Response of Phosphatidylcholine in the Gel and Liquid-Crystalline States to Membrane Surface Charges[†]

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ABSTRACT: The influence of membrane surface charge on the conformation of the choline head group of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was investigated in the gel and liquid-crystalline states by using ^{2}H NMR spectroscopy of specifically choline-deuterated DMPC. The surface charge was made progressively more negative through admixture of various proportions of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG). All membrane compositions showed nearly identical gel- to liquid-crystalline-phase transitions centered about 24 °C. The gel-state ^{2}H NMR spectra from all three choline head-group deutero-labeling positions (α, β, and γ) decreased in intensity and broadened relative to the liquid-crystalline-state spectra. These effects were not so severe that they masked the overriding influence of surface charge on the choline head-group conformation as reflected in the ^{2}H NMR spectra. Thus, in both the liquid-crystalline and gel states, the presence of negative surface charge caused the quadrupole splitting from DMPC-α-d_2 to increase while causing that from DMPC-β-d_2 and DMPC-γ-d_9 to decrease. These effects were progressive with increasing density of negative surface charge. Correlation plots of the quadrupole splittings obtained, under otherwise identical conditions, from different deutero-labeling positions were linear over most of the range of surface charge densities, in both the liquid-crystalline and gel states, for all three correlations (α–β, β–γ, and α–γ). At extreme surface charge densities, the α–β and α–γ correlations showed biphasic behavior in that, at high surface charge densities, the change in the quadrupole splittings from DMPC-α-d_2 became less pronounced. The β–γ correlation was linear over the entire range of surface charge densities, regardless of the phase state of the membrane. In terms of the present model of the conformational change undergone by the choline group in response to membrane surface charges, the biphasic α–β and α–γ correlations for negative surface charges indicate a steric hindrance to the tilting of the choline P–N vector toward the membrane surface. These results demonstrate the utility of the ^{2}H NMR approach for the investigation of surface charge effects in gel-state lipid membranes and indicate the potential for its exploitation in the study of other membrane-associated phenomena such as lipid lateral-phase segregation. In addition, we describe a model of the relationship between surface charge and ^{2}H NMR quadrupole splittings which reproduces both qualitatively and quantitatively all the major features as established experimentally. On the basis of the premise that the choline group tilts in response to surface charges, the model provides a context for understanding the particulars of the ^{2}H NMR response, and provides new predictions subject to experimental verification.

It is well established that, in its liquid-crystalline state, phosphatidylcholine senses and responds to the presence of electrical charges at the surface of a phospholipid bilayer (Seelig et al., 1987). This response involves a conformational change in the choline head group of phosphatidylcholine. Deuterium nuclear magnetic resonance (^{2}H NMR)[1] spectroscopy of phosphatidylcholine, specifically deuterated in the choline head group and incorporated into such bilayers, allows one to monitor this conformational change through its effects on the ^{2}H NMR quadrupole splittings, $\Delta\nu_Q$. By the same token, changes in $\Delta\nu_Q$ permit one to calculate the membrane surface charge density. The best available evidence suggests that the choline head group tilts with respect to the plane of the bilayer surface as its positively charged quaternary nitrogen is either attracted to or repelled by opposite or like surface charges, respectively (Scherer & Seelig, 1989). This effect, which is termed the "molecular voltmeter", has proved to be operative in all cases involving the deposition of charges at the surface of a membrane. The origin of these surface charges may be external to the membrane, as is the case for the binding of surface ligands like divalent cations or peptides, or internal to the membrane, as is the case when mixing in charged phospholipids or amphiphiles [for a recent review, see Seelig et al. (1987)].

All of the above studies were carried out at temperatures where the bilayer lipids were in a liquid-crystalline state, because of the ease with which the ^{2}H NMR spectra can be acquired and interpreted under such conditions, and because this is the physiologically relevant state.

We report here the first evidence that the choline head group of phosphatidylcholine when in the gel state responds to and, via ^{2}H NMR spectroscopy, reports on membrane surface charge densities for phospholipid bilayers. These findings support and extend the "molecular voltmeter" concept and point out new possibilities for its exploitation in exploring

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[1] Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; ^{2}H NMR, deuterium nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; DSC, differential scanning calorimetry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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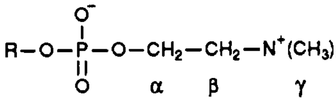
phenomena relevant to both biological membranes and other systems where surface electrostatics play an important role. In addition, we propose a model for the influence of surface charge on the choline head group, which reproduces all of the essential features of the dependence of the quadrupole splittings on surface electrostatics, and which provides new, experimentally verifiable predictions concerning the details of that dependence.

MATERIALS AND METHODS

The following nomenclature is employed for phosphatidylcholines labeled with deuterons at the indicated positions:



$$\mathrm{R{-}O{-}\overset{\overset{O^-}{|}}{\underset{\underset{O}{\|}}{P}}{-}O{-}\underset{\alpha}{CH_2}{-}\underset{\beta}{CH_2}{-}\underset{\gamma}{N^+(CH_3)_3}}$$

1,2-Dimyristoyl-*sn*-glycero-3-phosphate (DMPA), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were purchased from Avanti Polar Lipids (Birmingham, AL). DMPC was selectively deuterated at either the α, β, or γ positions starting from DMPA as described by Harbisson and Griffin (1984). The deuterated DMPC's were further purified by chromatography on CM-52 as described by Comfurius and Zwaal (1977). The purity of the products was checked by thin-layer chromatography, which on an overloaded plate showed a singlet spot migrating with an R_f identical with authentic DMPC, and by ^{1}H NMR, which provided spectra having all the expected resonances in the correct intensities.

^{2}H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz by employing the quadrupole echo technique (Davis et al., 1976), while using quadrature detection with complete phase cycling of the pulse pairs (Griffin, 1981). Particulars regarding 90° pulse lengths (2.0 μs), interpulse delays (40 μs), recycle delays (250 ms), spectral widths (100 kHz), data size (2K), and number of acquisitions (15 000) are those noted in parentheses, unless otherwise indicated.

Samples for NMR observation were prepared as follows. A volume of chloroform/methanol containing 25 μM deuterated DMPC plus the desired quantity of DMPG was dried under a stream of nitrogen, and any residual solvent was removed under high vacuum. The lipids were dispersed in excess deuterium-depleted buffer (150 mM NaCl/10 mM HEPES, pH 7.4) by repeated warming to 45 °C and gentle vortexing. Once a homogeneous suspension was obtained, the sample was centrifuged at 13000g for 20 min, and the pellet was taken for NMR measurement.

Quadrupole splittings were determined from the separation of the two maxima in the ^{2}H NMR spectrum. For liquid-crystalline-state membranes, these values are accurate to within ±50 Hz. For gel-state membranes, the positions of the maxima were determined from the zero-crossing points of dispersion-mode spectra. For such broad spectra, the errors are intrinsically larger (±150 Hz).

RESULTS

Phospholipid bilayers having different densities of surface charge were produced by mixing (zwitterionic) DMPC with various proportions of (negatively charged) DMPG. Both DMPC and DMPG undergo a gel- to liquid-crystalline-phase transition around 24 °C (Blume, 1983). Differential scanning calorimetry (DSC) measurements revealed, as expected, that these mixed phospholipid bilayers underwent a single, gel- to liquid-crystalline-phase transition centered at a temperature of 24 °C, regardless of the proportion of DMPC to DMPG.

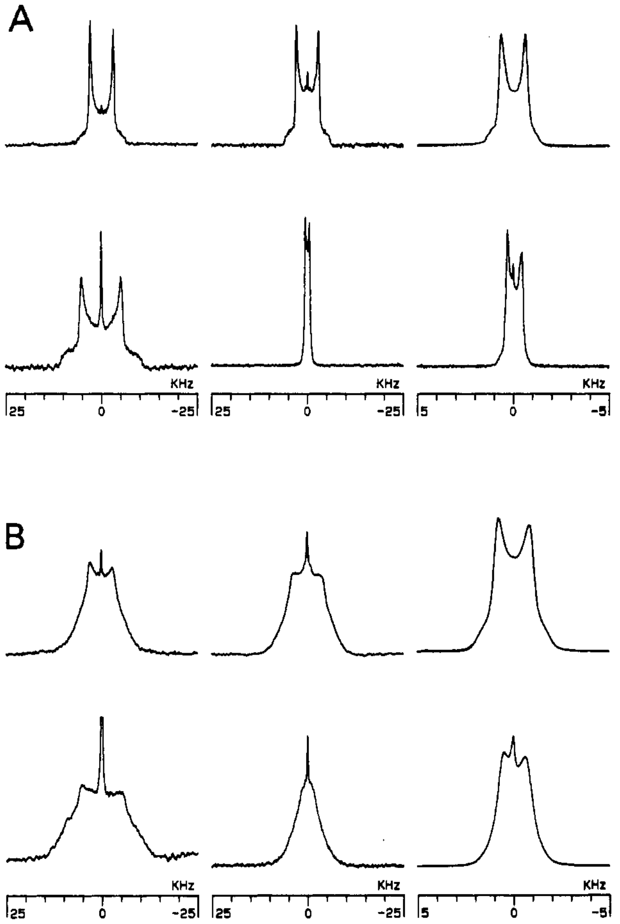


FIGURE 1: Deuterium NMR spectra of (A) liquid-crystalline-phase (30 °C) and (B) gel-phase (15 °C) choline-deuterated DMPC and mixed DMPC/DMPG membranes. In both (A) and (B), the upper three spectra were obtained with 100 mol % DMPC membranes, while the lower three spectra were obtained with mixed 20 mol % DMPC/80 mol % DMPG membranes. In each row of spectra, from left to right, the position of deuterium labeling changes from the α, to the β, to the γ segment of the choline head group of DMPC. In (B), all spectra were acquired by using a recycle delay of 500 ms and 50 000 acquisitions.

Figure 1A illustrates the changes which occur in the ^{2}H NMR spectra of liquid-crystalline, choline head-group-deuterated DMPC when a negative surface charge density is imposed through admixture of DMPG. These spectra were all acquired at 30 °C, i.e., at a temperature above the lipid gel- to liquid-crystalline-phase transition. They are each typical of the ^{2}H NMR spectrum expected for a random dispersion of liquid-crystalline phospholipids in a bilayer arrangement (Seelig, 1977).

The top three spectra in Figure 1A, from left to right, were acquired by using membranes consisting of 100 mol % either DMPC-α-d_2, DMPC-β-d_2, or DMPC-γ-d_9, respectively. The size of the quadrupole splitting (corresponding to the separation in hertz between the two maxima in the spectrum) was 6.1, 5.8, and 1.2 kHz for α-, β-, and γ-deuterated DMPC, respectively, in agreement with previously reported results [see, for example, Seelig and Macdonald (1987) and references cited therein].

The bottom three spectra in Figure 1A, from left to right, were acquired by using membranes consisting of mixtures of 80 mol % DMPG plus 20 mol % either DMPC-α-d_2, DMPC-β-d_2, or DMPC-γ-d_9, respectively. The presence of DMPG caused the size of the quadrupole splitting from DMPC-α-d_2 to increase (to 10.4 kHz), while decreasing the size of the quadrupole splitting from both DMPC-β-d_2 and DMPC-γ-d_9 (to 0.9 and 0.7 kHz, respectively). This counterdirectional change in the size of the quadrupole splittings from α- versus β-choline-deuterated phosphatidylcholines in the presence of surface charges is indicative of a concerted conformational change undergone by the entire choline head group in response to the altered surface charge density. The direction of the changes observed in the size of the quadrupole splittings in this case is that which is expected for the presence of negative surface charge density (Seelig et al., 1987).

Figure 1B illustrates the effects on the ^{2}H NMR spectra of converting the membrane lipids to a gel state. These spectra were all acquired at 15 °C, i.e., at a temperature below the lipid gel- to liquid-crystalline-phase transition. The conversion of the membrane lipids to a gel state was accompanied by an overall broadening of the spectra and a decrease in signal intensity. The onset of these changes occurred at 25 °C for each DMPC/DMPG mixture, regardless of the deutero-labeling position. At 25 °C, i.e., near the center of the phase transition, the ^{2}H NMR spectra consisted of superpositions of broad and narrow components arising from the separate contributions of gel-state and liquid-crystalline-state lipids. Such spectral changes as have been observed here are attributable to the decreased overall rates of motion sustained by the lipids in the highly ordered and less fluid gel state. These effects were not sensed equally at each choline segment. As may be ascertained by comparing the gel-state ^{2}H NMR spectra from the three choline deutero-labeling positions, the severity of spectral broadening and intensity loss diminished with increasing distance from the lipid phosphorus group.

The effects of the decreased rates of motion in the gel state were not so profound that they were able to mask the response of the choline group to the presence of DMPG as reflected in the ^{2}H NMR spectra. A comparison between the ^{2}H NMR spectra obtained at 15 °C for 100 mol % DMPC and for the mixture 20 mol % DMPC + 80 mol % DMPG shows that, in the gel state, the choline head group of DMPC undergoes a conformational change in response to the presence of negative surface charge which is qualitatively similar to that undergone in the liquid-crystalline state. Thus, for the 80 mol % DMPG plus 20 mol % DMPC mixtures at 15 °C, the residual quadrupole splitting increased relative to the 100 mol % DMPC membranes when the deuteron labels were located at the α position of the choline head group, and decreased when the deuterons were located at the β position or the γ position of the choline head group.

The sharp central spike observed in the ^{2}H NMR spectra from membranes containing 80 mol % DMPG is attributed to the presence of residual aqueous deuterium. Normally, excess water is removed during centrifugation of the lipid vesicles prior to acquisition of the NMR spectrum. However, at high surface charge densities, the swelling of the vesicles renders them difficult to pellet properly. ^{31}P NMR spectra of such samples show no isotropic resonance. In the gel-state spectra, the problem is exacerbated by the decreased intensity and broadening of the signal.

Figure 2 shows the variation of the quadrupole splittings with changing temperature for a range of representative DMPC/DMPG mixtures. In general, regardless of the particular proportion of DMPC/DMPG and for all deutero-labeling positions, lowering the temperature through the gel- to liquid-crystalline-phase transition brought about an increase in the size of the quadrupole splitting. For all membrane compositions, the increase in the size of the quadrupole splittings occurred, as expected, between 25 and 20 °C, again regardless of the deutero-labeling position. Figure 2 demonstrates that the effects of the transition to the gel state do not mask the effects of surface charge on the choline conformation as reported by ^{2}H NMR. On the contrary, the effects of surface charge on the deuterium quadrupole splittings are greater in magnitude than the effects of the transition from the liquid-crystalline to the gel state.

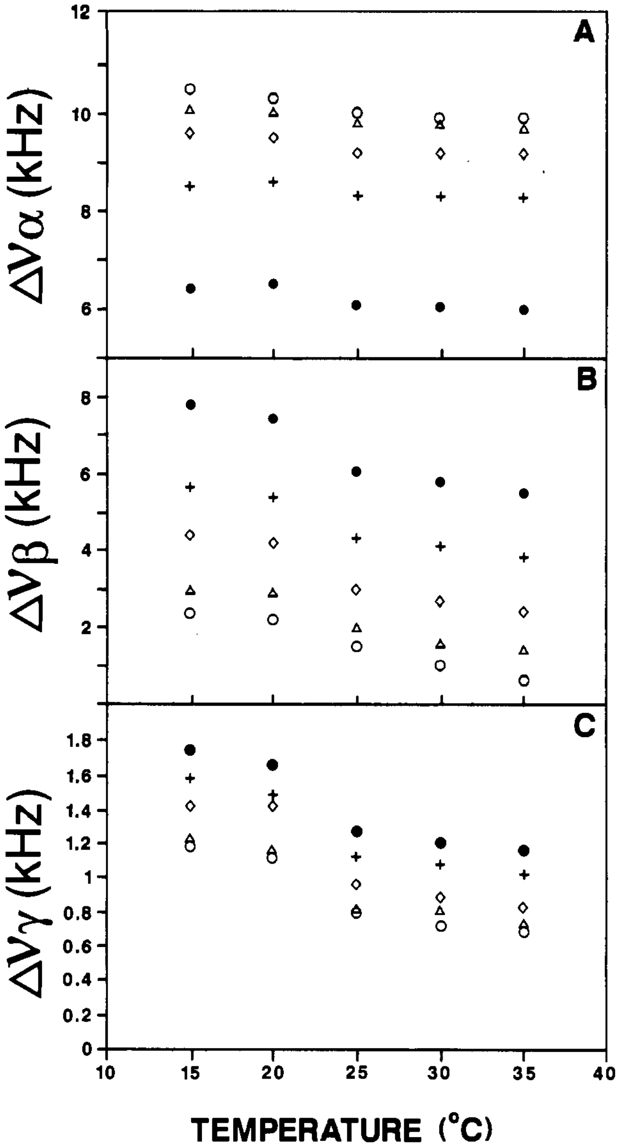


FIGURE 2: Effect of the transition from the liquid-crystalline to the gel state on the deuterium quadrupole splittings from representative (choline-deuterated) DMPC/DMPG mixtures. The choline head group of DMPC was deutero-labeled in (A) at the α, (B) the β, and (C) the γ position. Quadrupole splittings are plotted as a function of measuring temperature for membranes consisting of (●) 100 mol % DMPC and (+) 80/20, (◇) 60/40, (△) 40/60, and (○) 20/80 mol % DMPC/DMPG mixtures.

Figure 3 provides an even more striking illustration that the choline head group responds to surface charge effects regardless of the phase state of the membrane lipids. Here the quadrupole splittings have been normalized with respect to the value for a membrane containing 100 mol % DMPC for each deuteron-labeling position and for each individual temperature. The normalized ^{2}H NMR data from an entire series of DMPC/DMPG mixtures spanning the range from 100 mol % DMPC to 10 mol % DMPC + 90 mol % DMPG, in increments of 10 mol %, have been plotted as a function of the mole percent of DMPG contained in a particular mixture. The progressive changes in the size of the quadrupole splittings

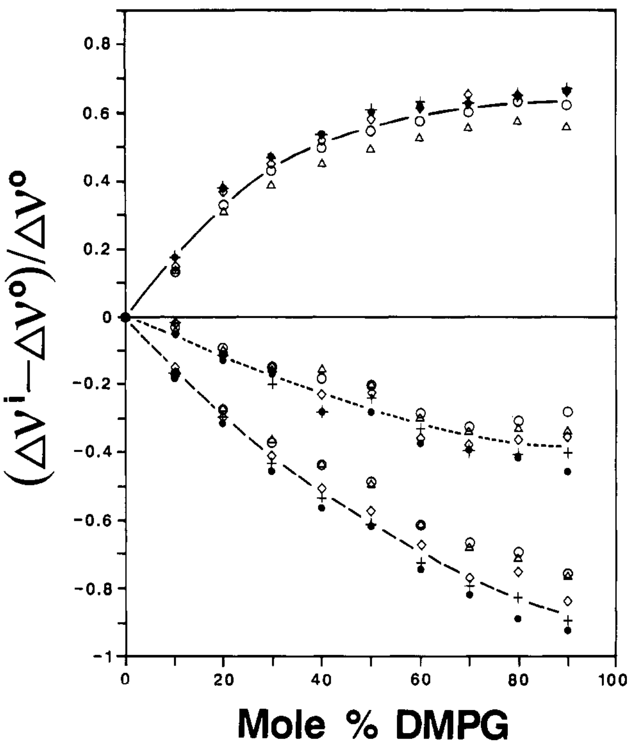


FIGURE 3: Comparison of the influence of surface charge on the deuterium quadrupole splittings from choline-deuterated DMPC in the liquid-crystalline and in the gel phase. The quadrupole splittings are normalized with respect to the value of the quadrupole splitting at a particular deutero-labeling position at a particular temperature for a membrane containing 100 mol % DMPC. Normalized quadrupole splittings are plotted as a function of the mole percent DMPG contained in the particular mixture. The data obtained from the α, β, γ positions are indicated by the solid, dashed, and dotted lines, respectively. (△) 35 °C; (○) 30 °C; (◇) 25 °C; (●) 20 °C; (+) 15 °C.

from choline-deuterated DMPC with increasing density of negative surface charge are particularly evident in Figure 3. The changes in the quadrupole splittings with increasing quantity of DMPG were not linear, but rather appeared to approach a limiting value characteristic of the particular deutero-labeling position. It is evident that the influence of surface charge on the geometry of the choline head group as reported by ^{2}H NMR is retained in its essential features upon conversion of the lipids to their gel state. Thus, the counterdirectional changes in the quadrupole splittings from α- versus β-deuterated choline-labeled DMPC, which are observed to accompany the addition of surface charge in the liquid-crystalline state, are reproduced both qualitatively and quantitatively in the gel state. Moreover, the quadrupole splittings from DMPC-γ-d_9 also responded in a similar fashion to the presence of negative surface charges, whether the membrane lipids were in the gel or the liquid-crystalline states.

As a further demonstration of the fidelity with which the response of the choline head group to surface charges in the gel state mimics that which is observed in the liquid-crystalline state, Figure 4 shows correlation plots of the quadrupole splittings from each of the choline head-group deuteron-labeling positions as a function of the quadrupole splittings from the other labeling positions. For clarity, only the correlations at 15 and 35 °C are shown. In all cases, the transition from the liquid-crystalline to the gel state had no influence on the quality of the correlations. The correlation plots involving the quadrupole splittings from DMPC-α-d_2 (α–β, α–γ) were biphasic. At lower surface charge densities (small mole fractions of DMPG), the α–β and α–γ correlations were quite linear, having slopes equal to −0.94 (−1.03) and −0.096 (−0.108) at 35 °C (15 °C), respectively. At the highest surface charge densities (large mole fractions of DMPG), these correlations became increasing nonlinear, apparently because the quadrupole splittings from DMPC-α-d_2 approached a limiting value more abruptly than did those from DMPC-β-d_2 or DMPC-γ-d_9. In contrast, the β–γ correlations were linear over the entire range of surface charge densities and at all temperatures, having slopes equal to 0.11 and 0.10 at 35 and 15 °C, respectively.

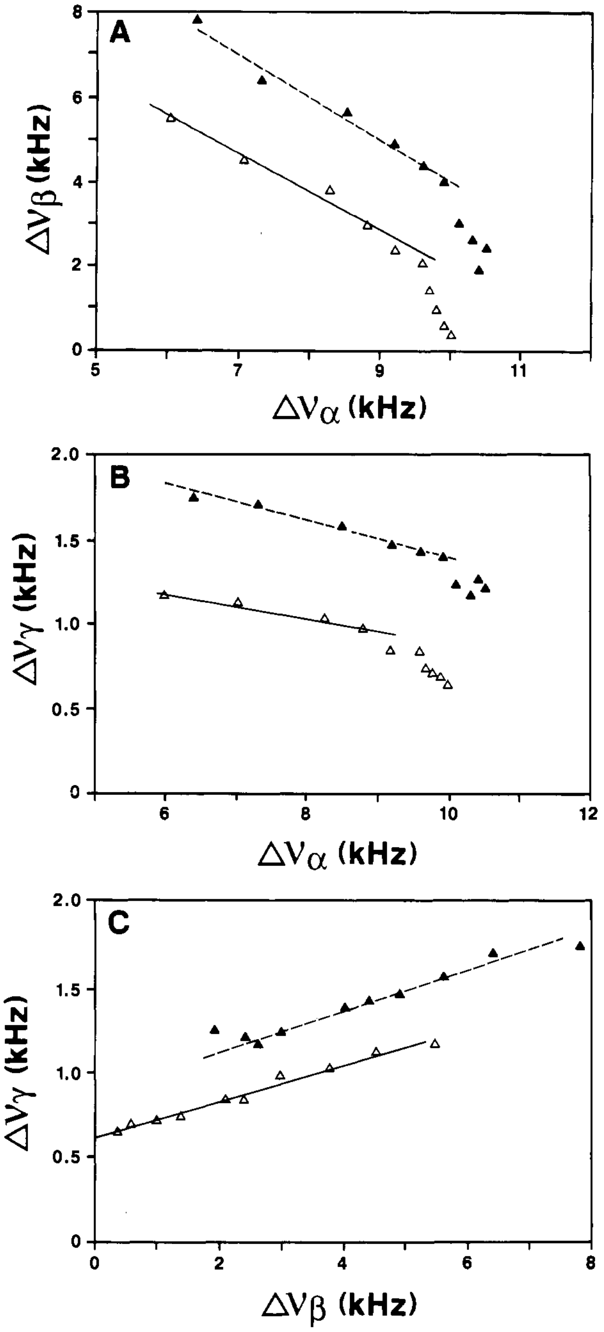


FIGURE 4: Correlation plots for DMPC mixed with various proportions of DMPG. The quadrupole splitting from a particular deutero-labeling position is plotted as a function of the value measured at another position under identical conditions. (A) α–β, (B) α–γ, and (C) β–γ correlations. Open symbols, 35 °C. Closed symbols, 15 °C.

DISCUSSION

The choline head group of phosphatidylcholine behaves as a "molecular voltmeter" in that it responds to and, via ^{2}H NMR, reports on the electrical charge present at the surface of lipid bilayer membranes. The results described in this paper demonstrate that, regardless of whether the membrane lipids assume a gel- or a liquid-crystalline-phase state, this "molecular voltmeter" effect is still operative. These findings have implications both for our understanding of the precise nature of the conformational change undergone by the choline group in response to surface charge influences and for the range of membrane-associated phenomena amenable to investigation via the "molecular voltmeter" technique.

The efficacy of the "molecular voltmeter" technique rests on the sensitivity of the ^{2}H NMR spectrum to molecular conformation as embodied in the relationship:

$$\frac{\Delta\nu_i}{\Delta\nu_Q} = \left|\frac{1}{2}(3\cos^2\phi - 1)S_f\right| \qquad (1)$$

where $\Delta\nu_Q \approx 125$ kHz, ϕ is the angle between the CD bond vector and the axis of motional averaging, and S_f is an order parameter representing the degree of off-axis "wobbling" about the motional axis. For this expression to be valid, the membrane lipids must undergo rotation about their long axes at rates which are fast on the ^{2}H NMR time scale. In liquid-crystalline bilayers this condition is readily met. In the densely packed, highly ordered gel state, rates of molecular motion are, in general, restricted. The consequences of motional deceleration for the ^{2}H NMR spectrum can range from a decrease in intensity and a distortion of the line shape at intermediate rates of motion to a complete absence of the desired conformational sensitivity in the spectrum as rates of motion decrease toward the static limit (Spiess, 1985). The ^{2}H NMR spectra obtained here show none of the line shape distortions characteristic of the "intermediate" time scale regime, nor do their widths approach the static limits expected for the "slow" motional regime. We conclude that rates of motion, in particular long axis rotations of the entire lipid molecule, are still sufficient, at the measuring temperatures employed here, to allow a straightforward interpretation of the ^{2}H NMR spectra in terms of molecular conformation. This conclusion is supported by numerous studies indicating that phosphatidylcholines in the gel state retain considerable motional freedom [e.g., see Campbell et al. (1979), Davis (1979), Marsh (1980), MacKay (1981), Wittebort et al. (1981), and Boroske and Trahms (1983). Inevitably, at lower temperatures, motional rates will decrease to the point of rendering the ^{2}H NMR spectrum intractable to analysis without resort to spectral simulations. This represents the lower temperature limit of the "molecular voltmeter" approach—that point at which the ^{2}H NMR spectrum is no longer sensitive to molecular conformational changes. For DMPC-containing bilayers, below 15 °C (i.e., at approximately the temperature of the pretransition in pure DMPC membranes, corresponding to the onset of rapid long axis rotations), spectral broadening and intensity loss are so severe as to make ready interpretation impractical.

A major conclusion drawn from the results reported here is that the conformational change undergone by the choline head group in response to surface charge influences is qualitatively and quantitatively similar both in the gel and in the liquid-crystalline phases. To understand how it is that, in the densely packed, highly ordered gel state, the choline head group remains free to respond to surface charge influences, we note, first, that membrane lipid thermotropic phase transitions, such as the gel to liquid-crystalline transition, are driven by cooperative interactions among the phospholipid fatty acyl chain segments. However, the response of phosphatidylcholine to surface charge is confined entirely to a conformational change in the phosphocholine head-group region of the molecule (Scherer & Seelig, 1989). In this sense, the choline polar head group is decoupled from the glycerol backbone and the fatty acyl chain regions of the phospholipid which are, all in all, oblivious to the presence or absence of surface charges. Second, it is not obvious that the dense molecular packing of the fatty acyl chain region in the gel state should prohibit the choline head group from responding to surface charge influences. For instance, in the case of a neutral membrane surface, the choline head group always adopts a conformation in which its P–N vector is aligned essentially perpendicular to the phospholipid's long molecular axis, whether in PC crystals, as determined by using X-ray crystallography (Pearson & Pascher, 1979), or in gel-state membranes or in liquid-crystalline membranes, as determined by using neutron diffraction [see Buldt and Wohlgemuth (1981) and references cited therein]. ^{2}H NMR data from liquid-crystalline phosphatidylcholine bilayers are also consistent with such a conformation of the choline head group (Seelig et al., 1977; Skarjune & Oldfield, 1979). Evidently, molecular packing considerations do not dictate the conformation preferred by the choline head group. NMR studies of phosphatidylcholines in the gel state indicate that, for the head-group region, the transition from the liquid-crystalline to the gel state reduces rates of motion but that this reduction occurs over many 10 of degrees [e.g., see Seelig (1978) and Campbell et al. (1979)]. The calorimetrically observable phase transition for pure DMPC bilayers can be as narrow as 1 °C or less (Mabrey & Sturtevant, 1978). So in this respect, as well, coupling between the head-group and hydrocarbon regions of the phospholipid is loose. Motional freedom implies conformational freedom. Third, it is apparent from earlier ^{2}H NMR studies in the liquid-crystalline state that surface electrical charge is the major determinant of the choline conformation (Seelig et al., 1987). A transition from the liquid-crystalline to the gel state will not substantially alter the membrane surface charge, provided the phospholipids remain thoroughly mixed. The present study shows that surface electrical charge remains a major determinant of the choline head-group conformation in the gel phase.

Our present understanding of the conformational change undergone by phosphatidylcholine in response to surface charge influences suggests that the entire choline head group tilts with respect to the plane of the membrane surface as the positively charged quaternary nitrogen is either attracted to or repelled by, respectively, opposite or like surface charges (Scherer & Seelig, 1989). For a neutral membrane surface, the choline head group lies nearly parallel (within 30°) to the plane of the membrane (Buldt et al., 1978). One might expect, therefore, that the response of the choline group to surface charge effects would be greater in the case of positive charges than negative charges. Due simply to steric effects, the ability of the choline group to approach close to the membrane surface will be limited, thereby opposing the electrostatic attraction between the positively charged choline quaternary nitrogen and the negatively charged surface. Indeed, Scherer and Seelig (1989) have shown that the response of the quadrupole splittings of head-group-deuterated phosphatidylcholine, as well as the corresponding change in the ^{31}P NMR chemical shift anisotropies, is far greater in the case of positively charged membranes than for negatively charged membranes. Nevertheless, for both positive and negative surface charges, the values of the quadrupole splittings do not change indefinitely but instead appear to reach some limiting value. This nonlinearity has, in fact, several possible origins. It may be due to steric hindrance (i.e., the angle of tilt of the choline group toward the plane of the membrane surface becomes prohibitively small), to errors in the estimation of the actual surface charge density (i.e., counterion condensation or p*K* changes occurring at high surface charge densities reduce the expected surface charge), to nonlinearity in the relationship between the angle of tilt of the choline group and the quadrupole splittings (i.e., this relationship is only approximately linear because the C–D bond vectors in choline are initially near to the "magic angle"), or to nonlinearity in the relationship between the angle of tilt of the choline group and the surface charge (i.e., the quaternary nitrogen of choline may respond to an exponentially decaying electrical potential located several angstroms distant from the plane of charge).

Since both positively charged and negatively charged amphiphiles produce similar limiting behavior in the response of the choline quadrupole splittings, steric effects cannot be the only explanation. Other laboratories, using electrophoretic mobilities as well as other means of measuring surface potentials in comparable membrane systems, have found good agreement between the surface potentials expected from Gouy–Chapman–Stern theory and those derived from experiment [e.g., see Winiski et al. (1989)], which argues that counterion condensation or p*K* changes at high surface charge densities do not account for the limiting values reached by the deuterium quadrupole splittings. In a later section, we present a model of the response of the choline head group to surface charge which correctly predicts the effect of charge on the size of the quadrupole splittings, and which shows that the limiting behavior at high surface charge densities is intrinsic to the nature of that response.

One may infer from the fact that DMPC, DMPG, and mixtures of DMPC/DMPG all have nearly identical gel- to liquid-crystalline-phase transition temperatures (Blume, 1983) that in both phase states the DMPC and DMPG components are thoroughly mixed. Since in both phases the choline group responds similarly to apparent equal surface charge densities, the ^{2}H NMR data support this contention. This leads to the important possibility that ^{2}H NMR of head-group-labeled phosphatidylcholines might provide a means of testing for lateral-phase separation of lipid species. Clearly, in the case of laterally segregated, charged phospholipid species, the effective local surface charge experienced by phosphatidylcholine will not equal that which would be calculated simply from a consideration of the global mole fraction of charged phospholipid species. The results reported here represent a case of ideal mixing and provide a base line for the investigation of the effects of charged phospholipids in cases of nonideal mixing, whether induced thermally or by the action of external agents such as membrane proteins. Regardless of the phase state of the laterally segregated species (gel or liquid-crystalline), the ^{2}H NMR technique should be capable of providing insights into the extent of lateral segregation via the accompanying effects on surface charge densities.

"Choline-Tilt" Model of the "Molecular Voltmeter". It is evident that changes in membrane surface charge density are transduced into changes in the quadrupole splittings from deuterated choline in PC-containing bilayers. However, the precise nature of the conformational response undergone by the choline head group is not yet certain. In the following, we describe a physical plausible model of this transduction mechanism which is consistent with the ^{2}H NMR data to date. We note beforehand that direct experimental evidence supporting this model is lacking. Our purpose in presenting the "choline-tilt" model is to delineate, and provide the impetus for, specific experiments which will clarify the effects of surface charge on the choline conformation.

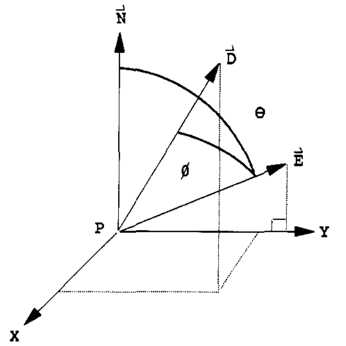


FIGURE 5: Schematic representation of the relative geometry of the electrical field vector **E**, the choline head-group vector **PN**, and the CD bond vector **D**. **E** is simultaneously the electrical field vector, the symmetry axis for head-group motion, and the normal to the plane of the bilayer.

Our model of the response of ^{2}H NMR quadrupole splittings is based on the premise that surface charges exert a torque on the choline head group, causing it to tilt with respect to the plane of the membrane surface. This in turn causes changes in the quadrupole splittings. This choline-tilt concept was proposed by Seelig and co-workers [e.g., see Scherer and Seelig (1989)] and has been placed on a quantitative footing by Bloom (Roux et al., 1989). The model which we describe here is based on that of Roux et al. (1989) with certain modifications. As will be shown, by considering the relationship between the electrical field at the membrane surface, the angle of tilt of the choline head group, and the geometry of deuterons attached to the choline, using this model one can predict all of the major features of the response of the quadrupole splittings to surface charge.

In Figure 5, the choline head group is represented as the vector **PN** ($\vec{PN}$ in figure). It is assumed to be essentially rigid and to be hinged about the C1–O torsion angle of the glycerophosphate C2–C1–O–P segment [the θ_1 torsion angle of Sundaralingam (1972)]. The angle between **PN** and the normal to the plane of the bilayer is denoted as θ. In the most general case, the electrical field generated by surface charges will consist of both axial and radial elements. However, bilayer lipids undergoing rapid ($\tau_r \sim 10^{-9}$ s) long-axis rotational motion will experience a net electrical field only in the direction parallel to the motional axis. Consequently, we assume that surface charges in hydrated lipid bilayers behave such that they produce a continuous electrical field originating at a plane level with the lipid phosphorus groups and directed normal to the membrane surface. In Figure 5, this electrical field is represented by the vector **E**. Note that the direction of **E** represents simultaneously the normal to the bilayer surface, the axis of motional averaging, and the electrical field at the surface. In this case, the torque, $\vec{\tau}$, exerted by the electrical field on a point charge q located at N corresponds to

$$\vec{\tau} = \mathbf{PN} \times \mathbf{F} \qquad (2)$$

where **F** is the electrical field force vector. The magnitude of this torque is then

$$\tau = qPN \sin \theta\sigma / 2\epsilon_0\epsilon_D \qquad (3)$$

where PN is the length of **PN**, σ is the surface charge density, ϵ_D is the dielectric constant of water, and ϵ_0 is the permittivity of free space. The surface charge density for a binary mixture of charged plus neutral lipids equals $X_f ze/D$, where X_f is the mole fraction of charged lipid of valence z and e is the unit charge, assuming that both charged and neutral lipids occupy identical cross-sectional areas, D.

Table I: Definition of the Angles in Figure 5 for Use as Direction Cosines in Equation 8

axis system	angle	axis system	angle
XPD	α	XPE	ϵ
YPD	β	YPE	λ
NPD	γ	NPE	θ
DPE	ϕ		

In the absence of surface charges, the choline head group prefers a conformation in which the PN vector lies nearly parallel (within 30°) to the membrane surface. This neutral equilibrium position represents the point at which all other forces acting on the choline head group sum to zero. These will include both intramolecular and intermolecular contributions. The conformational calculations of Pullman and Saran (1975) have shown that changes in the preferred torsion angle for θ_1 will be resisted primarily by the internal rotational potential arising from steric and other intramolecular interactions characteristic of the glycerophosphocholine group. Since the value of θ_1 (and hence θ) preferred at neutrality corresponds to the minimum of a symmetric potential energy well, we will model the resistance to changes in θ_1 (and θ) as originating with a countertorque **K** of magnitude

$$K = B \sin(\theta - 60) \tag{4}$$

which resists the electrical torque $\vec{\tau}$. B is the force constant measured in joules and may be estimated from the calculations of Pullman and Saran (1975).

At equilibrium, the torque/countertorque pair cancels such that

$$\vec{\tau} + \mathbf{K} = 0 \tag{5}$$

Letting $qPN/2\epsilon_0\epsilon_D = A$, the equilibrium position of the choline head group in the presence of surface charge is then readily shown to correspond to

$$\sin\theta = (1 + C^2)^{-1/2} \tag{6}$$

where

$$C = \frac{\sigma A B^{-1} + \cos 60}{\sin 60} \tag{7}$$

At neutrality ($\sigma = 0$), these functions give $\theta = 60°$. For positively charged surfaces ($\sigma > 0$), θ tends to decrease. For negatively charged surfaces ($\sigma < 0$), θ tends to increase.

The intermolecular contribution to resistance to changes in θ will be dominated by hard-core repulsions which become effective as θ approaches 90°. These we model as a Δ function of infinite height which simply restricts the **PN** vector to lie above the plane of the membrane surface so that in eq 6, for all $\theta > 90°$, $\theta = 90°$.

Having obtained an expression for the manner in which θ varies with σ, the next step is to derive the dependence of $\Delta\nu_Q$ on θ. In Figure 5, the CD bond vector **D** assumes an angle ϕ with respect to **E** such that the quadrupole splitting is determined by eq 1. For the most general case, the angle ϕ is related to θ via the expression:

$$\cos\alpha\cos\epsilon + \cos\beta\cos\lambda + \cos\gamma\cos\theta = \cos\phi \tag{8}$$

where the direction cosines of the vectors **D** and **E** are defined with respect to the axis system XYN and are listed explicitly in Table I. By taking **E** to lie in the YN plane, no loss in generality is suffered, while eq 8 reduces to

$$\cos\beta\sin\theta + \cos\gamma\cos\theta = \cos\phi \tag{9}$$

Substitution of eq 9 into eq 1 and employing eq 6 provide the relationship between the quadrupole splitting and the surface charge density:

$$\frac{\Delta\nu_i}{\Delta\nu_Q} = \left|\frac{1}{2}[3(\cos\beta_i\sin\theta + \cos\gamma_i\cos\theta)^2 - 1]S_f\right| \tag{10}$$

where the subscript i refers to a particular deutero-labeling position. A solution of eq 10 first requires estimating the values of β, γ, A, B, and S_f, all of which are assumed to be independent of the surface charge density.

Values for α, β, and γ may be obtained from X-ray crystallographic data (Pearson & Pascher, 1979). Alternately, reasonable values may be assumed and the model tested for its dependence on the particulars of those values. In taking the latter approach, as a first approximation we constrain the CD bond vector to lie at 90° to the **PN** vector of the choline head group. In this instance, eq 9 reduces to

$$\cos\beta\sin\theta = \cos\phi \tag{11}$$

Cos β may then be estimated from eq 1 by using the known values of the quadrupole splittings at neutrality and by assuming values at neutrality for θ and for S_f. θ is taken to be 60° as explained earlier. The size of S_f for the choline head group is problematic. It is certainly less than 0.66, which is the order parameter at the C1 position of the glycerol backbone (Gally et al., 1975). Moreover, it is likely that S_f decreases with increasing distance from the glycerol backbone. As a "first guess", we will set $S_f = 0.25$ for both the α and β positions of the choline head group.

When $\Delta\nu_\alpha = 6.0$ kHz, $\Delta\nu_\beta = 5.1$ kHz, $S_f = 0.25$, and $\theta = 60°$ are used, eq 1 and 11 yield two values each of cos ϕ and cos β, for each of the α and β positions. This is a consequence of the fact that ^{2}H NMR provides only the absolute value and not the sign of the quadrupole splitting. We arbitrarily choose those values of cos ϕ and cos β which allow the correct response of the quadrupole splittings upon changing the surface charge density, that is, cos $\theta = 0.679$ (0.474) and cos $\beta = 0.784$ (0.547) at the $\alpha(\beta)$ position at neutrality.

As a result of these approximations, the following expression dictates the dependence of the quadrupole splittings on the surface charge:

$$\Delta\nu_i = \left|\frac{1}{2}\{3[(1 + C^2)^{-1/2}\cos\beta_i]^2 - 1\}S_f\right|\Delta\nu_Q \tag{12}$$

where the subscripts i refer to the α or β position of the choline head group.

With $\epsilon_D = 80$, $\epsilon_0 = 8.8542 \times 10^{-12}$ C^2/Nm^2, $PN = 4.5$ Å, $e = 1.60219 \times 10^{-19}$ C, and $D = 70$ Å^2, the value of σA for a 100% charged lipid membrane ($X_f = 1$) equals 1.165×10^{-20} J. B is taken to be 6.95×10^{-21} J, corresponding to a potential energy barrier of 1 kcal/mol for rotations about the torsion angle θ_i of the magnitude involved here (Pullman & Saran, 1975).

Figure 6 shows the manner in which the quadrupole splittings changed with increasing mole fraction of either positive or negative charged lipids according to eq 12. Comparison of this figure with the data reported by Scherer and Seelig (1989) in their Figure 4 shows that our model is able to reproduce all of the major features of the dependence of the quadrupole splittings on surface charge density, both qualitatively and quantitatively. These include the counterdirectional changes in $\Delta\nu_\alpha$ versus $\Delta\nu_\beta$, the opposite effects of positive and negative charges on the quadrupole splittings from any one head-group position, the nonlinear dependence of the quadrupole splittings on the mole fraction of charged lipid as well as the limiting behavior at extreme surface charge densities, and the overall greater sensitivity of the quadrupole splittings to positive as opposed to negative surface charges. Moreover, as shown in Figure 7, the model provides a strictly

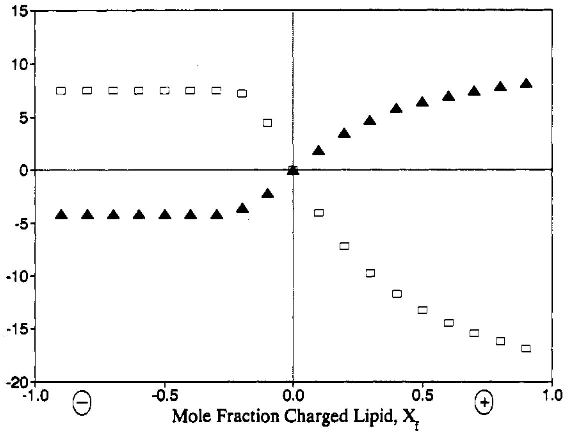


FIGURE 6: Variation of the deuterium quadrupole splittings of α (squares) and β (triangles) choline-deuterated phosphatidylcholine with the mole fraction of positively or negatively charged lipid as predicted by eq 12 (see text). The data are plotted in terms of the change in the quadrupole splitting for a particular charge state with reference to its value at neutrality.

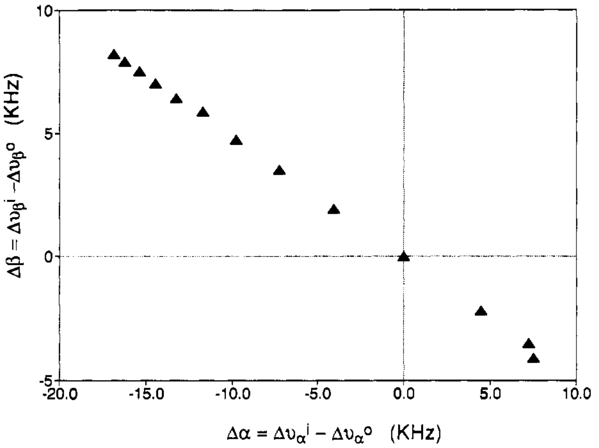


FIGURE 7: α–β correlation plots for the quadrupole splittings predicted via eq 12. The data are plotted in terms of the change in the quadrupole splitting for a particular charge state with reference to its value at neutrality.

linear α–β correlation plot, having a slope of −0.48, in excellent agreement with results reported by numerous investigators for various situations resulting in the deposition of positive charge at the membrane surface [e.g., see Seelig et al. (1987) and references cited therein; Roux et al., 1989]. However, such correlation plots for cases of deposition of negative charge at the membrane surface usually show slopes on the order of −1.0. The present model does not predict such a difference for positive and negative charges, the reasons for which will be discussed below.

The success of the choline-tilt model provides a context within which to understand the details of the relationship between surface charge and quadrupole splitting. The nonlinearities of this relationship, for example, are connected to the harmonic decrease in the magnitude of the electrical field force vector and the concomitant increase in the magnitude of the countertorque as the angle of tilt of the choline head group approaches the perpendicular the membrane surface. For negative surface charge densities, there exists the additional factor of the hard-core repulsions in the neighborhood of the membrane surface, leading to a more rapid desensitization of the dependence of the quadrupole splittings on surface charge density. This leads to the experimentally verifiable prediction that the limits to the change in the quadrupole splittings at large negative surface charge densities should differ according to the size of the head group attached to the negatively charged lipid. For example, the size of the head group of the lipids phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylinositol (PI) increases in the order PA < PG < PI. For positive surface charge densities, the size of the head group of the charge-bearing species should have little effect on the quadrupole splittings measured at large charge densities. In addition, the model suggests that there is really only one type of conformational change undergone by the choline head group in response to surface charge and that differences in the slopes of α–β correlation plots for positive versus negative surface charges are due to the additional hard-core repulsive potential which comes into effect for tilting of the choline group toward the membrane surface. A more realistic representation of this potential as other than a simple Δ function would likely improve the correspondence between experiment and model.

In choosing the parameters for the model, we have made a number of more or less valid approximations. Most questionable is the choice of the values for the angles β and γ. An equivocal choice for these angles is not possible from ^{2}H NMR alone. Rather, data from X-ray crystallography and NMR and other techniques must be considered together and a likely range of values assessed, in the spirit of Seelig et al. (1977) and Skarjune and Oldfield (1979). The success of the model is most dependent, however, on the choice of S_f. Too large a value of S_f leads to a strictly linear relationship between the quadrupole splittings and the surface charge density. This is because, for large order parameters, the changes in the quadrupole splittings correspond to small angular deviations of θ, while smaller order parameters correspond to large angular deviations. For small angular deviations from 60°, eq 3 and 4 are essentially linear. Another possible refinement to the model would be to consider, as well, differences in the location of the charges. We have considered only the case in which charges were distributed in a plane level with the lipid phosphate groups. Roux et al. (1989) have presented a model in which this aspect was a consideration, but no attempt was made to predict values for quadrupole splittings.

Some further comments are warranted concerning the interpretation of the angle of tilt of the choline group suggested by our model. The ^{2}H NMR quadrupole splittings (and, hence, the angle ϕ in eq 1 and any angles derived therefrom) represent a time and ensemble average. In addition to rapid long-axis rotations, the phosphatidylcholine experiences wobbling about its long molecular axis, while the choline head group itself experiences additional internal motional freedom. The order parameter S_f in eq 1 is a product of the order parameter representing the overall off-axis motion of the molecule and the internal order parameter for the choline head group. Since the order parameters reflect the statistical distribution of instantaneous orientations about a prefered orientation, averaged over time and all the molecules in the sample, one cannot define a unique angle θ describing the tilt of the choline group. Rather there is a statistical distribution of orientations about some average. It is the average orientation of the choline group which changes in the "choline-tilt" model.

One benefit of the "choline-tilt" model is that it provides a context within which to consider alternate explanations of the effects of surface charge on the ^{2}H NMR quadrupole splittings. One might argue, for example, that all of the observed effects are due to molecular density changes at the surface (i.e., head-group packing constraints) and that the effects of surface charge density are secondary. However, head-group packing constraints must necessarily increase S_f at all positions in the choline group, leading to increased values of the quadrupole splittings at all positions. The counterdirectional changes in the size of the quadrupole splittings from α- versus β-deuteriocholine argue against this interpretation. An interesting test to differentiate these possibilities would be to examine the response of the "molecular voltmeter" in the presence of cholesterol, which is known to decrease packing density at the surface and to cause the quadrupole splittings from all choline deutero-labeling positions to decrease (Brown & Seelig, 1978).

The nonlinear "α–β" and "α–γ" plots obtained here for DMPC/DMPG mixtures (Figure 4) are readily interpreted if the present model of the conformational change undergone by the choline group in response to surface charge effects is accepted as being correct. The linearity of such plots reveals the concerted nature of the conformational change undergone by choline; i.e., the entire head group undergoes the conformational change. The slope of such an "α–β" plot reveals the differential effects of the charge carrier on the choline α and β segments.

The α segment of the choline group, being closest to the "backbone" of the phospholipid, will experience any steric hindrance to a greater degree than the β- or γ-choline segments, which are more distant. As the choline moiety tilts further toward the membrane surface in response to the increasing negative surface chare, the α segment abruptly encounters steric hindrance to further conformational change. These steric effects are not yet sufficient to hinder a response at the β or γ positions which continue to show changes in their quadrupole splittings even at very high surface charge densities. Since the conformational change of the choline group at high surface charge densities is no longer a concerted one, it is necessary to invoke changes in the torsion angles along the choline carbon backbone in order to account for the observed quadrupole splittings. In modeling the conformation of the choline head group, it is generally held that free rotation occurs about the N–C(γ) and C(β)–N bonds (Seelig et al., 1977; Skarjune & Oldfield, 1979). Other torsion angles are not uniquely defined but tend instead to occur in restricted ranges which provide "quasi-conformations". A change in the range of torsion angles about the C(α)–C(β) bond would be consistent with the observed quadrupole splittings at high negative surface charge density. In all probability, some change in the torsion angle about the O–C(α) bond occurs as well, since the quadrupole splittings from the α position continue to change with increasing surface charge density, albeit with an altered dependence on the charge density.

In conclusion, it has been shown in this report that the response of the choline head group of phosphatidylcholine to surface charges is qualitatively and quantitatively similar both in the liquid-crystalline and in the gel state of membrane lipids. ^{2}H NMR of specifically choline-deuterated phosphatidylcholine reports on this response and can be exploited to monitor membrane surface charge effects in either phase state. In addition, we have presented a model of the response of the choline head group to surface charges in general which allows one to predict the effect on ^{2}H NMR quadrupole splittings and which provides a number of experimentally verifiable predictions of the correctness of the model.

Registry No. DMPC, 18194-24-6; DMPG, 61361-72-6; DMPA, 28874-52-4.

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