Westwick, J., Li, S. W., & Camp, R. D. (1989) *Immunol. Today 10*, 146-147.
Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., & Axel, R. (1979) *Cell 16*, 777-785.
Wolpe, S. D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D. G., Nguyen, H. T., Moldawer, L. L., Nathan, C. F., Lowry, S. F., & Cerami, A. (1988) *J. Exp. Med. 167*, 570-581.
Wood, W. I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spencer, S. A., Helmiss, R., Martin, J. L., & Baxter, R. C. (1988) *Mol. Endocrinol. 2*, 1176-1185.
Yoshimura, T., Matsushima, K., Oppenheim, J. J., & Leonard, E. J. (1987) *J. Immunol. 139*, 788-793.

Effect of Increased Lipid Packing on the Surface Charge of Micelles and Membranes†

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ABSTRACT: We have investigated the responsiveness of micelle and bilayer surfaces to changes in bulk pH through titrations, and to changes in lipid packing through the application of high hydrostatic pressure using two fluorescent, pH-sensitive surface probes. In micelles, the surface is very sensitive to bulk pH while in phosphatidylcholine and phosphatidic acid bilayers the surface charge changed little through a large pH region. Application of pressure on micelles causes proton dissociation due to the volume reduction achieved from the contraction of water around the charges (electrostriction). However, in bilayers, the effect of electrostriction is greatly reduced, most likely due to the energy needed to expand and hydrate the surface. The sign and amount of change in dissociation the probe undergoes with pressure depend on the initial degree of probe dissociation, which is in turn dependent on the particular surface rather than the charge of the lipid head groups comprising the bilayer. This finding may limit the use of fluorescent probes to determine the exact surface potential. By assuming the change in ΔV for proton dissociation from the probe is constant for a given pH, we can calculate the changes in local pH that occur under pressure relative to a neutral or uncharged system. In doing so, we find that the local pH around the probe in bilayers changes very little ($\sim$0.1 pH unit or less) in the first 2000 bars. Also, if pressure data are coupled with titration curves, we can determine the change that the bulk pH must undergo to produce the observed change in dissociation seen under pressure. Here, we find that raising the pressure from 1 to 2000 bar has no effect on dioleoylphosphatidylcholine bilayers whereas raising the pressure of dioleoylphosphatidic acid bilayers is equivalent to decreasing the bulk pH from 7 to 3. Although this appears to be a large effect, the actual change in surface charge in this pH region is quite low.

The physical state of a membrane bilayer is the result of the particular interactions occurring on the surface and in the interior. Since lipid packing may regulate the aggregation and function of integral membrane proteins, it is important to understand the role lipid–lipid interactions play in determining membrane properties. While previous work has mainly focused on the influence of hydrocarbon packing on the phase and fluidity of bilayers, there is very little information about how packing modulates the interactions between surface groups (Cevc, 1987). The goal of this study is to determine the effect of lipid packing on the surface charge.

In vivo, the ambient temperature and pressure surrounding a cell are relatively constant, and lipid packing is controlled by careful regulation of the composition. Here, packing will be varied by hydrostatic pressure since altering the membrane composition makes analysis on the chemical level difficult and varying the temperature produces changes that are due to a combination of both volume and kinetic energy. Many membrane systems have been investigated using high pressure [see Heremans (1982)], and it has been found that pressure increases the gel-to-liquid-crystal-phase transition by approximately 20 °C/kbar (1 bar = 1.013 atm). The effect of high hydrostatic pressure on bilayers surface has not yet been characterized (MacDonald, 1984).

While many naturally occurring lipid head groups have no net charge, many are negatively charged and have a tendency to repel each other. This repulsion could be partially relieved by hydration, counterion binding, and, where possible, hydrogen bonding. We are specifically interested in determining whether increasing chain packing through high pressure could destabilize the bilayer due to increased repulsion between head groups. To accomplish this, we will use fluorescent pH indicators that are sensitive to changes in surface pH and thus charge. Previously, pH probes have been used extensively to characterize the surface potential of various membranes (Fromherz, 1973; Fromherz & Masters, 1974). Using one of these probes embedded in phosphatidylethanolamine (PE)[1]

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[1] Abbreviations: PE, phosphatidylethanolamine; DECNA, 6-decanoylnaphthol; HEXCO, 3-hexadecanoylcoumarin; OG, *n*-octyl glucoside; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DMPA, dimyristoylphosphatidic acid; DMPS, dimyristoylphosphatidylserine; DOPG, dioleoylphosphatidylglycerol.

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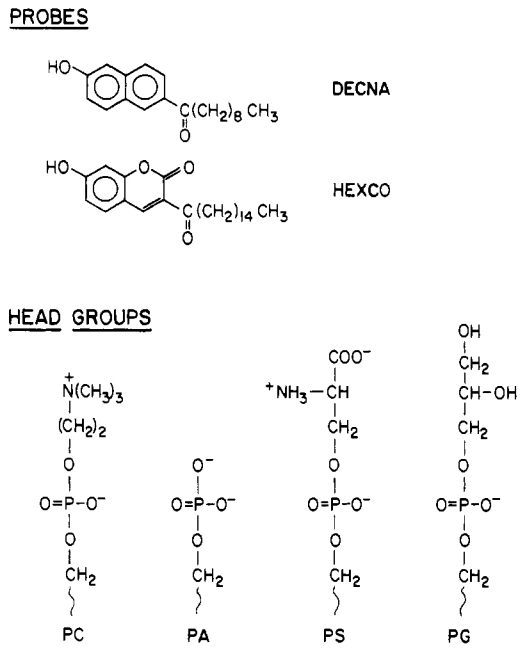

FIGURE 1: Chemical structures of the probes and head groups used in this study.

monolayers, Soucaille et al. (1988) found a small shift in the pK of the probe with increasing lateral pressure. This shift greatly decreased when NaCl was added to the subphase at a final concentration of 0.1 M. The authors also showed that the probe did not change orientation with compression. Interestingly, the pK of their probe did not change when a negatively charged lipid was substituted for a neutral one, and it was concluded that the influence of the polar heads on the pK of the probe was small. Here, we will use probes that are more responsive to the electrical properties of the surface and investigate the effects of hydrostatic pressure on membrane bilayers rather than monolayers.

To fully isolate effects due to membrane structure from those due to pressure, we will initially characterize the surfaces of positively charged, negatively charged, and neutral micelles. These results will be compared to data obtained for a variety of different bilayers. A thorough investigation will be done using one pH probe, and a second will be employed to verify the trends seen with the first. The structure of the two probes as well as the head groups of the lipids is presented in Figure 1. These lipids were chosen for their charge characteristics. Phosphatidylcholine (PC) head groups are zwitterionic. The phosphatidic acid (PA) head group is capable of dissociating two protons, and thus the charge on each molecule can vary from 0 to −2. Between the fully protonated and fully dissociated state is a region where the protons are delocalized on the bilayer surface through a hydrogen-bonding network that has the effect of greatly stabilizing the bilayer (Eibl, 1983; Eibl & Blume, 1979). At neutral pH, the polar groups of phosphatidylserine (PS) and phosphatidylglycerol (PG) have a net −1 charge. Thus, by comparing different charged surfaces, we can determine the relation between surface charge and compression. In order to gauge the amount of change occurring with pressure, we will compare the pressure data to pH titration curves obtained at atmospheric pressure. These curves will not only help us understand the effects of pressure effects on membrane surfaces but also help us determine the response of surfaces to changes in bulk pH.

Materials and Methods

6-Decanoylnaphthol (DECNA) and 6-propionylnaphthol (PRONA) were generous gifts from Professor Gregorio Weber at the University of Illinois. The extinction coefficient of DECNA in ethanol was found to be 12 000 M^{-1} cm^{-1} at 360 nm. 3-Hexadecanoylcoumarin (HEXCO) and 3-butanoylcoumarin (BUCO) were purchased from Molecular Probes, Inc. (Eugene, OR), and used without further purification. All lipids were from Avanti Polar Lipids, Inc. (Birmingham, AL). Small unilamellar vesicles were prepared by sonication without the addition of probe (Scarlata, 1988). Vesicles were labeled by addition of a small aliquot of a concentrated probe solution in ethanol to give either 0.1 mol % DECNA or 0.5% HEXCO. Micelle solutions were prepared and used at concentrations at least twice and usually 4 times the critical micelle concentration. On the basis of the reported number of detergent molecules comprising the particular micelles (Neugebauer, 1988), each solution had approximately one probe per eight micelles.

DMPA, DMPS, and DMPC samples were run at 37 °C, and all others were at 22 °C. All materials were dissolved in 50 mM Hepes and 160 mM KCl or NaCl, pH 7.0, except where indicated. Fluorescence emission spectra were taken on an ISS Greg-PC spectrofluorometer (ISS Inc., Champaign, IL). The probes were excited at the maxima of their protonated peaks (315 nm for DECNA and 360 nm for HEXCO). Samples were subjected to elevated pressures on a home-built apparatus based on the cell of Paladini and Weber (1981). All samples were checked for reversibility after pressure release. Fluorescence data were analyzed in terms of the intensity ratio (IR) of the protonated emission maximum over the unprotonated where IR(DECNA) = $I(428)/I(500)$ and IR(HEXCO) = $I(430)/I(460)$. These maxima were the same in all systems studied. From the IR, we can calculate the degree of proton dissociation (α) in which a value of 1 is completely dissociated and a value of 0 is completely protonated through the equation:

$$\alpha = [\mathrm{IR(obs)} - \mathrm{IR(OH)}]/[\mathrm{IR(O^-)} - \mathrm{IR(obs)}] \quad (1)$$

where IR(obs) is the observed intensity ratio of that sample and IR(OH) and IR(O^-) are the ratios for the fully protonated and fully dissociated species, respectively. The intensity ratio of the fully protonated form was determined from the emission spectra in dilute hydrochloric acid or SDS micelles at pH 7 (both gave identical spectra) while the fully deprotonated form was from the probes in CTAB micelles at pH 13.0.

In our analysis, we write the reaction as being in the direction toward dissociation:

$$K = [\mathrm{H^+}][\mathrm{A^-}]/[\mathrm{HA}]$$

Except where indicated, the experiments took place in buffered solutions at constant pH and thus

$$K_{app} = [\mathrm{A^-}]/[\mathrm{HA}] = \alpha/(1 - \alpha) \quad (2)$$

At constant pH and temperature, the volume change is related to the change in the apparent equilibrium constant with pressure by

$$\Delta V = -\mathrm{d}(RT \ln K_{app})/\mathrm{d}P \quad (3)$$

Results

Ground-State Studies

DECNA. pH titrations were accomplished by monitoring the ratio of the absorption maxima of the protonated and deprotonated species (Table I). The pK values were determined in neutral (OG and Triton), negatively charged (SDS), and positively charged (CTAB) micelles. The results are listed in Table II. To measure the pK of the DECNA head group in water, a soluble propionyl analogue of DECNA, called

Table I: Absorption and Emission Maxima of the Two Probes[a]

	DECNA	HEXCO
absorbance max OH	313	363
absorbance max O^-	362	430
emission max OH	428	435
emission max O^-	500	461

[a] The data are in nanometers.

Table II: Ground- and Excited-State pK Values of the Probes[a]

	DECNA		HEXCO	
	pK	pK^*	pK	pK^*
OG	8.9	1.7	6.5	1.9
SDS	10.9	11.9	8.6	7.8
CTAB	8.0	CBD	4.0	CBD
calcd		1.0		0.6

[a] CBD indicates a value that cannot be determined.

PRONA, was used, and a pK of 8.8 was obtained. This value matches the pK reported for the closely related fluorophore β-naphthol (Becker, 1969). The pK of DECNA in neutral micelles was found to be 8.9. We note that the spectrum of DECNA in neutral micelles is the same as that of PRONA in water which indicates that both are in similar dielectric environments and thus very little of the neutral form of DECNA buries itself in the micelle. When DECNA was added to charged micelles, the pK shifted to 8.0 in CTAB and to 10.9 in SDS. Since the surface charge is affecting the pK to such a large extent and the spectra and pK values of PRONA and DECNA under neutral conditions are so close, we conclude that a significant portion of the probe head groups lie on the micelle surface. The pK of DECNA in DOPC bilayers was found to be 10.2 while in DMPA two proton equilibria were observed having pKs of 1.9 and 9.0 which correspond to the two proton dissociations of phosphatidic acid.

HEXCO. pH studies using the commercially available probe HEXCO were done in order to verify the results obtained for DECNA. In neutral micelles, the pK of HEXCO was found to be 6.5 which is similar to previous reports (Pal, 1983). In CTAB, the pK shifts to 4.0 while in SDS it shifts to 8.6. Thus, while SDS causes a similar pK shift for both DECNA and HEXCO, CTAB appears to affect the pK of HEXCO to a greater extent.

Excited-State Studies

DECNA. The excited-state pK or the pK^* of DECNA can be calculated from the fluorescence spectra of both species (Figure 2) through the following [see Becker (1969)]:

$$pK - pK^* = 0.0021\bar{\nu}$$

where $\bar{\nu}$ is the frequency difference in wavenumbers of the 0–0 bands of the protonated and deprotonated species. The above equation yields a pK^* of 1.0 for DECNA.

Figure 3 presents the degree of dissociation (eq 1) of DECNA as a function of pH for the three types of micelles. The titration curve of DECNA in OG micelles shows a sharp rise in α between pH 1.7 (the lowest point taken) and pH 4. Thereafter, a smooth, more gradual increase is seen until pH 11 where α equals 1, indicating full dissociation. If we extrapolate to the midpoint, we obtain an approximate pK^* that is close to the calculated value of 1.0. The DECNA–CTAB micelles are 90% dissociated from pH 2 to 7, and afterward the dissociation increases to 100%. Thus, the positive charge of CTAB lowers both the ground- and excited-state pK values, and this effect is greater in the excited state.

DECNA–SDS micelles display a more complex pH behavior than OG or CTAB. At low pH values, DECNA is completely dissociated. As the pH increases, DECNA becomes protonated until its full value is reached at pH 5, and thereafter it dissociates. The reason why DECNA is unprotonated at low pH values is not clear. It may be that at low pH values the head groups of SDS are protonated and the molecules no longer form a micelle. To test whether this is the case, we measured the aggregation of SDS at high and low pH. This was done using the method of Chattopadhyay

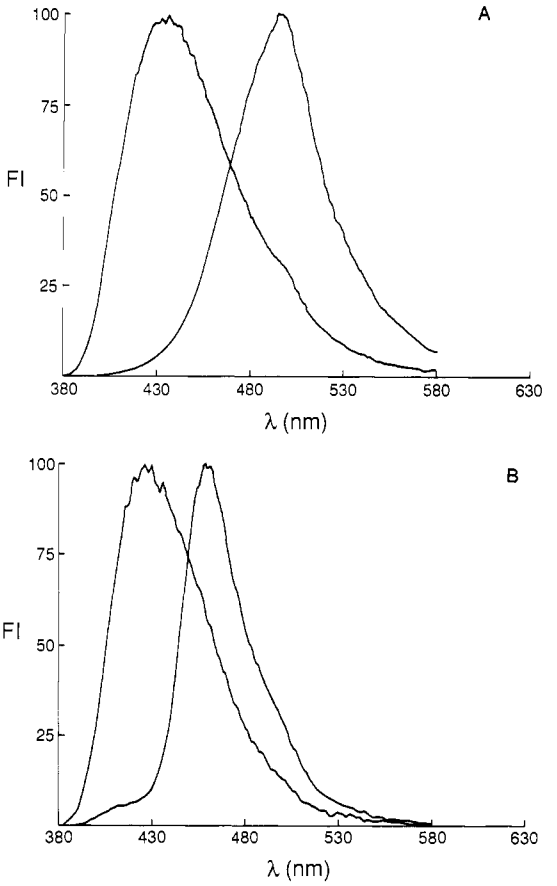

FIGURE 2: Normalized fluorescence spectra of the undissociated (lower energy) and dissociated (higher energy) forms of (A) DECNA and (B) HEXCO.

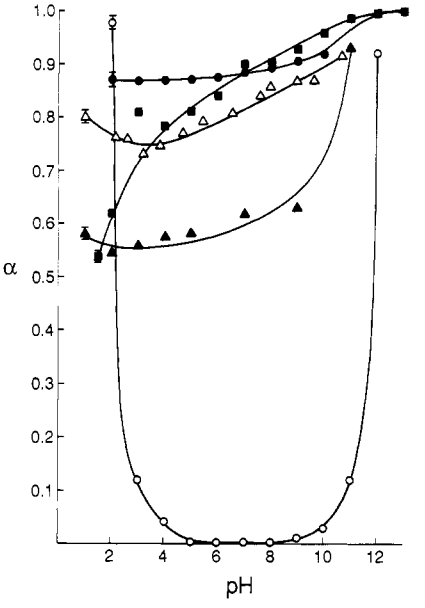

FIGURE 3: Degree of DECNA dissociation (α) as a function of pH in (■) OG, (●) CTAB, and (○) SDS micelles and in (▲) DOPC and (△) DOPA bilayers.

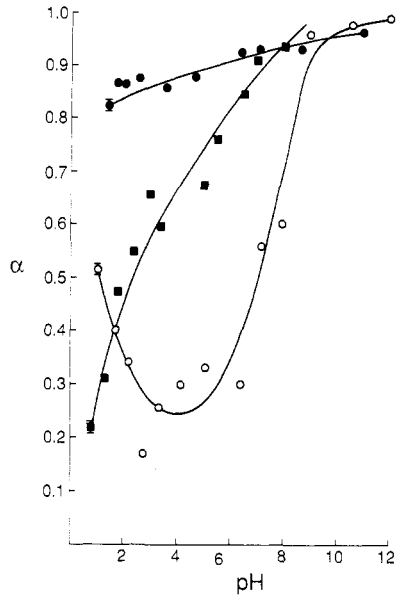

FIGURE 4: Degree of HEXCO dissociation (α) as a function of pH in (■) OG, (●) CTAB, and (○) SDS micelles.

and London (1984) in which the critical micelle concentration is determined by the large intensity increase in 1,6-diphenyl-1,3,5-hexatriene that occurs upon aggregation due to the probe inclusion into the micelle. The results were also checked by light scattering. We found that under our conditions (50 mM SDS, 0.16 M Na^+) the fatty acid is aggregated at all pH values.

We tested the dissociation behavior of DECNA in DOPC (zwitterionic) and DOPA (negatively charged) small unilamellar vesicles. To avoid any perturbation of the bilayer due to the presence of probe, low probe to lipid ratios were used (~0.1% mol/mol). The titration curves are presented in Figure 3. In DOPC, DECNA is always more than 50% dissociated even at very low pH values. In DOPA, DECNA shows little change with pH, staying close to 85% dissociation through most of the titration.

HEXCO. pH titrations using the probe HEXCO show similar behavior as those of DECNA (Figure 4). Because the absorption band of the HEXCO anion and the emission band of the protonated HEXCO overlap and the protonated form is able to excite the anion, we worked at low probe concentrations to avoid energy transfer, and thus the data are more scattered than those of DECNA. The calculated pK^* of HEXCO is only 0.65. In CTAB, the promotion of proton dissociation due to the positive charge is pushed so low that the pK^* cannot be measured. This was also the case with DECNA in CTAB micelles. As with DECNA in OG micelles, a steep increase is observed for HEXCO–OG at very low pH values (between 0.8 and 2.0 in this case) followed by a more gradual change. However, the pK^* in OG is found to be higher than the calculated value. The titration curve of SDS also has a similar shape as DECNA except that the curve is shifted toward lower pH values and at no time is there ever 100% protonation. Both of these differences are due to the lower pK^* of the probe.

Pressure Experiments

DECNA. We tested the behavior of DECNA in the three types of micelles from 1 to 1250 bar as higher pressures destabilize the micelle phase. The samples were run at pH 7 in a pressure-insensitive buffer (HEPES). The fluorescence intensities and center of masses of both the protonated (DECNA in HCl) and deprotonated (DECNA in CTAB, pH 13) species were found to be essentially constant with pressure, and therefore their atmospheric values were used in analysis. Pressure data were treated by putting the observed intensity ratios in terms of the degree of dissociation (eq 1) and then ln K_{app} (eq 2) to obtain the apparent volume change (ΔV_{app}). Linear plots were obtained in all cases unless otherwise noted. The results are listed in Table III.

Table III: Apparent Volume Changes[a]

	DECNA	ΔpH_D	HEXCO	ΔpH_H
OG	−5.2	0.0	CBD	
SDS	−18.9	0.50	−9.6	CBD
CTAB	CBD		CBD	
DOPC	0.0	0.0	0.0	0.0
DOPC, pH 2.2	−2.7	0.10	−2.3	0.08
DMPC	0.0	0.0	0.0	
DOPA	+2.5	−0.09	+3.5	−0.13
DOPA, Li^+	+2.5	−0.09		
DOPA, NH_4^+	+2.7	−0.10		
DMPA	+0.7	−0.2		
DMPS	−0.9	0.03		
DOPG	−4.1	0.15		

[a] All apparent volume changes are in milliliters per mole and have a maximum error of 12%. Samples were run at 22 °C except DMPC, DMPA, and DMPS which were at 37 °C. Unless otherwise indicated, the counterion was K^+ or Na^+. ΔpH values were calculated with respect to OG for micelles and DOPC for bilayers. The subscripts D and H refer to DECNA and HEXCO, respectively. CBD indicates that the particular value cannot be determined.

In OG micelles, DECNA tended to further dissociate with pressure. This behavior is completely expected as pressure tends to promote dissociation of weak acids due to electrostriction. The apparent volume change for proton dissociation was determined to be −5.2 ± 0.4 mL/mol. This value is approximately equal to the volume change of the propionyl analogue PRONA in buffer (−5.5 ± 0.4 mL/mol). DECNA in SDS shows a large change in dissociation with pressure, giving a volume change of −18.9 mL/mol. In this case, the apparent volume change was only determined from the linear region of the data from 0.2 to 1.25 kbar as significant curvature was observed at lower pressure. Since there is such a small amount of deprotonated species, the apparent volume change calculated may be artificially high. In CTAB where DECNA is over 90% dissociated at neutral pH, the application of pressure shows no significant effect on the dissociation state.

We then measured the pressure behavior of DECNA in SDS micelles at pH 2.2 where DECNA was approximately 50% dissociated and a volume change of −8.1 mL/mol was obtained. However, we note that at this pH we could not find a pressure-insensitive buffer and ran the experiment in unbuffered water. The lack of buffer causes several problems. First, high pressure could promote the dissociation of water, thus lowering the pH and shifting the probe equilibrium (Neuman et al., 1973). This would cause the observed volume change to become more positive. To test the differences between buffered and unbuffered solvents, we took the water-soluble butyl analogue of HEXCO called BUCO and measured its dissociation as a function of pressure in HEPES buffer and in water at pH 7. It behaved identically in both solvents, showing very little change with pressure. This indicates that the observations made without buffer reflect primary changes in the system and are not due to water dissociation. Second, at lower pH values, proton dissociation from SDS may occur. In order to estimate the amount of proton dissociation arising from SDS, BUCO was added to SDS micelles at pH 2.2, and the pressure behavior was compared to BUCO in water, OG micelles, and DOPC and DOPA bilayers at pH 2.2. Atmospheric values of α show that BUCO

incorporated into micelles and bilayers. The apparent volume changes are −4.8 ± 0.6 mL/mol for BUCO–SDS, −3.0 ± 0.4 mL/mol for BUCO in water, −3.6 ± 0.4 mL/mol for BUCO–OG, and −3.5 ± 0.4 mL/mol for BUCO in DOPC. The plot of ln K_{app} versus pressure for DOPA was nonlinear and a volume change could not be determined. The similarity of the apparent volume changes of BUCO in water, OG, and DOPC, when coupled to the experiment above at pH 7, shows that in the neutral micelles and zwitterionic bilayers the volume changes at lower pH values can be compared to the pH 7 data. Also, as will be shown below, decreasing the pH results in more negative volume changes in DECNA–DOPC and DECNA–OG. If significant proton dissociation from water or phosphates occurred, then increasing the pressure would decrease the pH and favor proton association to the probe, resulting in more positive volume changes. The larger apparent volume change obtained for BUCO–SDS at pH 2.2 indicates that proton dissociation from SDS occurs, and, because this equilibria is coupled to that of DECNA, an exact volume change cannot be determined.

The pressure behavior of DECNA embedded in different small unilamellar vesicles was monitored from 1 to 2000 bar, and the results are listed in Table III. It is possible that pressure would affect the pK values of the charged groups on the lipid heads, complicating our analysis. If a chemical reaction has a net change in the number of charged species, high pressure will promote dissociation due to electrostriction and decrease the pK (Hamann, 1980). At neutral pH and atmospheric pressure, the carboxylic acid of PS and the phosphates of PC, PS, and PG are fully dissociated and cannot dissociate further. The same is true for the head groups of SDS. The amino group of PS will not be affected by pressure since there will be no net change in the number of charged species upon dissociation. The only lipid head group whose pK may be perturbed by pressure is the second proton dissociation of PA (atmospheric pK = 9.0). On the basis of the work of Neuman et al. (1973), we would expect a maximum change in pK of 2 units in this pressure range. Since plots of ln K_{app} versus pressure for PA systems did not show curvature at higher pressures and since we obtained volume changes that correspond to proton association (see below), we feel that any change in pK of the surface head groups with pressure is not observable in our measurements. The volume changes of the isolated probes show that pressure does perturb their pK values and our titration data show that these pKs are highly dependent on the local environment. The primary effect of pressure on the local surface charge can be isolated from changes in pK by comparisons of the volume changes in neutral and uncharged systems (see Discussion).

Pressure has no effect on DECNA in zwitterionic DOPC bilayers. In most negatively charged bilayers, small changes in the equilibrium toward dissociation are seen. In phosphatidic acid bilayers, proton association is observed, and the resulting apparent volume change is independent of counterion. All dimyristoyl samples were run at higher temperatures (37 °C) in order to elevate the pressure at which the gel-to-liquid-crystalline-phase transition occurs. We note that in passing through the phase transition, we only observed smooth, reversible changes in α. Lowering the pH causes the apparent volume change of DECNA in DOPC to become more negative.

HEXCO. In order to check the behavior of DECNA, we repeated several of the pressure runs with HEXCO. In buffer or water at pH 7, the equilibrium of the short-chained analogue BUCO was not affected by pressure, with α fluctuating between 0.92 and 0.94. In both CTAB and OG micelles at pH 7.0, the probe is almost completely dissociated, and pressure did not affect the equilibria (Table III). In SDS micelles, pressure promoted dissociation, yielding an apparent volume change of −9.6 mL/mol. When the pH was lowered to 2.2, the plot of ln K_{app} versus pressure showed significant curvature, and a volume change could not be determined. When DOPC bilayers were labeled with HEXCO, no changes in the amount of dissociated species were observed. Like DECNA in DOPC, lowering the pH resulted in a reduced apparent volume change. In DOPA, pressure favors protonation of HEXCO, and lowering the pH reduces this value. Thus, the trends of the apparent volume changes measured for HEXCO are the same as those of DECNA.

Discussion

The goal of this work was to determine the effect of lipid packing on the surface charge of bilayers. In order to view changes in surface charge, we used two fluorescent pH probes whose atmospheric pK values are very different (8.8 and 6.5). The first was used in a large variety of systems for thorough investigation while the second served to check the behavior of the first. In all cases, the trends seen for both probes were comparable which indicates that the observed behavior is due to the effects of pressure on the membrane surface rather than changes specific to the probe.

pH Titrations. The purpose of the pH titrations is 2-fold: to determine the sensitivity of the surface to changes in bulk pH and to be able to assess the changes in surface charge that may occur under pressure. Initially, we determined the shift in pK of the probes when they are embedded in neutral (OG), negative (SDS), or positive (CTAB) micelles. Using the behavior of the probe in OG micelles as a reference, we find that SDS shifts the probe dissociation up by 2 pH units due to the stabilization of the protonated state by the negative charge of the micelle. CTAB, on the other hand, promotes the formation of the anion and lowers the pK. The pK of HEXCO is more perturbed by the CTAB surface than DECNA. This behavior is due either to the particular properties of the probes and their interactions with the micelle surface or to different occupancy sites of the probes in the micelle. Our data cannot distinguish between these two possibilities.

From the shift in pK, we can calculate the potential of the CTAB and SDS interface at neutral pH (Fromherz, 1973; Fromherz & Masters, 1974):

$$E = 2.3kT/e[pK(\text{neutral}) - pK(\text{sample})] \quad (4)$$

Using this equation, we obtain a potential of −120 mV for both probes in SDS and 50 mV for DECNA in CTAB and 150 mV for HEXCO in CTAB with an approximate error of 25 mV.

In the excited state, the dipole of a fluorophore is larger than in the ground state, and hence the pK of a dissociating proton in the excited state (pK^*) is usually much lower (Becker, 1969). This larger dipole in the excited state is thus more strongly influenced by its immediate environment as seen in the SDS data in Table II.

Although one would expect to observe the classic titration curves of the ground state simply shifted to lower pH values in the excited state due to the lower dissociation constant, in all cases different behavior was seen (Figures 3 and 4). In SDS, both probes show an unexpected proton association at low pH values, and we found that under our conditions SDS is still aggregated. Proton association at very low pH values could be due to an alternate phase in the micelle that is not easily detectable by our spectroscopic methods and/or an initial pulling away of the probe protons by the partially deprotonated

sulfate groups. In OG, DECNA shows a sharp dissociation up to ~75% at low pH values, followed by a gradual rise to complete dissociation. In positively charged micelles, the primary pK^* was too low to be determined while a small secondary dissociation appears at higher pH values, accounting for 10% of the total species. The species that dissociate at higher pH values could be located at more internal positions and less prone to dissociate or, alternately, could be hydrogen bonded to a surface group. Fluorescence anisotropy data of the anion indicate that the latter is more likely the case (Scarlata and Rosenberg, unpublished data). Hydrogen bonding of the probe proton to a surface group would explain why the measured pK^* of HEXCO is higher than the calculated one.

Titrations of DECNA in bilayers also gave unexpected results. In DOPC, the degree of dissociation was above 0.5 throughout the entire pH range (1.0–11.1), and so a pK^* could not be determined. The curve showed a very small dip at low pH values which, based on the SDS results, is most likely due to the influence of the phosphate group. From pH 3 to 9, small increases in dissociation were observed followed by a sharp increase at pH 9.5. The hovering of the degree of dissociation around 60% through such a large pH region shows that the two charged groups of DOPC serve to hold the effective pH on the surface fairly constant. Thus, in PC membranes, even if the bulk pH changes by a large amount, dissociable groups on the surface are buffered by the surrounding lipid heads.

Because DMPA and DOPA are negatively charged through a large pH range, one might expect the DECNA dissociation behavior would be similar to that observed in SDS. Instead, we observe a curve similar to that of DOPC (Figure 3). Eibl (1983) has shown that in bilayers after the first ionization, phosphate protons stabilize the negative surface through an extensive hydrogen-bonding network in which the protons are delocalized over two phosphate groups. We expect the proton of DECNA to participate in this network, and since the phosphate groups are more electronegative, DECNA protons will be localized more toward the phosphates, causing DECNA to be mostly dissociated even at low pH values. Thus, like DOPC, the bilayer surface greatly changes the pK of dissociable groups on the surface and tends to buffer surface groups from large changes in the bulk pH.

High-Pressure Results. High-pressure spectroscopy was used to determine the effect of increased chain packing on the surface charge of the membrane. Pressure may perturb the orientation and position of the probes although previous investigations on monolayers using a similar probe have shown the probe orientation to be constant with increasing lateral pressure (Soucaille, 1988). Because our probes have such long hydrocarbon tails, it is unlikely that they are eliminated with pressure. If only the head groups were pushed out with pressure, then we would expect a large proton dissociation from the probe, which we do not see, and increases in the head group rotation, which we do not see (Scarlata and Rosenberg, unpublished results). Alternately, if the probes were pushed into the membrane, a positive volume change would be observed for all our samples, and a positive volume change is only found for PA bilayers. Although we can eliminate the possibility of pressure causing gross orientational changes, pressure may produce subtle changes which alter probe–head group interactions. While this possibility certainly exists, we note that the same behavior was seen using both probes.

Initially, we studied the probes in micelles in order to determine the role that bilayer structure plays in the pressure perturbation of surface charge. In neutral micelles at pH 7,

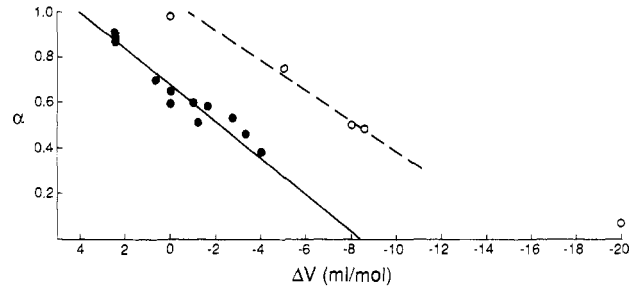

FIGURE 5: Degree of dissociation at atmospheric pressure (α_0) as a function of the apparent volume change for DECNA in micelles (○) and bilayers (●).

pressure caused DECNA to dissociate due to electrostriction or the contraction of water around the newly ionized species. This effect is observed for reactions that result in an increase in the number of charged species (Neuman et al., 1973; Hamann, 1980). At pH 7, HEXCO is initially over 90% dissociated, and increased pressure did not affect its equilibrium. In SDS, pressure promotes proton dissociation of both probes with a large volume change. This large volume change was surprising because one would expect that the negative charge of the SDS surface would tend to decrease the amount of proton dissociation under pressure.

In order to assess the changes in surface charge that occur in the probe environment under pressure, we compared the amounts of dissociated and undissociated probe molecules. This yields an apparent equilibrium constant that does not take into account changes in hydrogen ion concentration. Because our probe is subjected to the potential of the micelle surface, the local pH may be different from the bulk. Since the equilibrium constant is related to the apparent equilibrium constant by $K = K_{app}[H^+]$, the volume change of probe dissociation (ΔV_p) is composed of the apparent volume change and a volume change due to changes in proton concentration:

$$\Delta V_p = -RT\, \mathrm{d} \ln K/\mathrm{d}p = -RT[\mathrm{d}(\ln K_{app})/\mathrm{d}p + \mathrm{d} \ln [H^+]/\mathrm{d}p]$$

For the free probes in buffer, the apparent volume change equals the volume change of the probe dissociation. When the probe is in an alternate environment, the magnitude of ΔV_p should remain unchanged, and the difference between the apparent volume change and ΔV_p is related to the change in the local proton concentration with pressure. Thus, we find similar volume changes for PRONA in buffer and DECNA in neutral OG micelles. Comparing the apparent volume change for SDS and OG micelles at pH 7, we find that the SDS surface changes by 0.5 pH unit. We note that we cannot differentiate between surface charge and surface pH.

In contrast to micelles, bilayers at pH 7 showed both positive and negative apparent volume changes. In trying to understand this behavior, we found that the magnitude of the volume change depends on the initial degree of dissociation: the more protonated the probe, the greater its tendency to dissociate under pressure. The relation of the apparent volume change to the initial degree of DECNA dissociation can be seen in Figure 5 where we find that bilayers generally fall on one line and micelles fall on a similar line that is displaced to the right of the bilayer by ~−6 mL/mol. The reason for this linear dependence is not yet clear. Some of the different values of α_0 were obtained by subjecting DECNA–OG and DECNA–DOPC to various pH values. From Figure 5, we see that the only way micelles can show a zero apparent volume change is if they are initially 100% dissociated. As the sample becomes more protonated at atmospheric pressure, ΔV_{app} decreases

Table IV: Comparison of DECNA Dissociation in Different Systems at pH 7[a]

	charge/lipid	species	α_0	α_2	ΔV_{app} (mL/mol)
micelles	−1	SDS	0.0	0.30	−18.9
	0	OG	0.89	0.93	−5.2
DO bilayers	−1.2	DOPA	0.88	0.76	+2.5
	−1.0	DOPG	0.39	0.48	−4.1
	0	DOPC	0.67	0.67	0.0
DM bilayers (37 °C)	−1.2	DMPA	0.70	0.68	+0.7
	−1.0	DMPS	0.51	0.65	−0.9
	0	DMPC	0.59	0.59	0.0

[a] Where DO stands for dioleoyl and DM for dimyristoyl; α_0 and α_2 are the dissociation at atmospheric pressure and 2 kbar. The charge/lipid values of DOPA and DMPA were calculated by using the measured pK values of 1.9 and 9.0 (see Results).

because the protonated surface will allow for dissociation under pressure due to electrostriction of water around the dissociated probe. The displacement of the micelle data from the bilayer in Figure 5 reflects the difference in the apparent volume change that occurs when DECNA dissociates in these two systems. Since the separation between the two lines is similar to the volume change of the probe due to electrostriction (−5.2 mL/mol in OG micelles), then this result indicates that the drive to dissociate due to electrostriction is greatly inhibited in bilayers.

The dependence of the degree of dissociation on the apparent volume change accounts for the lack of observable effect with different counterions. If the volume change was highly dependent on head group spacing, then changing the counterions may greatly affect its magnitude. However, we have found that at ionic strengths greater than 0.01 M, the charge to mass ratio of the counterion has only a very small effect on the degree of DECNA dissociation (Scarlata and Rosenberg, unpublished data).

While we can interpret the apparent volume change observed in different bilayers on an individual basis (i.e., pressure promotes protonation in PA bilayers due to surface repulsion etc.), the data in Figure 5 show that there is an overall process that inhibits the effects of electrostriction. One possibility may be the limited expansion of the bilayer surface: When a proton dissociates from a probe on the surface of a micelle and water contracts around the ions, the surface can accommodate the increased volume taken up by the ion–water complex. The surface of a bilayer, on the other hand, either has limited or has no expansion since the hydrocarbon interior is compressing rapidly with pressure [e.g., see Eisinger and Scarlata (1987)]. To support this interpretation, we have found that the addition of cholesterol, which has the effect of increasing head group distances, promotes proton dissociation from DECNA in DOPC bilayers and reduces the amount of proton association of DECNA in DOPA bilayers (Scarlata and Pang, unpublished data).

In viewing the apparent volume changes listed in Table III, we find that the difference between DECNA in DOPC and OG is similar to the displacement of the micelle and bilayer line in Figure 5. This implies that the surface charge around DECNA in DOPC remains unchanged with pressure and will allow for the change in surface pH (ΔpH) of the other bilayers to be calculated with respect to DOPC. These data are listed in Table III. Because of the small apparent volume changes, these data show that the local pH of bilayers changes little with pressure. If we do not assume that the local surface charge around DECNA in DOPC remains constant with pressure, then we can compare its apparent volume change to that of DECNA–OG and calculate a pH change of 0.19 unit in 2000 bar. The values for the other bilayers will then be lowered by this amount. In Table III, the change in local pH around HEXCO was calculated with respect to the apparent volume change in DOPC.

In light of the above results, we would like to characterize what surface features determine the degree of DECNA dissociation. The data in Table IV show that in micelles going from a charge per molecule of −1 to 0 the degree of dissociation of DECNA increases from 0 to ~90%. Bilayers, on the other hand, do not show a clear trend. This can only be due to the different interactions unique to a particular head group. In some cases (DOPG, DMPS), the effect of the surface may be similar to that of micelles whereby the negative charge stabilizes the protonated form of the probe. In PA lipids, DECNA and HEXCO protons become delocalized due to the surface hydrogen-bonding network. These data show that the potential one calculates by using this method may not reflect the true surface charge of the membrane but rather the particular interactions between probes and head groups.

In order to determine the changes that the bulk pH must undergo to produce the observed changes in α with pressure, we must couple the titration curves in Figures 3 and 4 to the pressure results. In doing so, we find that raising the pressure from 1 to 2000 bar does not affect the surface charge of PC bilayers while the surface of PA bilayers change from an external bulk pH equivalent of pH 7 to 3. Although this may seem like a large change, the titration curve shows it to be a relatively small change in dissociation. This result agrees with the small changes in local surface pH with pressure and supports the notion that the surface charge of a membrane is very stable. While the data in Table IV limit a general treatment of increased packing effects based solely on surface charge, they do give us an appreciation for head group complexities in regards to how different surfaces may affect the dissociation properties of small molecules and proteins on the membrane surface.

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Registry No. DECNA, 129422-99-7; HEXCO, 69377-68-0; OG, 29836-26-8; SDS, 151-21-3; CTAB, 57-09-0; DOPC, 4235-95-4; DMPC, 18194-24-6; DOPA, 14268-17-8; DMPA, 30170-00-4; DMPS, 64023-32-1; DOPG, 62700-69-0.

REFERENCES

Becker, R. (1969) *Theory and Interpretation of Fluorescence and Phosphorescence*, pp 239–243, Wiley Interscience, New York.

Cevc, G. (1987) *Biochemistry 26*, 6305–6310.

Chattopadhyay, A., & Landon, E. (1984) *Anal. Biochem. 139*, 408–412.

Eibl, H. (1983) *Membrane Fluidity in Biology* (Alaoia, Ed.) Vol. 2, pp 217–236, Academic Press, New York.

Eibl, H., & Blume, A. (1979) *Biochim. Biophys. Acta 553*, 476–488.

Eisinger, E., & Scarlata, S. (1987) *Biophys. Chem. 28*, 273–281.

Fromherz, P. (1973) *Biochim. Biophys. Acta 553*, 476–488.

Fromherz, P., & Masters, B. (1974) *Biochim. Biophys. Acta 356*, 270–275.

Hamann, S. (1980) *Rev. Phys. Chem. Jpn. 50*, 147–168.

Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.

MacDonald, A. G. (1984) *Philos. Trans. R. Soc. London, B 304*, 74–68.

Neugebauer, J. (1988) *A guide to the properties and uses of detergents in biology and biochemistry*, Calbiochem Corp., La Jolla, CA.
Neuman, R., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.
Pal, R., Petri, W., Barenholz, Y., & Wagner, R. (1983) *Biochim. Biophys. Acta 729*, 185–192.
Paladini, A., & Weber, G. (1981) *Rev. Sci. Instrum. 52*, 419–427.
Scarlata, S. (1988) *Biophys. J. 55*, 1215–1223.
Soucaille, P., Prats, M., Tocanne, J. F., & Teissie, J. (1988) *Biochim. Biophys. Acta 939*, 289–294.

A Combination of H_2O_2 and Vanadate Concomitantly Stimulates Protein Tyrosine Phosphorylation and Polyphosphoinositide Breakdown in Different Cell Lines†

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ABSTRACT: Treatment of four cell lines [rat hepatoma (Fao), murine muscle (BC3H-1), Chinese hamster ovary (CHO), and rat basophilic leukemia (RBL)] with a combination of 3 mM H_2O_2 and 1 mM sodium orthovanadate markedly stimulates protein tyrosine phosphorylation, which is accompanied by a dramatic increase (5–15-fold) in inositol phosphate (InsP) formation. H_2O_2/vanadate stimulate best formation of inositol triphosphate while their effects on the mono and di derivatives are more moderate. In the presence of 3 mM H_2O_2, both protein tyrosine phosphorylation and InsP formation are highly correlated and manifest an identical dose–response relationship for vanadate. Half-maximal and maximal effects are obtained at 30 and 100 μM, respectively. This stimulatory effect of H_2O_2/vanadate is not mimicked by other oxidants such as spermine, spermidine, $KMnO_4$, and vitamin K_3. In RBL cells, the kinetics of inositol triphosphate formation correlate with tyrosine phosphorylation of a 67-kDa protein, while tyrosine phosphorylation of a 55-kDa protein is closely correlated with both inositol monophosphate formation and serotonin secretion from these cells. Taken together, these results suggest a causal relationship between tyrosine phosphorylation triggered in a nonhormonal manner and polyphosphoinositide breakdown. Furthermore, these results implicate protein tyrosine phosphorylation in playing a role in the stimulus–secretion coupling in RBL cells.

Tyrosine phosphorylation of proteins has been implicated both in mitogenic signal pathways and in oncogenic transformation (Hunter, 1987; Yarden & Ullrich, 1988). Recent reports have indicated the possibility of a positive cross-talk between tyrosine phosphorylation and polyphosphoinositide breakdown. For example, transformation of a mink lung epithelial cell line (CCL64) with the tyrosine kinase encoding oncogenes v-*fms* or v-*fes* results in enhanced rate of polyphosphoinositide turnover (Jackowski et al., 1986). Similarly, growth factors whose receptors comprise an intrinsic tyrosine kinase activity, such as FGF, PDGF, and EGF, stimulate inositol phosphate (InsP)[1] formation (Tilly et al., 1988) and potentiate thrombin-induced phosphoinositide breakdown in hamster fibroblasts (Paris et al., 1988). Vanadate and molybdate, which are potent inhibitors of protein tyrosine phosphatases (PTPases) (Tonks et al., 1988), increase protein tyrosine phosphorylation of a 50-kDa protein and induce the generation of InsP in electropermeabilized platelets (Lerea et al., 1989). Finally, phospholipase C-γ (PLC-γ) undergoes in vivo tyrosine phosphorylation in response to stimulation with EGF or PDGF (Wahl et al., 1988, 1989a,b; Margolis et al., 1989), and it also serves as an in vitro substrate for both the EGF and PDGF receptor kinases (Nishibe et al., 1989; Meisenhelder et al., 1989). Taken together, these observations point to a tight association between protein tyrosine phosphorylation and phosphoinositide breakdown although the molecular basis for this linkage is presently unknown.

We have recently shown (Heffetz et al., 1990) that treatment of different cell lines with a combination of H_2O_2 and vanadate leads to a 6–20-fold increase in intracellular protein tyrosine phosphorylation. The action of H_2O_2 and vanadate is mediated, at least in part, through inhibition of PTPases and through the activation of receptor tyrosine kinases (Heffetz et al., 1990). Since the combination of H_2O_2/vanadate acts as a nonhormonal stimulus, it serves as a useful tool to investigate the role of tyrosine phosphorylation in the regulation of polyphosphoinositide breakdown. This is particularly important in cells where physiological stimuli that induce tyrosine phosphorylation have not yet been identified. In the present study, H_2O_2/vanadate were employed to examine the consequences of enhanced tyrosine phosphorylation on polyphosphoinositide hydrolysis in several cell types. This approach led to the conclusions that (a) increased protein tyrosine phosphorylation and enhanced polyphosphoinositide breakdown are tightly coupled, (b) this phenomenon is general and occurs in several different cell lines, (c) in one of these lines, the rat basophilic leukemia (RBL) cells, inositol triphosphate ($InsP_3$) and inositol monophosphate ($InsP_1$) formation closely correlates with tyrosine phosphorylation of 67- and 55-kDa proteins,

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[1] Abbreviations: InsP, inositol phosphate; PTPases, protein tyrosine phosphatases; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; PLC-γ, phospholipase C-γ.

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