left[-aA\left(\frac{1}{y} - y\right)\right] \\ &\approx K'c_{H^+}y^2\exp\left[-aA\left(\frac{1}{y} - y\right)\right]\end{aligned} \tag{A13}$$

and

$$K' \equiv K\exp[(a - b)(\Delta\phi - \chi)C_{ht}].$$

The approximation in Eq. A13 is justified because $a - b$ is of the order of a few cm^2 μC^{-1}, whereas RTC_{ht}/F equals 0.044 μC cm^{-2}. Following the same procedure as in Eqs. A2–A7 with Eq. A10 replacing Eq. A2, C_d is now given by

$$\frac{1}{C_d} = \frac{1/C^{GC}}{1 + \frac{F}{2RT}\frac{\sigma_{max}}{C^{GC}}\frac{z}{(1 + z)^2}\left(2 + \frac{aA}{y} + aAy\right)}. \tag{A14}$$

APPENDIX II

Let us consider an x mol% PS-PC monolayer and let us assume for simplicity that the density N of the phospholipid molecules in the monolayer is the same both for a pure PC monolayer ($x = 0$) and for a pure PS monolayer ($x = 1$). At pH < 9, the amino group of PS can be regarded as fully protonated. Hence, PS will be considered to be present in the monoanionic form PS^-, in the neutral forms PSH and PSK and in the monocationic form PSH^+_2, whereas PC will be considered to be present in the neutral form PC and in the protonated form PCH^+. Let us define the equilibrium constants:

$$K = \frac{[PCH^+]}{[PC]c_{H^+}y^2}; \qquad K_1 = \frac{[PSH]}{[PS^-]c_{H^+}y^2};$$

$$K_2 = \frac{[PSH^+_2]}{[PSH]c_{H^+}y^2}; \qquad K_M = \frac{[PSK]}{[PS^-]c_{K^+}y^2},$$

with

$$y \equiv \exp\left(-\frac{F\psi}{2RT}\right).$$

Here the symbols within square brackets denote surface concentrations, ψ is the surface ionic potential, c_{H+} and c_{K+} are the bulk concentrations of the H^+ and K^+ ions, whereas $c_{H+}y^2$ and $c_{K+}y^2$ are the corresponding concentrations on the lipid surface. Upon assuming that the H^+ and K^+ ions bind to the polar heads according to Langmuir isotherms, the charge densities σ_{PC} and σ_{PS} due to the PC and PS polar heads are given by

$$\sigma_{PC} = (1-x)\sigma_{max}\frac{Kc_{H+}y^2}{1+Kc_{H^+}y^2};$$
$$\sigma_{PS} = x\sigma_{max}\frac{K_1K_2c_{H^+}^2y^4 - 1}{1+(K_1c_{H^+}+K_Mc_{K^+})y^2+K_1K_2c_{H^+}^2y^4}, \quad \text{(A15)}$$

with $\sigma_{max} \equiv$ FN. Application of the GC theory yields

$$\sigma_M + \sigma_{PC} + \sigma_{PS} = A(1/y - y), \quad \text{(A16)}$$

with

$$A \equiv \sqrt{\frac{RT\epsilon(c_{K^+}+c_{H^+})}{2\pi}}; \qquad \sigma_M \equiv \left(\Delta\phi - \chi + \frac{2RT}{F}\ln y\right)C_{ht}. \quad \text{(A17)}$$

Here σ_M is the charge density on the metal surface, $\epsilon = 78$ is the dielectric constant of the solvent, $\Delta\phi$ is the potential difference across the whole interphase, χ is the surface dipole potential, and $C_{ht} \approx 1.7\ \mu F\ cm^{-2}$ is the differential capacity of the hydrocarbon tail region. If we assume that χ remains approximately constant at constant E with varying the composition of the solution and we use the GC theory to estimate ψ, independent measurements of σ_M permit us to ascribe a value of about -0.2 V to the quantity $(\Delta\phi - \chi)$ at $E = -0.5$ V/*SCE*. Equation A16, with σ_{PC}, σ_{PS}, and σ_M given by Eqs. A15 and A17, expresses y implicitly as a function of K, K_1, K_2, K_M, $(\Delta\phi - \chi)$, C_{ht}, c_{H+} and c_{M+}, and is readily solved using the Newton-Raphson iterative procedure.

By the same procedure used in Appendix I, the diffuse-layer capacity C_d is given by

$$\frac{1}{C_d} \equiv \frac{d\psi}{d\sigma_M} = \frac{1/C^{GC}}{1 - \frac{1}{C^{GC}}\frac{d(\sigma_{PC}+\sigma_{PS})}{d\psi}},$$

with

$$\frac{1}{C^{GC}} \equiv \frac{d\psi}{d(\sigma_M+\sigma_{PC}+\sigma_{PS})} = \left[\frac{FA}{2RT}\left(y+\frac{1}{y}\right)\right]^{-1}$$

and

$$\frac{d(\sigma_{PC}+\sigma_{PS})}{d\psi} = -\frac{F}{RT}\sigma_{max}c_{H^+}y^2\left[\frac{(1-x)K}{(1+Kc_{H^+}y^2)^2} + x\frac{4K_1K_2c_{H^+}y^2 + BK_1K_2c_{H^+}y^4 + B/c_{H^+}}{(1+By^2+K_1K_2c_{H^+}^2+y^4)^2}\right].$$

Here $B \equiv K_1c_{H^+} + K_Mc_{K^+}$.

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