Deuterium NMR Studies of Surface Electrostatics

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Contents

I. Abstract

The utility of ^{2}H NMR spectroscopy of headgroup deuterated amphiphiles as a tool for measuring charge density at the surface of lipid bilayer membranes and polymer colloids is described. Attention is given to current ideas regarding the physical origin of the spectral changes observed in the presence of surface charges, and to several novel applications of the technique including determination of the pK_a of surface bound ionizable groups, measurement of equilibrium ligand-surface binding isotherms, observation of lateral phase separations of charged membrane species, resolution of charge differences at the inner versus outer monolayer surfaces of a lipid bilayer, and estimation of the electrostatic charge at the surface of a colloidal particle.

II. Why Lipid Bilayer Membranes are "Squishy Solids"

The distinct advantages of broadline deuterium NMR spectroscopy for characterizing molecular conformation and dynamics have long been recognized and have lead to a profusion of applications in virtually every area of the chemical sciences, and in biological membranes in particular. The architecture of biomembranes is unique: they consist of a continuous bilayer of amphiphilic lipids, wherein the hydrophobic lipid acyl chains are sequestered toward the bilayer interior while the hydrophilic lipid polar headgroups face the aqueous exterior (1). This self-assembled supramolecular aggregate provides a two-dimensional fluid matrix for the membrane proteins which are embedded in, and traverse the lipid bilayer, and which perform most of the active func-

tions of biological membranes.

This is not to suggest that the role of the lipid components is an entirely passive one. There are many classes of naturally-occurring lipid structures and the large variability of chemical structure within classes suggest specific roles for specific lipids, such as the second-messenger role of phosphatidylinositol (2). However, the vast majority of membrane lipids appear to serve primarily as modulators of the overall physico-chemical properties of the membrane and, thus, to influence membrane functioning only indirectly.

The large size and hindered motions of membrane lipids and proteins mean that orientation-dependent quantities such as the chemical shift anisotropy, the quadrupolar coupling, and the dipole-dipole interactions are not averaged to zero as they are in liquid-state NMR spectroscopy. Consequently, to study conformation and dynamics in membranes using NMR spectroscopy one resorts to solid-state NMR techniques. Nevertheless, biomembranes are a "fluid-mosaic" wherein individual molecules enjoy considerable freedom of whole-body translational and rotational diffusion, in addition to their internal rotational and vibrational motions (1). The two-dimensional bilayer architecture imposes an anisotropy on translational and rotational motions which permits only partial averaging of orientation-dependent interactions. In this sense biomembranes can be regarded, from an NMR perspective, as being squishy solids. The residual anisotropy which is manifest in the NMR spectrum of membrane species contains, therefore, a wealth of information on molecular conformation and dynamics. The ^{2}H NMR spectrum is dominated by a single interaction, the quadrupolar coupling, which means that the details of the average conformation and rates of conformational averaging are readily extracted from the spectrum. This fact, combined with the ability to specifically locate deuterium labels virtually anywhere within any biomolecule in an non-perturbing fashion, has propelled ^{2}H NMR into the forefront of techniques for investigating biomembrane structure and function.

The earliest ^{2}H NMR membrane investigations were concerned with describing conformation and flexibility in the acyl chain region of the lipid bilayer, quantifying the effects of the lipid gel-to-liquid-crystalline thermotropic phase transition, determining the impact of cholesterol, and examining lipid-protein interactions (3). A well-known and fundamental finding of such studies is that the conformation of the methylene segments of the acyl chain region of a lipid bilayer is characterized by a profile of decreasing orientational order with increasing distance from the bilayer surface. More recently, attention has focussed on the headgroup region of the lipid bilayer which, being proximal to the aqueous surroundings, is the first point of contact with ions, drugs, peptides, structural proteins, and any and all other species associating with and acting upon lipid bilayers (4). Perhaps the most profound finding of these latter ^{2}H NMR studies is that the conformation of lipid headgroups is strongly influenced by surface electrostatic charge.

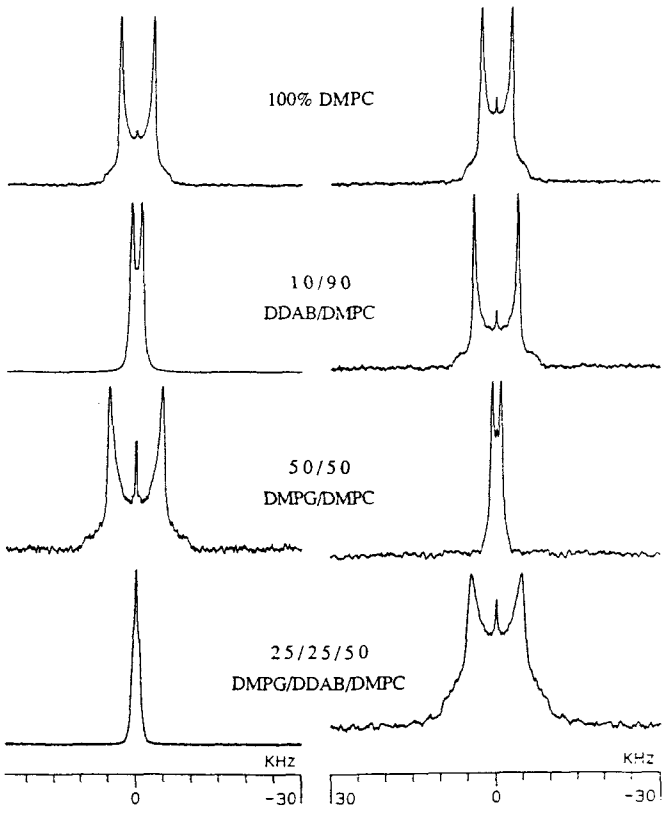


Figure 1: Surface charge effects on the ^{2}H NMR spectra of DMPC-α-d_2 (left) and DMPC-β-d_2 (right). All spectra were acquired at 35 °C in multilammellar vesicles having the molar compositions of, from top to bottom, 100% DMPC, 10:90 DDAB:DMPC, 50:50 DMPG:DMPC and 25:25:50 DMPG:DDAB:DMPC.

This article provides an overview of recent ^{2}H

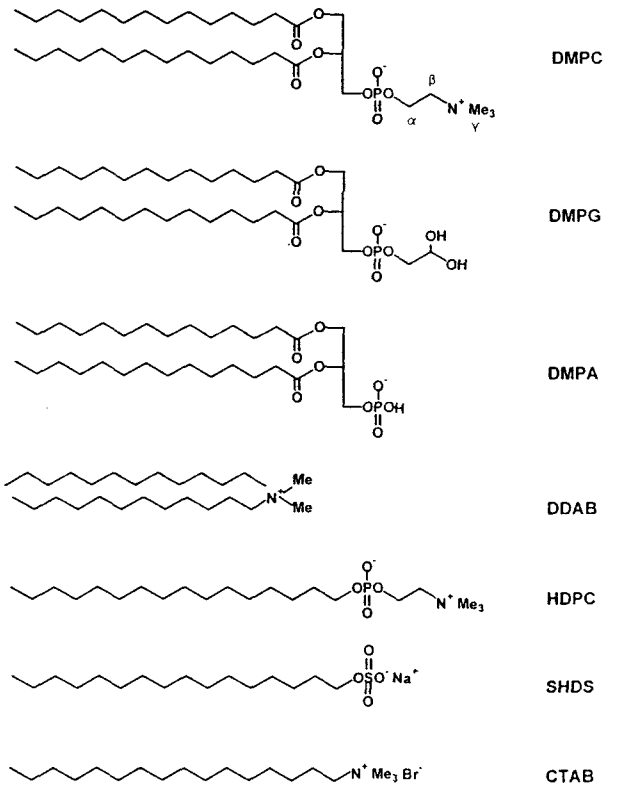


Figure 2: Chemical structures of the various amphiphiles of relevance in this report.

NMR studies of surface electrostatics in model membranes and in polymer colloids. First, the ^{2}H NMR evidence pointing to a specific conformational response of particular phospholipid headgroups to surface charge will be reviewed, and current thinking regarding the physical origin of this phemomenon will be discussed. Next, the exploitation of this response as an investigative tool will be illustrated with recent examples probing structure-function relationships in membranes. Finally, the manner in which the ^{2}H NMR surface charge "technology" can be transferred to study surface electrostatics in a non-biological system, such as polymer colloids, will be demonstrated.

III. How the "Molecular Voltmeter" Got Its Name

The fundamental ^{2}H NMR observation suggesting a specific conformational response of phospholipid headgroups to surface charge is illustrated in Figure 1. These are a series of ^{2}H NMR spectra of the zwitterionic phospholipid DMPC (dimyristoylphosphatidylcholine) deuterated at either the alpha or the beta methylene segments of the choline headgroup (5). The DMPC is assembled into a lipid bilayer membrane containing the anionic amphiphile DMPG (dimyristoylphosphatidylglycerol) and/or the cationic amphiphile DDAB (didodecyldimethylammonium bromide). The chemical structures of these and other amphiphilic molecules of concern in this article, along with choline headgroup deuterium-labelling nomenclature, are shown in Figure 2. The ^{2}H NMR spectrum for a 100% DMPC membrane consists of an axially-symmetric Pake pattern characterized by a single quadrupolar splitting, corresponding to the separation, in Hz, between the two maxima or "horns" in the spectrum. In lipid membranes above their gel-to-liquid-crystalline phase transition temperature the lipids experience rapid anisotropic motional averaging about their long molecular axes. Consequently, the residual quadrupolar splitting, $\Delta\nu_i$, is then conveniently related to molecular conformation according to equation (1),

$$\frac{\Delta\nu_i}{\Delta\nu_0} = |(1/2)(3\cos^2\theta - 1)|S_f \qquad (1)$$

where $\Delta\nu_0$ is the static quadrupolar splitting (125 kHz for aliphatic deuterons), θ is the angle between the C-D bond vector and the director axis of motional averaging (taken to lie parallel to the bilayer normal and, hence, to the long lipid axis), and S_f is an order parameter ($0 \leq S_f \leq 1$) quantifying the degree of off-axis wobbling of the choline group.

When the membrane surface is negatively charged as a result of mixing DMPC with DMPG the quadrupolar splitting from DMPC-α-d_2 increases while that from DMPC-β-d_2 decreases. When positive charges are deposited at the membrane surface by mixing in DDAB the quadrupolar splitting from DMPC-α-d_2 decreases while that from DMPC-β-d_2 increases. This counter-directional change in the quadrupolar splittings for the alpha versus the beta deutero-labelling positions suggests that the entire choline headgroup undergoes a specific, concerted, conformational change in response to the presence of surface charges. It cannot be due merely to a generalized increase or decrease in orientational order within the choline headgroup since this would increase or decrease the quadrupolar splittings from both deutero-labelling positions simultaneously. Moreover, the fact that positive and negative charges have opposite effects

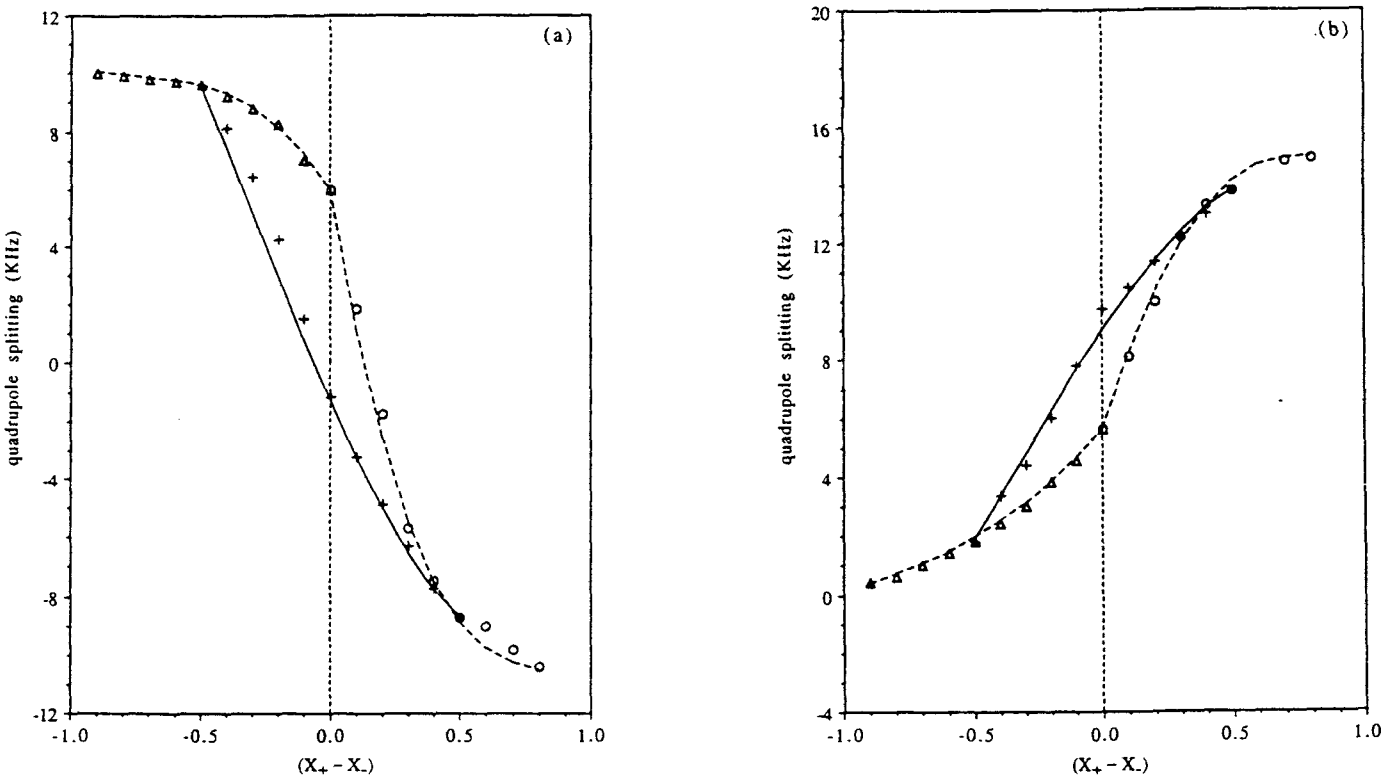


Figure 3: Surface charge effects on the ^{2}H NMR quadrupolar splittings of DMPC-α-d_2 (a) and DMPC-β-d_2 (b). All values were recorded at 35 °C. Quadrupolar splittings are plotted versus the net mole fraction of charged amphiphile (X_+ - X_-), where X_+ and X_- correspond to the mole fractions of DDAB and DMPG, respectively. The vertical dotted line indicates nominal charge neutrality (i.e. $X_+ = X_-$). (o) DDAB:DMPC mixtures. ($\triangle$) DMPG:DMPC mixtures. (+) DDAB:DMPG:DMPC mixtures. The solid lines are fits to the ternary mixtures data calculated as described in the text.

indicates that it is specifically the surface charge which is triggering the change in the quadrupolar splittings.

Figure 3 illustrates in detail how the quadrupolar splittings from DMPC-α-d_2 and DMPC-β-d_2 depend on the net mole fraction of charge in either binary lipid mixtures of DMPC+DMPG and DMPC+DDAB, or ternary mixtures of DMPC+DMPG+DDAB. Focusing for the moment on the binary lipid mixtures, it is evident that the essential features of the response of the choline headgroup to surface charge as manifest in the ^{2}H NMR spectra are reproduced at all mole fractions of charged species, as first reported by Scherer and Seelig (6). Furthermore, the changes are progressive with increasing mole fraction of the charged species, leading to differences between the extremes on the order of 20 kHz (the quadrupolar splittings are precise to within 100 Hz).

Such effects are of more than academic interest because a multitude of charged species which associate with membrane surfaces induce a near identical response in the choline headgroup. These include charged phospholipids (7-9), ions (7,8,10-13), anaesthetics (14,15), and peptides (16-20): a group embracing some of the most potent bioeffector molecules currently identified. All share two features in common: .they bind to lipid membranes and in doing so they induce a surface charge. In contrast, neutral species such as cholesterol or the neutral glycolipids, although they associate strongly with lipid bilayers, induce no such response observable via ^{2}H NMR. Charged species which fail to bind to lipid bilayers likewise fail to elicit this characteristic change in the quadrupolar splittings from choline headgroup deutero-labelled PC. Thus, the choline headgroup is described as behaving like a "molecular voltmeter", responding universally to any and all changes in membrane surface charge regardless of their detailed origin.

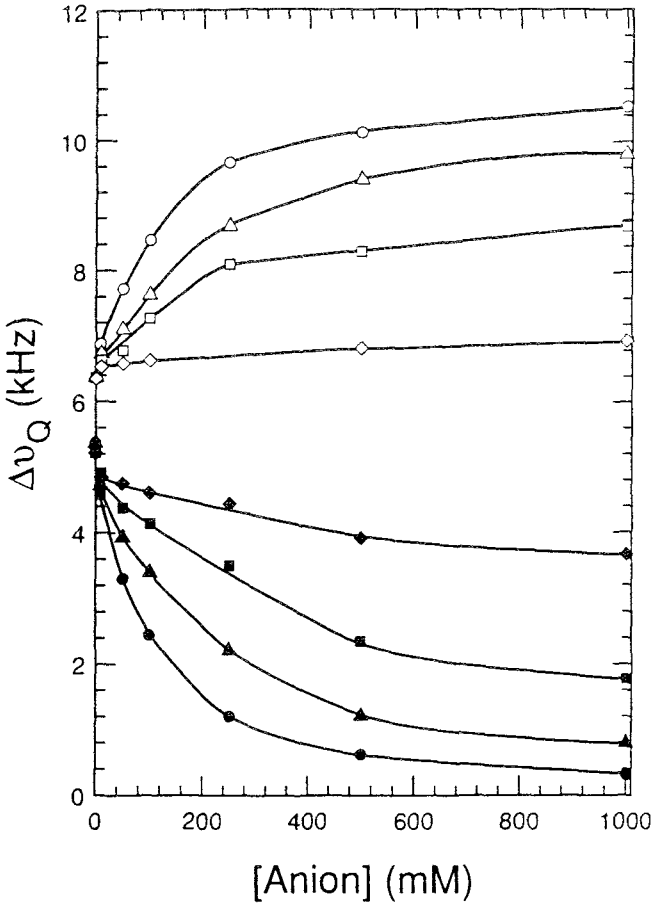


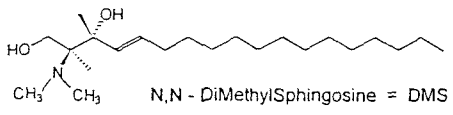
Figure 4: Anion binding effects on the quadrupolar splitting from POPC-α-d_2 (open symbols) and POPC-β-d_2 (closed symbols). Perchlorate ($\circ$) K_a = 115 M-1. Thiocyanate ($\triangle$) K_a = 80 M^{-1} Iodide ($\square$) K_a = 32 M^{-1} Nitrate ($\diamond$) K_a not determined.

IV. How the "Choline Tilt" Model Came and Went

In seeking an explanation for the conformation change apparently undergone by the choline headgroup of DMPC in response to the presence of surface charges ones attention is immediately drawn to the zwitterionic character of the phosphocholine moiety. Both theory and experiment indicate that the phosphocholine headgroup possesses a large dipole moment, on the order of 20 Debyes (21,22). Akutsu and Seelig (11) first proposed that the choline group tilts with respect to the plane of the bilayer surface under the influence of an electrical field emanating outwards from that surface. The hinge about which this tilt occurs would be the gycerol carbon-oxygen-phosphorus bond (6). Since the angle of tilt of the P-N vector of the PC headgroup for a nominally neutral membrane surface is about 15 degrees above the surface (23), positive charges would cause it to tilt upwards and away from the surface while negative surface charges would cause it to tilt downwards and towards the surface. This "choline tilt" model was formalized by Bloom and co-workers (20) who expressed the equilibrium angle of tilt of the choline group in terms of the net torque-countertorque experienced due to the action of opposing forces seeking to align the choline dipole in the direction of the surface electrical field versus resistance due to inter- and intra-molecular steric considerations. Macdonald *et al.* (24) demonstrated that by considering the internal torsion angles of the choline headgroup, available from X-ray crystallography data (25), such a "choline tilt" model reproduces all of the essential features of the dependence of the ^{2}H NMR quadrupolar splittings on surface charge in binary lipid mixtures, including the counter-directional changes for the alpha versus beta deutero-labelling positions, the opposite effects of positive versus negative surface charges, and even the qualitatively greater impact of a given level of positive versus negative surface charge on the absolute change in the value of the quadrupolar splittings.

Further support for this physical description comes from studies of other phospholipids such as phosphatidylserine, wherein the serine headgroup with its large dipole moment yields an analogous ^{2}H NMR response to surface charges (20). Phosphatidylglycerol, on the other hand, undergoes no comparable concerted conformation change observable via ^{2}H NMR (21). Calculations of the size of the dipole moment associated with the glycerol headgroup indicate that it is a fraction of that of the choline headgroup (21).

More recent studies, however, force one to reconsider the details of the "choline tilt". For example, if one considers ternary mixtures of DMPC+DMPG+DDAB, the 50/25/25 mixture will be nominally overall neutral. However, the corresponding ^{2}H NMR spectra, shown at the bottom in Figure 1, are very different from those obtained with 100% DMPC-α-d_2 or DMPC-β-d_2 lipid bilayers. In Figure 3 the ^{2}H NMR quadrupolar splittings for various ternary mixtures of DMPC+DMPG+DDAB are illustrated for the case that the mole percent of DMPC remains constant at 50% while the propor-



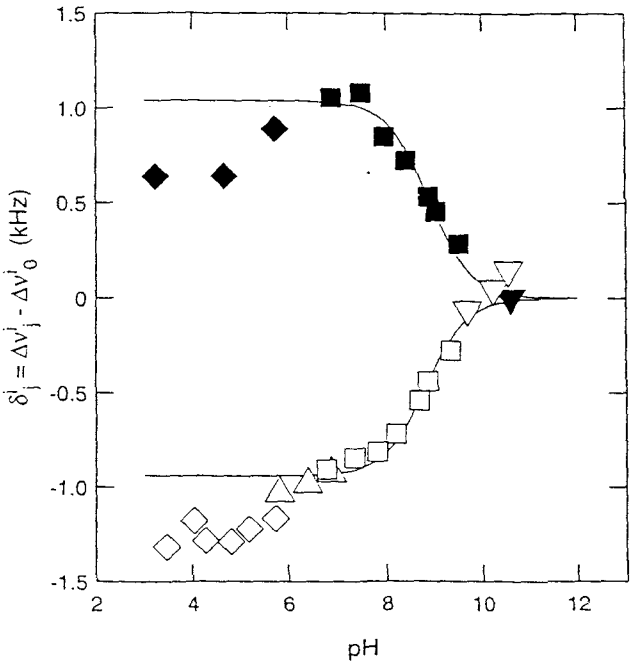


Figure 5: Experimental and simulated ^{2}H NMR DMS titration curves. The quadrupolar splittings are expressed as the net difference plus or minus 5 mole% DMS at the indicated pH. The different symbols denote the different buffers used over different ranges of pH: ◇ for citrate, △ for MES (2-[N-morholino]-ethanesulfonic acid), □ for BTP (3-bis[tris(hydroxy-methyl)-methylammino]propane), and ▽ for CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid). Open symbols correspond to experimental values obtained with POPC-α-d_2 and closed symbols to the experimental values obtained with POPC-β-d_2 The solid lines are simulations of the titration curves using equation (2) as per the text, with the values of k^α and k^β corresponding to the slopes of the DMS-$\Delta\nu$ calibration curves determined independently, and the pK_a of DMS equal to 8.8 for both deuteron-labelling positions.

tions of the anionic and cationic species are varied inversely. Obviously, the values of the quadrupolar splittings from binary versus ternary mixed lipid membranes of nominally identical surface charge do not correspond.

Reconciliation of this difficulty is achieved when one recognizes that the *perturbation* of the quadrupolar splitting for a given ternary mixture relative to the value measured for a 100% DMPC membrane is the *sum of perturbations* associated with the corresponding binary mixtures. Specifically, if one wishes to predict the change in the quadrupolar splitting for the ternary DMPC+DMPG+DDAB (50/25/25) mixture relative to that for 100% DMPC, one simply adds together the changes observed for the binary mixtures DMPC+DMPG (75/25) and DMPC+DDAB (75/25). By applying this principle to the data in Figure 3 one obtains a quite satisfactory correspondence between experiment and prediction, as shown by the solid line overlying the data for the ternary mixtures. One must conclude that, rather than responding to the net surface charge, the choline group is engaging in a series of one-on-one encounters with the different species present in its immediate surroundings, each of which perturbs the choline conformation in a particular fashion, and each of which contributes statistically to the observed average conformation as manifest in the ^{2}H NMR quadrupolar splittings. The further significance of these studies of ternary mixtures is that they demonstrate how to deal with ^{2}H NMR data from complex mixtures more closely approximating those found in real biological membranes.

As to the nature of the conformational perturbation experienced by the choline group, several recent reports have cast new light on this question. The counter-directional change in the size of the quadrupolar splittings from the alpha versus beta deutero-labelling position characteristic of the response of the choline group to surface charge can be reproduced by controlled dehydration of 100% PC membranes (26), or by increasing hydrostatic pressure on such membranes (27), which likewise should dehydrate the membrane surface. Moreover, a recent simulation study of the response of the conformation of the choline headgroup to surface charge effects, employing a sophisticated Langevin dynamics routine, concluded that the choline headgroup must undergo changes in its internal torsion angles,

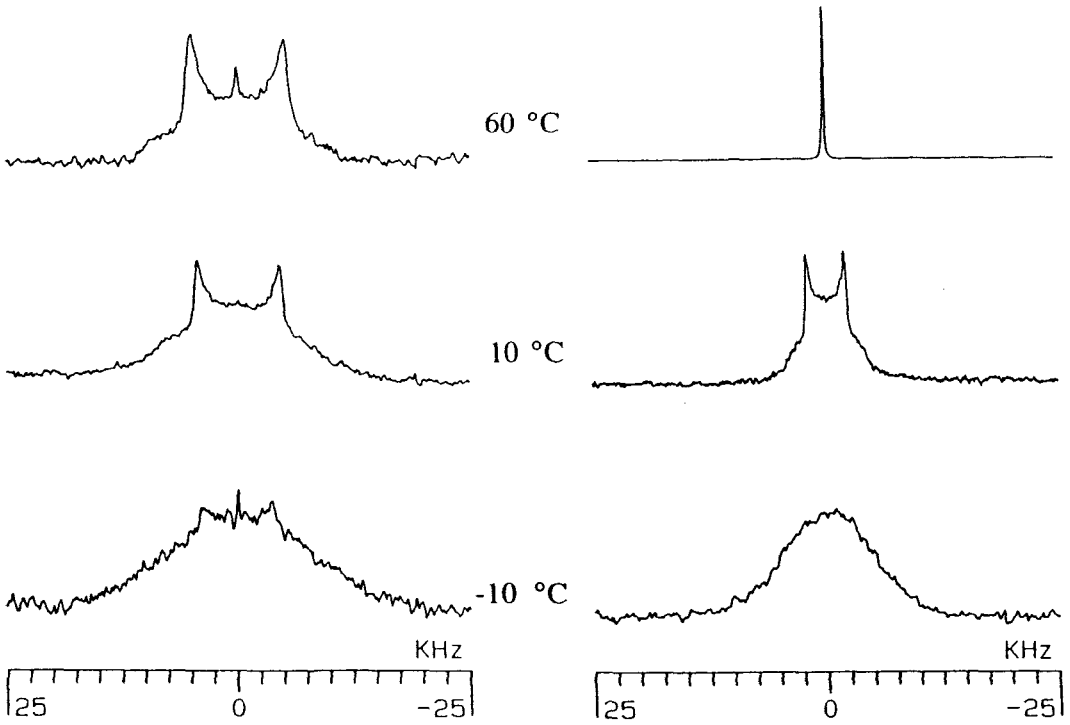


Figure 6: Temperature dependence of the ^{2}H NMR spectra of POPC-α-d_2 (left) and POPC-β-d_2 (right) mixed 50:50 with DMPA. At 60 °C (top spectra) POPC and DMPA are both above their respective phase transition temperatures and the lineshapes and quadrupolar splittings reflect a homogeneous fully-mixed fluid phase. At -10 °C (bottom spectra) POPC and DMPA are both below their respective phase transition temperatures as reflected by the broad ill-defined spectral lineshapes. At 10 °C (middle spectra) partial phase separation of DMPA-enriched gel-like domains has occurred and the spectra consist of a superposition of gel-like and fluid-like components. Note that the quadrupolar splitting of the fluid-like component is decreased for POPC-α-d_2 and increased for POPC-β-d_2 relative to the value measured at 60 °C.

in addition to an overall tilting relative to the surface plane, if the observed quadrupolar splittings are to be explained (28).

Finally, it should be noted that to date there has been no experimental evidence supporting the "choline tilt" model beyond ^{2}H NMR. Since the model predicts changes in displacement of the choline quaternary methyls of about 5 Å with respect to the plane of the phosphorus atoms between the extremes of highly negatively and positively charges surfaces, it may be possible to observe such a displacement using neutron diffraction techniques. Such studies are currently underway (K.R. Jeffrey, personal communication). Recent advances in solid state NMR techniques for defining ^{13}C-^{1}H, ^{31}P-^{1}H and ^{1}H-^{1}H dipolar couplings in phospholipids can be used to define conformation and could be applied to the question of the conformation change undergone by choline in response to surface charge (29).

V. When Ions Bind

The great utility of the "molecular voltmeter" is its ability to monitor membrane surface charge regardless of the origin of those charges. Many bioactive substance such as drugs, anaesthetics, ions, peptides, and proteins bind to membranes in the course of exerting their activities. Using ^{2}H NMR insight may be obtained regarding how such species interact with membranes and how their binding affects, and is affected by, surface charge. Many examples now exist in the literature, but the case of the Hofmeister series of anions fully illustrates the potential of the technique (14). Figure 4 shows the dependence of the quadrupolar splittings from membranes composed of POPC-α-d_2 or POPC-β-d_2 on the concentration of various aqueous anions present in the aqueous bathing medium, all as the sodium salts. POPC corresponds to 1-palmitoyl-2-oleoyl phosphatidylcholine, which re-

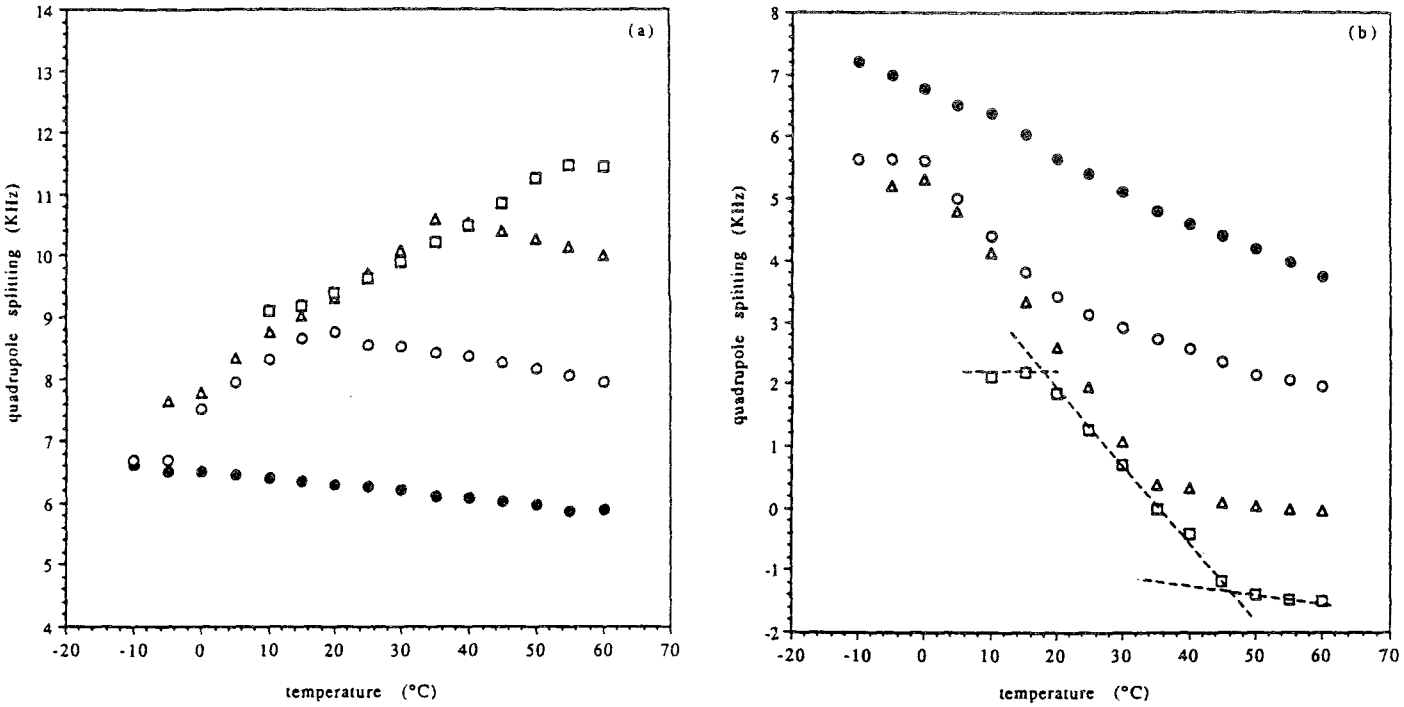


Figure 7: Temperature dependence of the ^{2}H NMR quadrupolar splittings of POPC-α-d_2 (a) and POPC-β-d_2 (b) in various binary mixtures with DMPA. (•) 0% DMPA. (○) 20% DMPA. (△) 50% DMPA. (□) 80% DMPA. The dashed lines show the manner in which the high-temperature and the low temperature breakpoints were determined.

tains the "molecular voltmeter" response of DMPC but displays a much lower and more physiologically-relevant thermotropic gel-to-liquid-crystalline phase transition temperature. The anions constitute a so-called Hofmeister series in which water-structure-breaking, or chaotropic, propensity increases in the order $NO_3^- < I^- < SCN^- < ClO_4^-$ (30). As Figure 4 demonstrates, this is the same order of sensitivity of the quadrupolar splittings to the presence of such anions. The direction in which the quadrupolar splittings change (alpha increasing and beta decreasing) is indicative of the accumulation of negative charge at the surface of the lipid bilayer.

The process of extracting association constants from such data can be presented here only in outline. First, the sensitivity of the quadrupolar splittings to the presence of a known level of binding of a particular charged ligand must be calibrated, over at least a limited range, using an independent assay of ligand binding levels. Interestingly, when a comparison is made of such sensitivities across a series of ligands it reveals that those compounds expected to penetrate most deeply into the interior of the lipid bilayer are those most effectively sensed via the "molecular voltmeter" (14). This confirms a prediction from Bloom and coworkers' torque-countertorque model (20). Next, with this calibration in hand one converts quadrupolar splittings to levels of ligand binding and, thence, to surface charge densities. Using Guoy-Chapman-Stern theory one next estimates the surface electrical potential. Subsequently, one may employ the Boltzmann equation to estimate the concentration of the particular ligand present in the aqueous layer immediately adjacent to the surface where it is available to bind to sites on the surface. Finally, having in hand both the levels of bound ligand and the relevant interfacial concentration of free ligand, one may proceed to fit model binding isotherms and extract association constants.

The association constants for binding of the Hofmeister anions in Figure 4 were obtained by fitting a simple Langmuir binding isotherm to the ^{2}H NMR data. They reveal that affinity for the membrane surface increases in the same order as increasing chaotropic properties. In effect the ability to loose waters of hydration, which correlates with chaotropism, is intrinsic to any membrane surface binding event. Moreover, the ability to penetrate

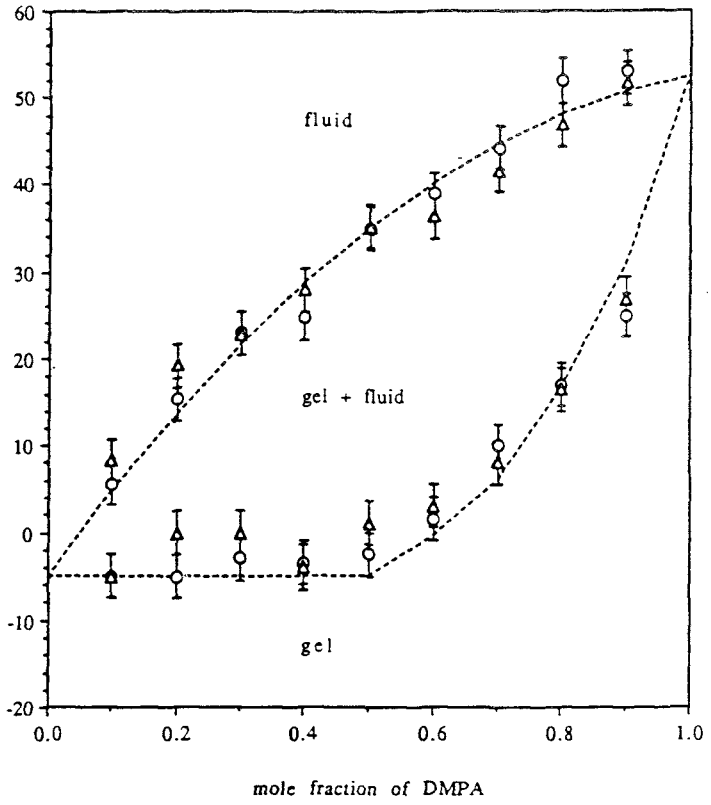


Figure 8: Temperature-composition phase diagram of POPC:DMPA mixtures obtained from the high- and low-temperature breakpoints of the quadrupolar splittings temperature dependence. (○) POPC-α-d_2 (△) POPC-β-d_2

the organized water layer coating all membrane surfaces, likewise correlated with chaotropism, will favour binding. If the membrane surface is made initially positively charged, for instance by mixing POPC with DDAB, anion binding levels increase radically (12). When surface charge effects are properly accounted for one discovers that the intrinsic affinity of the anions has not been altered by the presence of the cationic surface charges. It is apparent then that surface charge and ligand binding are two sides of the same coin, the one influencing and regulating the other. The potential for biological control through such a mechanism is enormous, but as yet can only be described in dim outline.

VI. A Question of Equilibrium (Acid-Base)

Another capability provided by the ^{2}H NMR technique is the measurement of the pK_a of ionizable groups present at a membrane surface, as first demonstrated by Watts and Poile (31). We have carried out a ^{2}H NMR-based determination of the pK_a of DMS (dimethyl-D-sphingosine), a potent inhibitor of the regulatory enzyme protein kinase C (PKC) (32). Positive charge and an eighteen carbon chain are the two prerequisites for inhibitors of PKC, but DMS is more potent even than its non-methylated parent compound sphingosine. One possible explanation for this difference is that the membrane-bound pK_a of DMS differs from that of sphingosine.

Figure 5 shows the pH dependence of the quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2 in the presence of 5 mole% DMS (dimethyl-D-sphingosine) (33). Addition of DMS at pH 7.0 decreases the quadrupolar splitting obtained from POPC-α-d_2 and increases that obtained from POPC-β-d_2. This is the behavior expected in the presence of a species which induces a positive surface charge. One immediately concludes that DMS associates with bilayer lipid membranes and orients such that its cationic dimethyl amino group is located at the membrane surface. When the pH of the aqueous solution bathing the lipid bilayers is varied in the range from 3 to 12, the ^{2}H NMR quadrupolar

splitting from both POPC-α-d_2 and POPC-β-d_2 in the presence of DMS changes progressively. In the absence of DMS the quadrupolar splittings are virtually independent of the solution pH in this range, with the exception of values below pH 5.0. The values of the quadrupolar splittings shown in Figure 5 are expressed as the difference in the quadrupolar splitting with versus without 5 mole% DMS at any one particular pH. At all pH values below 7.0 the quadrupolar splittings are characteristic of the presence of cationic surface charges. At all pH values above 10.0 the quadrupolar splittings indicate a neutral membrane surface. The pH dependence at intermediate pH values is consistent with a titration of a weak base, such as that of the dimethyl amino group of DMS, from its protonated (cationic) form at low pH to a deprotonated (neutral) form at high pH. Consequently, the quadrupolar splitting is a good measure of the degree of protonation of DMS, and provides a means to calculate the corresponding pK_a.

One may predict the change in the quadrupolar splitting due to the presence of a given level of DMS for a given deuteron labelling position and a given pH, assuming a particular pK_a, using equation (2):

$$\delta_j^i = \Delta\nu_j^i - \Delta\nu_o^i = \frac{k^i[T]_j}{1 + K_a/[H^+]} \qquad (2)$$

where the superscript i delineates the alpha versus beta deutero-labelling position, the subscript j designates a particular total DMS concentration, and k^i is the calibrated sensitivity of the quadrupolar splittings to DMS. The line-of-best-fit, as judged by nonlinear least squares fitting of the predicted to the experimental POPC-α-d_2 and POPC-β-d_2 quadrupolar splittings is shown in Figure 5, and corresponds to the case $pK_a = 8.8$. The poor fit at low pH values is attributed to buffer specific, rather than pH specific, effects since anomalies are observed at low pH with or without DMS. One concludes that at physiological pH over 90% of DMS exists in a cationic form. However, sphingosine, with its membrane-bound pK_a of 8.5 also exists largely as a cationic species. Thus, the differences in their relative potencies as inhibitors of PKC does not lie in differences in their cationic versus neutral populations. Rather, one must look to specific stereochemical binding to a binding site on PKC.

VII. Its Just a Phase They're Going Through

It is known that in biological membranes there can exist long-lived domains, or phases, of distinct composition, supporting distinct biological functions. One of the challenges of membrane studies is to describe such heterogeneities and to evaluate their functional consequences. Recently, it has been shown that the "molecular voltmeter" provides a new means of exploring phase separation and phase composition in lipid membranes (34). To make such a demonstration one requires a system in which lateral phase separation into domains of distinct composition, and surface charge, can be controlled at will. Binary mixtures of POPC plus DMPA (dimyristoylphosphatidic acid) permit one to control the degree of phase separation of the high-melting DMPA (52 °C) from the low-melting POPC (-5 °C). For instance, differential scanning calorimetry (DSC) on such POPC+DMPA mixtures reveals the presence of distinct endothermic events for the two lipids. Thus, at temperatures above the phase transition of both POPA and DMPA, both lipids are fluid-like and are fully and randomly mixed with one another. At temperatures below the phase transition of both POPC and DMPA, both lipids are solid-like and largely demixed from one another. At temperatures in between the phase transitions of the two lipids, fluid-like and solid-like phases coexist, the former enriched with respect to POPC and the latter enriched with respect to DMPA.

Figure 6 shows a series of ^2H NMR spectra from POPC-α-d_2 and POPC-β-d_2 mixed 50:50 with DMPA. The three temperatures shown correspond to temperatures above (60 °C), in between (10 °C), and below (-10 °C) the thermotropic phase transitions of POPC and DMPA. At 60 °C the spectra are characteristic of a fluid-like, ideally-mixed, homogeneous, liquid-crystalline lipid bilayer, in that one observes single-component, axially symmetric Pake patterns with residual quadrupolar splittings reflecting the presence of 50 mole% negatively-charged DMPA (i.e. 10 kHz for POPC-α-d_2 and $\sim$ 0 kHz for POPC-β-d_2). At -10 °C the spectra are characteristic of a solid-like, motionally- restricted gel phase, in that one observes broadened, poor signal-to-noise, ill-defined spectral lineshapes. At +10 °C the spectra are characteristic of co-existing fluid-

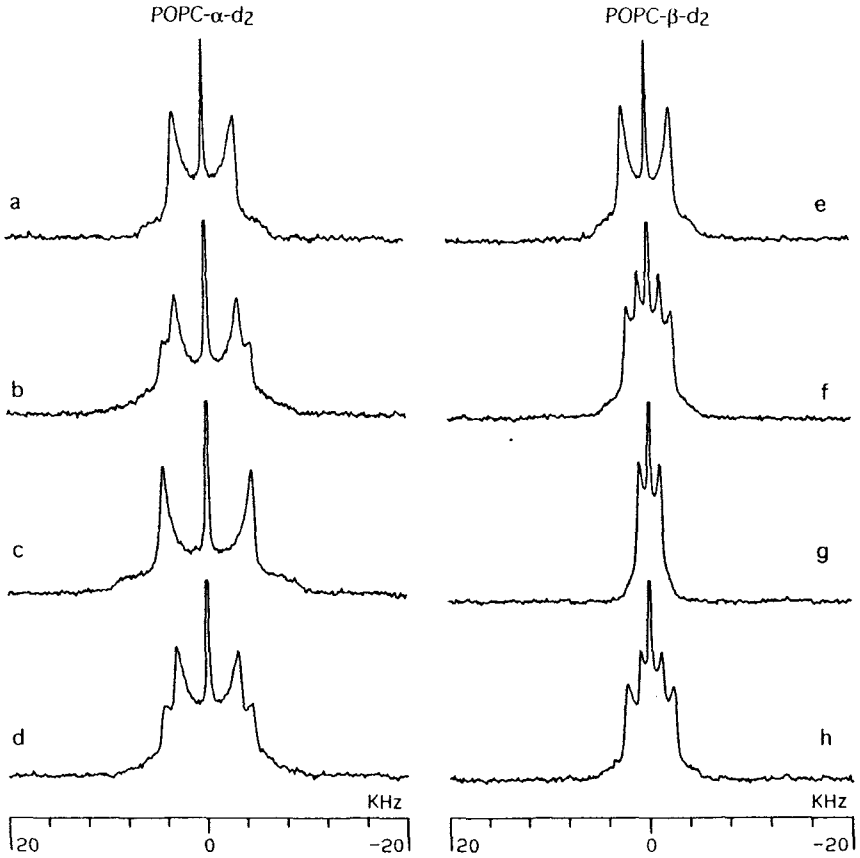


Figure 9: Resolution of the inner and outer monolayers of the lipid bilayer in Giant Unilamellar Vesicles (GUVs). An asymmetric perchlorate ion distribution was imposed by exchanging the exterior vesicular solution isotonic for $NaClO_4$ in GUVs composed of POPC:CHOL, 70:30. Spectra on the left were recorded using POPC-α-d_2 while those on the right were recorded using POPC-β-d_2 (a and e) Initial spectra prior to perchlorate addition. (b and f) 150 mM perchlorate added to the vesicle exterior. (c and g) End point spectra after freeze-thaw. (d and h) Addition spectra generated from the 1:1 addition of the initial and end point spectra (a+c and e+g).

like and solid-like phases with only slow-exchange of lipid between the two, in that they consist of superposition of solid-like and fluid-like components. The latter point may be proven definitively using an inversion recovery sequence with an appropriately chosen delay. It is highly significant that the quadrupolar splitting of the fluid-like spectral component in the phase co-existence region does not simply correspond to that of the 60 °C spectrum, but instead indicates a much lower surface charge density of DMPA.

The detailed dependence of the quadrupolar splittings on temperature for several POPC+DMPA mixtures, as shown in Figure 7, reflects the depletion of the DMPA content of the remaining fluid-like phase domains in the co-existence region following the onset of phase separation of solid-like, DMPA-enriched domains. As temperature is decreased, one first observes a generalized increase in the quadrupolar splitting from the POPC+DMPA mixtures for both POPC-α-d_2 and POPC-β-d_2 which mirrors the temperature dependence observed with 100% POPC and merely reflects the temperature dependence of the order parameter S_f in equation (1). At a particular temperature characteristic of a given POPC+DMPA mixture there is a sudden shift, or breakpoint, in the temperature dependence of the quadrupolar splittings towards values characteristic of lower amounts of negative charge. In the POPC+DMPA mixtures both deutero-labelling position behave indentically in this regard while the 100% POPC membranes show no such effect. It seems sensible to attribute this breakpoint to the onset of lateral phase separation of DMPA-

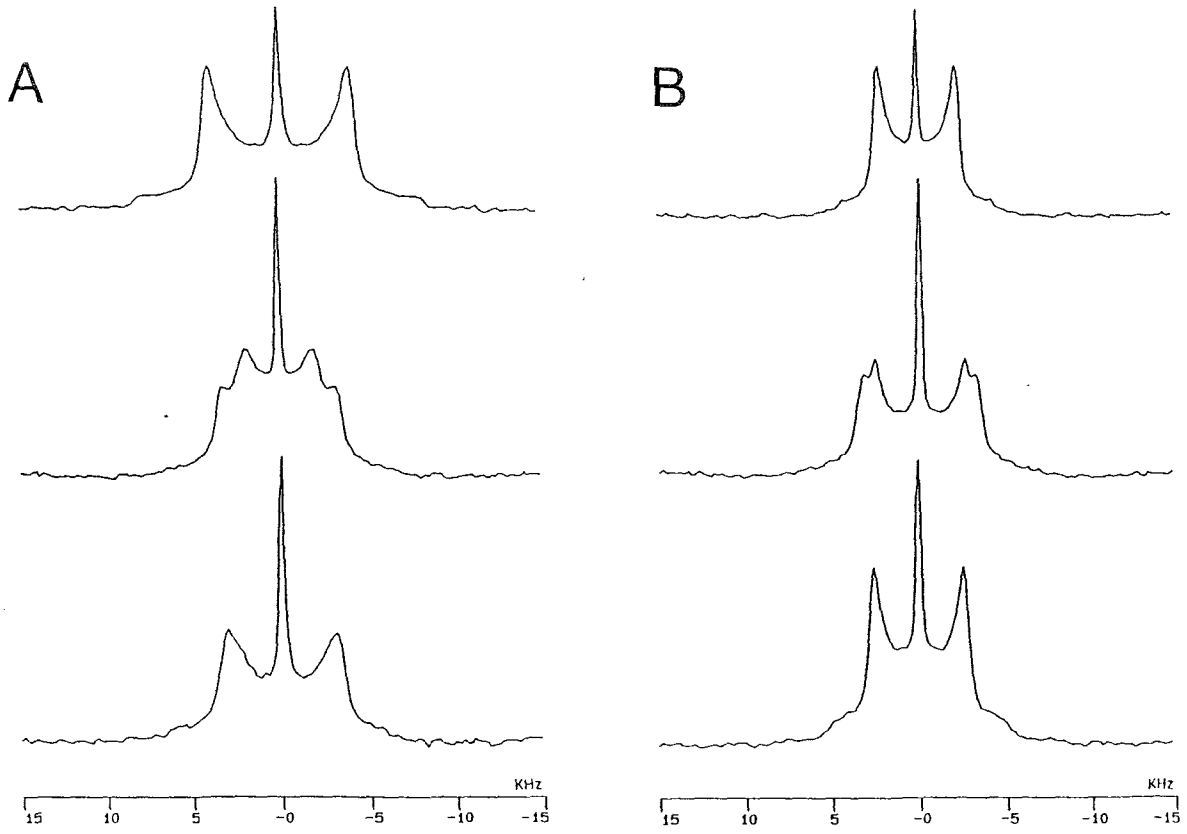


Figure 10: Experimental verification of the predicted effects of a transmembrane-potential-driven TPP^+ redistribution on ^2H NMR quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2. GUVs were composed of POPC/POPG/CHOL (70/10/20), total volume (V^o + V^i) = 600 uL, and $\Delta\Psi_{tm}$tm = -200 mV. Spectra in (A) were recorded with POPC-α-d_2 and spectra in (B) were recorded with POPC-β-d_2 Top spectra, $\Delta\Psi_{tm}$ = -200 mV, no TPP^+ Middle spectra, $\Delta\Psi_{tm}$ = -200 mV, 10 mM TPP^+ Bottom spectra, $\Delta\Psi_{tm}$ = 0 mV (due to freeze-thaw), 10 mM TPP^+.

enriched solid-like domains. Likewise, at a much lower temperature, again characteristic for a particular POPC+DMPA mixture, the temperature dependence of the quadrupolar splittings is lost. This breakpoint would seem to signify the end of further lateral phase separation of DMPA. If one plots these breakpoints for a range of POPC+DMPA compositions, as shown in Figure 8, one obtains the POPC/DMPA temperature-composition phase diagram.

The significance of this demonstration lies not so much in the fact of being able to obtain a phase diagram, although this in itself is no trifling accomplishment, but in the resulting certaintly that the "molecular voltmeter" is sensitive to charge distribution as well as global composition.

Lateral phase separations may be induced not only thermotropically but also electrostatically. The quadrupolar splittings for ternary DMPC+DMPG+DDAB mixtures shown in Figure 3 demonstrate, therefore, that no long-lived electrostatic complex between DMPG (anionic) and DDAB (cationic) is formed, but rather that the three lipid components remain thoroughly mixed. An electrostatic complex, composed of DMPG+DDAB (1:1) and largely excluding DMPC, should yield quadrupolar splittings overlying those of the corresponding binary mixtures, since DMPC would sense only the excess charge not involved in the complex. As discussed previously, this is clearly not the case and the results force us to recognize not only the limitation of the "choline tilt" model, but also that in aqueous media monovalent charged species will not tend to associate strongly even when confined to the two-dimensional geometery of a lipid bilayer. One expects polyvalent charged species,

conversly, to avidly seek out electrostatic associations.

VIII. A Tale of Two Monolayers

With few exceptions deuterium and phosphorus NMR studies of lipid bilayer membranes have employed multilamellar vesicles (MLVs) as model systems, for the very good reason that MLVs form spontaneously and yield large, essentially planar lipid bilayers simply upon dispersing the lipids in water. Unfortunately, because of their onionskin-like architecture, in MLVs one cannot study important membrane-associated phenomena such as the transmembrane potential, transbilayer lipid and/or protein asymmetries, or asymmetric interactions with the interior versus exterior vesicular solutions. In short, using MLVs one cannot distinguish inside from outside.

All of these aspects of biological membranes can be modelled and studied via ^{2}H NMR in unilamellar lipid vesicles, provided the vesicles can be made large enough that overall vesicle tumbling and lipid lateral diffusion do not adversely affect the NMR spectral lineshape. Giant unilamellar vesicles (GUVs) having diameters exceeding 500 nm can be prepared using detergent dialysis methods and provide ^{2}H and ^{31}P NMR spectra indistinguishable from their MLV counterparts (35). When DMPG is added to the GUV lipid mixture the quadrupolar splittings respond in a manner identical to MLVs, indicating that the "molecular voltmeter" is fully functional.

Can we use ^{2}H NMR in GUVs to resolve differences in the surface charge at the inner versus the outer monolayer surface of the vesicle's bilayer? A simple means of generating a transbilayer surface charge asymmetry is to expose the exterior of GUVs to $NaClO_4$ so that the perchlorate anion binds and induces a negative surface charge. Since the lipid bilayer is impermeable to perchlorate, only the exterior vesicle surface is affected. Figure 9 illustrates the effects of adding 150 mM $NaClO_4$ on the ^{2}H NMR spectra from GUVs containing POPC+CHOL (70:30). (Cholesterol, or CHOL, is added to enhance bilayer cohesion and mechanical stability so that such large unilamellar vesicles may be produced). In the absence of perchlorate the ^{2}H NMR spectra (a and e) consist of single Pake patterns, indicating a common environment for the lipids in both monolayers of the vesicle bilayer. Adding 150 mM $NaClO_4$ to the external solution leads to the appearance of a second overlapping Pake pattern in both the POPC-α-d_2 and POPC-β-d_2 spectra (b and f). The quadrupolar splitting from one Pake component remains unchanged from that measured in the absence of perchlorate, indicating that it arises from a population of lipids sequestered from exposure to perchlorate. The quadrupolar splitting from the second Pake component differs in a manner indicative of the presence of negative surface charges, indicating that this second population of lipids is exposed to perchlorate. The presence of two overlapping Pake patterns in the ^{2}H NMR spectra and, hence, the distinction between the two lipid populations - one exposed to and one sequestered from perchlo-

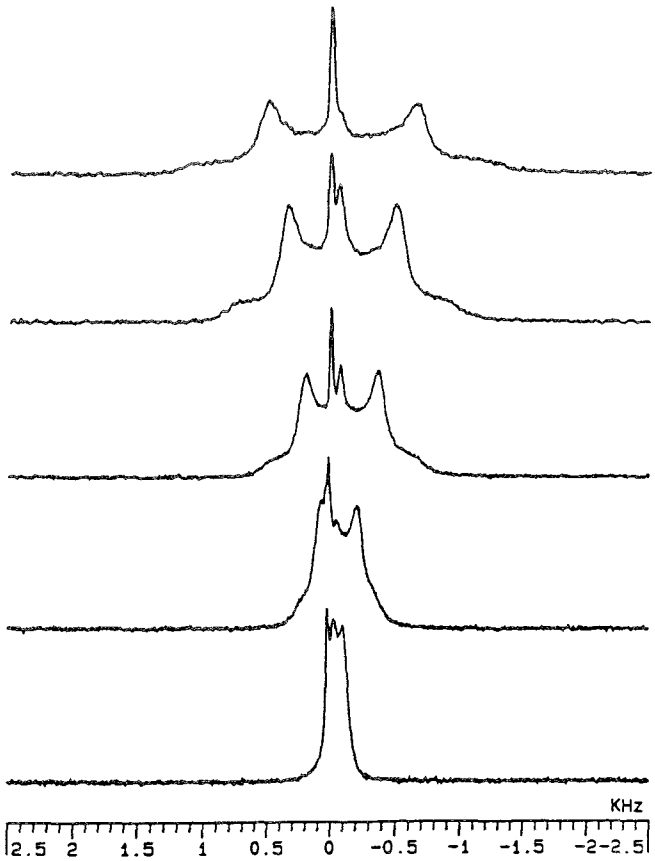


Figure 11: ^{2}H NMR spectra of HDPC-γ-d_6 mixed with various proportions of either a cationic surfactant (CTAB) of and anionic surfactant (SHDS) and bound as a monlyaer at the surface of polystyrene particles of diameter 500 nm. (A) HDPC:CTAB, 20:80. (B) HDPC:CTAB, 50:50. (C) 10050:50. (E) HDPC:SHDS, 20:80.

rate - persists for at least 12 hours. When the integrity of the lipid bilayer as a permeability barrier is compromised by freeze-thawing the vesicle preparation and allowing the inner and outer solutions to equilibrate, the resulting ^{2}H NMR spectra (c and g) again consist of single Pake patterns, and the size of the quadrupolar splittings indicates that this single populations of lipids is entirely exposed to perchlorate ion binding. If we add together the two "single population" spectra (a+c or e+g) in a 1:1 ratio we recreate the "two population" spectra (b and f) in every essential detail. We conclude that the two populations correspond to the inner and the outer monolayers of the lipid bilayer of the unilamellar vesicles.

The ability to resolve the differenes beween the two monolayers of a lipid bilayer paves the way for investigation of an entire range of phenomena related to transmembrane asymmetry not previously accessible to ^{2}H NMR. Some examples which spring to mind include the topography of protein insertion into membranes, transmembrane ion or pH gradients and their effects on protein conformation or lipid-protein interactions or the asymmetery of transbilayer lipid distribution, the mechanism of vesicle fusion, or the mechanical properties of membranes which influence lipid orientational order, such as bilayer undulations.

An innovative application of this ^{2}H NMR technique was described by de Kruijff and coworkers (36) who showed convincingly that in GUVs ^{2}H NMR detects the presence of a transmembrane potential indirectly through its effects on the transbilayer distribution of the potential sensitive, surface binding dye TPP^+ (tetraphenylphosphonium). We have developed a quantitative model for this experiment which allows us to predict the difference in the quadrupolar splittings at the inner versus the outer monolayer surface. The model permits us to optimize the resolution between these the two surfaces as a function of experimentally-controlled variables such as the transmembrane potential, the inner versus outer vesicular volume, the TPP^+ concentration, and the mole fraction of anionic phospholipid incorporated into the GUVs bilayer (37). Figure 10 shows some examples of ^{2}H NMR spectra of POPC-α-d_2 and POPC-β-d_2 incorporated into GUVs where we have imposed a transmembrane electrical potential. The transmembrane potential alone has little or no effect on the headgroup conformation of POPC, as shown by the top spectra, since we observe only a single quadrupolar splitting unchanged from control values in the absence of any transmembrane potential. If TPP^+ is added to the external solution it rapidly equilibrates across the bilayer membranes and distributes in a fashion dictated by the Nernst equation. In the case shown here, with the transmembrane potential negative inside, the TPP^+ tends to concentrate in the vesicle interior. Since the TPP^+ concentration in the two aqueous compartments differs, so does its degree of binding to the respective membrane surface and the surface charge so produced. The ^{2}H NMR spectra show two quadrupolar splittings reflecting this difference between the two surfaces, and the size of the quadrupolar splittings correspond with the values predicted with our model (37). Under optimal experimental conditions the two monolayer surfaces are readily resolved. Finally, when the transmembrane potential is collapsed any differences inside versus outside likewise collapse.

IX. The "Molecular Voltmeter" Meets Polymer Colloids

Surface electrostatics play a decisive role in determining the stability of colloidal dispersions. A detailed understanding of the critical effects of flocculants and coagulants on surface electrostatics is currently lacking, primarily because of the difficulty of using classical methods such as electrophoretic mobilities to obtain meaningful measurements of surface charge in condensed, as opposed to dispersed, particulate states. A transfer of the "molecular voltmeter" technology to study surface electrostatics in colloidal dispersion clearly would be desirable. Any strategy to accomplish this end must involve localizing the phosphocholine group to the surface of a colloidal particle. One tactic for doing so is to package phosphocholine in surfactant form (38), and to adsorb it onto the hydrophobic surface of polystyrene latex particles in aqueous dispersion (39). HDPC, or hexadecylphosphocholine, binds with great avidity and yields the ^{2}H NMR spectrum shown in Figure 11 when deuterons are located on the quaternary methyls of the choline nitrogen (HDPC-γ-d_6). The narrow resonance lines at 0 Hz and -79 Hz are assigned to natural abundance deuterium in water and to "free" HDPC-γ-d_6 , respectively, while the

Pake pattern with quadrupolar splitting of 574 Hz is assigned to "bound" HDPC-γ-d_6. Note that only when the surface of the polystyrene particle is saturated with HDPC-γ-d_6 such that a closely packed monolayer of approximately erect surfactants results does one obtain this lineshape indicative of anisotropic motional averaging. As shown in the figure, when this surface monolayer of zwitterionic HDPC is mixed with cationic surfactants such as CTAB (cetyltrimethylammonium bromide) or anionic surfactants such as SHDS (sodium hexadecysulfate), the quadrupolar splitting increases, or decreases, in direct proportion to the mole fraction of charged surfactant in precisely the manner observed in lipid bilayers (40). Hence, the "molecular voltmeter" appears fully operational in such circumstances.

As one finds in lipid bilayers, any species which binds to the aqueous interface of the polystyrene particle, and which in so doing alters the surface charge, induces a change in the quadrupolar splitting. Of practical interest in polymer colloids are the polyelectrolyte flocculants such as anionic PSSS (poly sodium styrenesulfonate) (41). The effects of PSSS addition on the quadrupolar splittings from polystyrene particles coated with a surface monolayer of HDPC+CTAB in various ratios are shown in Figure 12. In the absence of PSSS the quadrupolar splittings increase with increasing proportion of CTAB as expected for increasing positive surface charge. With increasing PSSS levels the quadrupolar splittings decrease once again, indicating that PSSS binds to the particle surface, in the process neutralizing to some extent the initial positive surface charge. Note that when no CTAB is present PSSS has a negligible effect in the quadrupolar splitting, so PSSS binding is largely due to electrostatic rather than any intrinsic affinity for the particle surface. Moreover, even at the highest PSSS levels it is not possible to obtain quadrupolar splittings as low as those of 100% HDPC surface monolayers. Most importantly, independent rheology measurements show that the levels of PSSS required to flocculate the dispersions fall well below the levels necessary to neutralize the particle surface charge. Thus, destabilization of the colloidal dispersion occurs long before surface charge neutralization. One scenario consistent with these finding is that the polyelectrolyte binds to two of more particles simultaneously in a process described as "bridging" flocculation. Alternately, the topography of the bound polyelectrolytes may permit loops of polymer to extend outwards from the surface, thereby reducing the interparticle potential to the point of allowing flocculation without requiring complete charge cancellation.

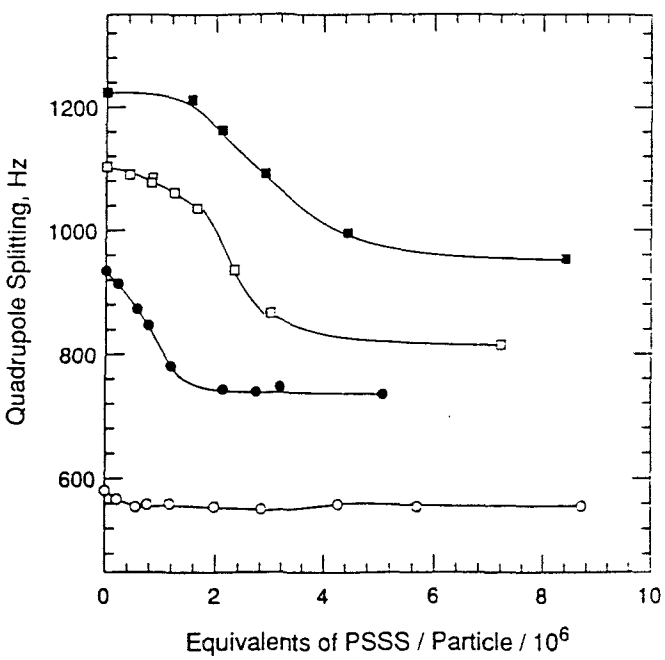


Figure 12: Effect of the anionic flocculating agent PSSS (poly sodium styrene sulfonate) on the quadrupolar splitting of HDPC-γ-d_6 mixed with various proportions of CTAB and bound as a monolayer at the surface of polystyrene particles. The PSSS concentration is expressed in terms of the number of PSSS monomer equivalents per particle in order to eliminate differences due to sample size variations. Key:(○) 100% HDPC. (●) 25:75 CTAB:HDPC. (□) 50:50 CTAB:HDPC. (■) 75:25 CTAB:HDPC.

X. The End is Near

As this article has attempted to demonstrate, during the last several years the horizons for ^{2}H NMR studies of surface electrostatics have widened considerably as the possibilities inherent in the technique have been elaborated. While further efforts in this direction are desirable and necessary, a consolidation stage in which the emphasis is more upon exploiting these newly-realized possibilities to gain

insights into structure-function issues, is likewise imperative. There seems little doubt in either case that the future contributions of the "molecular voltmeter" will be both numerous and notable.

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