Conformational Changes of Phospholipid Headgroups Induced by a Cationic Integral Membrane Peptide As Seen by Deuterium Magnetic Resonance[†]

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ABSTRACT: Deuterium nuclear magnetic resonance (^{2}H NMR) was used to study the interaction of a cationic amphiphilic peptide with pure DMPC membranes and with mixed bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). The choline and serine headgroups were selectively deuteriated at the α and β positions. The amphiphilic peptide, with 20 leucine residues in the hydrophobic core and two cationic hydrophilic lysine residues at each end, spanned the lipid bilayer. Although ^{2}H NMR experiments using DMPC with perdeuteriated fatty acyl chains showed that the average order parameter of the hydrophobic region was not significantly modified by the incorporation of the amphiphilic peptide, for either DMPC or DMPC/DMPS (5:1) bilayers, large perturbations of the quadrupolar splittings of the choline and serine headgroups were observed. The results obtained with the DMPC headgroup suggest that the incorporation of the cationic peptide in both DMPC and DMPC/DMPS (5:1) bilayers leads to a structural perturbation directly related to the net charge on the membrane surface. The magnitude of the observed effect seems to be similar to those observed previously with other cationic molecules [Seelig, J., MacDonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541]. Two of the three quadrupolar splittings of the PS headgroup exhibited large variations in the presence of the amphiphilic peptide, while the third one remained unchanged. Our data have led us to propose a model describing the influence of membrane surface charges on headgroup conformation. In this model, the surface charge is represented as a uniform charge distribution. The electric field due to the charges produces a torque which rotates the polar headgroups. This simple model is shown to be capable of accounting quantitatively for the dependence of headgroup quadrupolar splittings on a variety and range of membrane-associated charges.

Deuterium nuclear magnetic resonance (^{2}H NMR)[1] has been widely used to investigate the phospholipid interactions with membrane proteins in reconstituted liposomes. Several studies have demonstrated that integral proteins produce relatively small changes of the order parameters of phospholipid acyl chains [for review, see Seelig and Seelig (1980), Devaux (1983), Davis (1983), and Bloom and Smith (1987)]. More recent work involving headgroup deuteriated lipids indicates that the polar group order parameters are more strongly influenced by the interactions of the bilayer with peptides or proteins. Furthermore, the observed headgroup perturbations depend on the lipid and protein composition (Tamm & Seelig, 1983; Sixl et al., 1984; Sixl & Watts, 1985; Devaux et al., 1986; Roux et al., 1988). Yet, the interpretation of the NMR results obtained with natural proteins is limited by our incomplete knowledge of the three-dimensional structure of the polypeptide chain in the bilayer and how such structure is related to the peptide sequence. In this respect, ^{2}H NMR studies of deuteriated phospholipids in membranes containing synthetic well-characterized peptide derivatives, designed as simple models of extrinsic or intrinsic proteins, would help to establish a comprehensive set of ^{2}H NMR data useful in the analysis of more complex membrane systems.

We have already obtained results (Roux et al., 1988) with pentalysine showing that this extrinsic peptide, which interacts superficially at the membrane surface, does not induce large perturbations of the headgroup order parameters of either DMPC or DMPS. In the present study, we ask whether a related peptide, i.e., bearing cationic lysine residues, designed to be a model of integral proteins is capable of inducing more significant perturbations than pentalysine. The simplest model of an integral protein is a single α-helical hydrophobic peptide chain spanning the lipid bilayer, with hydrophilic amino acids at each end to ensure the transmembrane orientation. A previous study of Davis et al. (1983) has reported the chemical synthesis of such a model peptide containing leucine residues in the hydrophobic core and lysine dimer at each extremity. The complete sequence is Lys-Lys-Gly-Leu$_n$-Lys-Lys-Ala-CONH$_2$, which we shall refer to as peptide K$_2$GL$_n$K$_2$A in the following. Circular dichroism has shown that this leucine-containing cationic amphiphilic peptide ($n = 24$) has an α-helical structure in methanol, in detergent micelles (Ammonyx L$_0$), and in phospholipid vesicles (DPPC and EPC) (Davis et al., 1983). The α-helical structure of the peptide chain in DPPC liposomes was confirmed by a deuterium NMR study
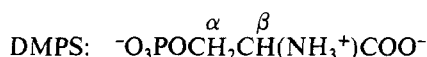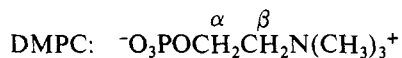
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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

of its exchangeable proton sites (Pauls et al., 1985). The usefulness of this peptide as a model of an integral protein, in the investigation of lipid/peptide interactions in bilayer structures, is enhanced by its five positive charges arising from the four lysine side chains and the N-terminal extremity. Such positive charges could lead to binding of negatively charged phospholipids with integral membrane proteins in biological membranes.

Deuterium NMR data were recorded from mixed bilayers of DMPC and DMPS prepared with various amounts of such a peptide containing 20 leucine residues ($n = 20$). After a qualitative discussion of the data (Qualitative Discussion), we attempted a more quantitative approach in which the measured values of the quadrupolar splitting are used (i) to estimate the nature and the amplitude of the geometrical perturbation of the lipid headgroups (Model for a Quantitative Interpretation of the Headgroup Quadrupolar Splittings) and (ii) to relate this perturbation to the interaction of the headgroup charges with an electric field generated by the positive charges of the peptide molecules (Continuum Model for the Interaction between Charged Particles and Polar Headgroups).

MATERIALS AND METHODS

Lipids and Peptides. DMPC was purchased from SIGMA. Headgroup-deuteriated DMPC and DMPS (monosodium salt) were respectively prepared according to the procedures described by Roux et al. (1983) and Roux and Neumann (1986). The following nomenclature is employed for the deuteriation sites of the choline and serine headgroups:

$$\overset{\alpha \quad \beta}{\text{DMPC: } ^-O_3POCH_2CH_2N(CH_3)_3{}^+}$$

$$\overset{\alpha \quad \beta}{\text{DMPS: } ^-O_3POCH_2CH(NH_3{}^+)COO^-}$$

The amphiphilic cationic peptide Lys-Lys-Gly-Leu$_{20}$-Lys-Lys-Ala-CONH$_2$ was synthesized according to the method described by Davis et al. (1983).

Sample Preparation. Liposomes which contain typically 93 mg of DMPC and 20 mg of DMPS were prepared by mixing organic solutions of DMPC (in chloroform), DMPS (in chloroform/methanol, 9:1), and peptide K$_2$GL$_{20}$K$_2$A (in methanol). Solvents were removed by evaporation under reduced pressure, and the solid residue was dried under high vacuum for 15 min. The residue was then dissolved in pure chloroform, the solvent was evaporated, and the resulting lipids were dried under high vacuum (10^{-2} mmHg) for 12 h. The lipids were then dispersed in 200 μL of Hepes buffer (50 mM in deuterium-depleted water, pH 7.5, 40 mM NaCl, 1 mM EDTA) at 40 °C, with continuous vortexing. After stepwise addition of 3.8 mL of excess buffer (total volume 4 mL), the resulting samples were submitted to five freezing (liquid nitrogen) and thawing (40 °C) cycles and then centrifuged at 30 °C (45 000 rpm, 2 h), and the resulting pellet was transferred directly into the NMR tube.

Calorimetry. Calorimetric traces of the membranes used in the NMR experiments were run on a Perkin-Elmer DSC 4 apparatus, with a 10 °C/min scanning rate, and are showed elsewhere (Roux, 1986). As reported previously by Silvius and Gagné (1984), the DMPC gel to liquid-crystalline phase transition and pretransition are still detected after the incorporation of DMPS, but the presence of DMPS causes the transition temperatures to increase. The incorporation of increasing amounts of amphiphilic peptide K$_2$GL$_{20}$K$_2$A in the mixed DMPC/DMPS bilayers leads to a progressive broadening and slight shift of the main transition peak. The changes in the thermal properties of DMPC/DMPS (5:1) mixtures
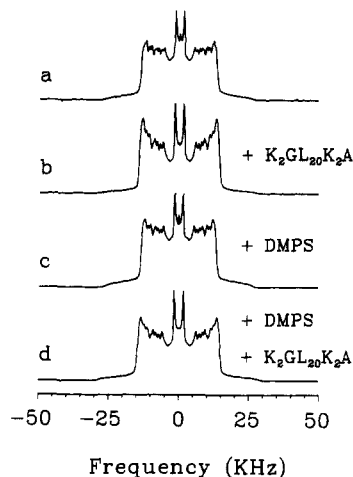

FIGURE 1: ^{2}H NMR spectra at 46 MHz of chain-perdeuteriated DMPC recorded from DMPC (a, b) or DMPC/DMPS (5:1 M/M) bilayers (c, d) either in the absence (a, c) or in the presence (b, d) of amphiphilic peptide K$_2$GL$_{20}$K$_2$A with a molar lipid to peptide ratio of 6:0.2.

produced by peptide K$_2$GL$_{20}$K$_2$A are similar to those reported for DPPC bilayers in the presence of similar peptides (Davis et al., 1983; Morrow et al., 1985). The main purpose of the calorimetric studies was to indicate the temperature range in which the lipid samples are in the liquid-crystalline phase with and without peptide. Following the calorimetric studies, it was decided to carry out all the NMR experiments at 34 °C, at which temperature all of the samples were in the liquid-crystalline phase.

NMR Experiments. ^{2}H NMR experiments were done at 46 MHz on a Bruker MSL-300 spectrometer. Spectra were acquired with a dwell time of 6 μs (spectral width of 83.3 kHz) and a repetition time of 100 ms. A quadrupolar echo pulse sequence (Davis et al., 1976) was employed with a pulse length of 5.5 μs and a pulse separation τ of 100 μs. The free induction decay was shifted by some fraction of the dwell time to ensure that its effective starting time for the Fourier transform corresponded to the top of the quadrupolar echo (Davis, 1983). Oriented ^{2}H NMR spectra were obtained by the numerical De-Paking procedure described by Sternin et al. (1983).

Spin–lattice relaxation times T_1 were measured by an inversion–recovery sequence, the intensity of the signal being measured with the area of the echo peak. The quadrupolar echo decay rate T_{2e} was measured by plotting the intensity of the echo peak, as a function of 2τ.

RESULTS

Acyl Chain Perdeuteriated DMPC. The quadrupolar splittings of the ^{2}H NMR spectra of DMPC-d$_{54}$-containing membranes (Figure 1) are hardly affected by the addition of DMPS and peptide K$_2$GL$_{20}$K$_2$A. De-Paked data (Figure 2) confirm this observation, although a small increase of the outer quadrupolar splitting of the plateau region associated with the methylene groups close to the glycerol backbone can be detected in the presence of peptide (26 to 28 kHz for DMPC, 26 to 29 kHz for DMPC/DMPS).

Headgroup-Deuteriated DMPC. Figure 3 displays ^{2}H NMR spectra (46 MHz) of headgroup-deuteriated DMPC at the α and β positions, either pure (a, b) or mixed with DMPS (e, f) and/or amphiphilic peptide K$_2$GL$_{20}$K$_2$A (c, d, g, h). ^{2}H NMR spectra recorded from DMPC/DMPS (5:1) without peptide (Figure 3e and also Figures 4a and 5a) exhibit line shapes characteristic of a nonisotropic distribution of membrane orientations. Upon the addition of the amphiphilic
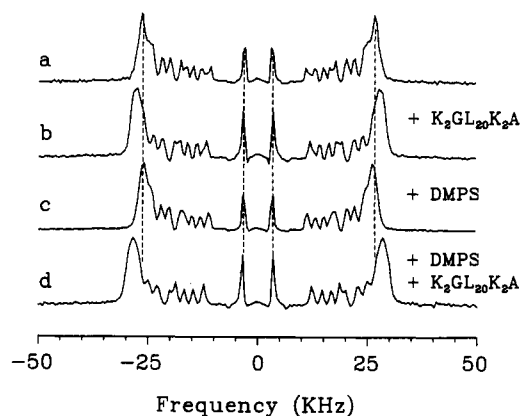
FIGURE 2: De-Paking of the spectra presented in Figure 1 provides the oriented spectra (0°). Note that the splittings are twice those obtained from the 90° edges of the powder pattern. DMPC (a, b) and DMPC/DMPS (5:1 M/M) (c, d) bilayers prepared in the absence (a, c) or the presence (b, d) of amphiphilic peptide $K_2GL_{20}K_2A$ with a molar lipid to peptide ratio of 6:0.2.

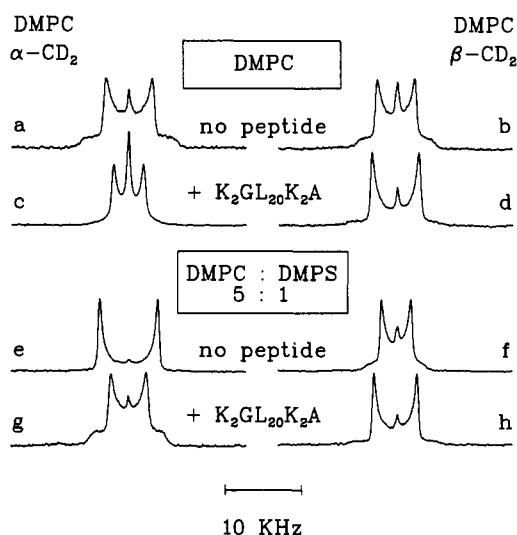FIGURE 3: ^{2}H NMR spectra at 46 MHz of DMPC α-CD$_2$ and DMPC β-CD$_2$ recorded from DMPC (a–d) or DMPC/DMPS (5:1 M/M) bilayers (e–h). (a, b) Pure DMPC; (c, d) DMPC/K$_2$GL$_{20}$K$_2$A (6:0.2 M/M); (e, f) DMPC/DMPS (5:1 M/M); (g, h) DMPC/DMPS/ K$_2$GL$_{20}$K$_2$A (5:1:0.3 M/M/M). Measuring temperature was 34 °C. A line broadening of 100 Hz was used.

peptide, the spectra return to a more classical Pake doublet powder pattern. The atypical line shapes without peptide may be due to a magnetic field-induced orientation of the lipid bilayers, and this has already been discussed in another paper[2] (Roux et al., 1988). As previously observed (Sixl & Watts, 1983; Roux et al., 1988), incorporation of DMPS in head-group-deuteriated DMPC bilayers induces an increase of the choline α-CD$_2$ quadrupolar splitting (Figure 3e) and a decrease of the β-CD$_2$ splitting (Figure 3f). An opposite effect is detected after incorporation of cationic peptide K$_2$GL$_{20}$K$_2$A, the α and β quadrupolar splitting exhibiting respectively a smaller and a larger value in the DMPC/peptide (6:0.2 M/M) bilayers (spectra c and d) than in pure DMPC membranes. Qualitatively similar variations are observed when the peptide is incorporated in DMPC/DMPS bilayers (spectra g and h).

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[2] Since De-Paking calculations fit a powder-type line shape, results are affected by line-shape changes, and distortions of the De-Paked spectrum base line have effectively been observed. Nevertheless, the quadrupolar splittings measured from such De-Paked data are not significantly altered.

Table I: Deuterium Quadrupolar Splitting (kHz) of Headgroup-Deuteriated DMPC Obtained from either De-Paked or Non-De-Paked (Value in Parentheses) Liquid-Crystalline Phase Spectra[a]

	DMPC α-CD$_2$	DMPC β-CD$_2$
DMPC/K$_2$GL$_{20}$K$_2$A		
1:0.0	6.2 (5.8)	5.1 (4.8)
6:0.2	4.1 (3.7)	6.2 (6.0)
DMPC/DMPS/K$_2$GL$_{20}$K$_2$A		
5:1:0.0	7.6 (7.2)	3.9 (3.8)
5:1:0.2	5.4 (5.2)	5.1 (4.9)
5:1:0.3	4.8 (4.4)	5.7 (5.4)

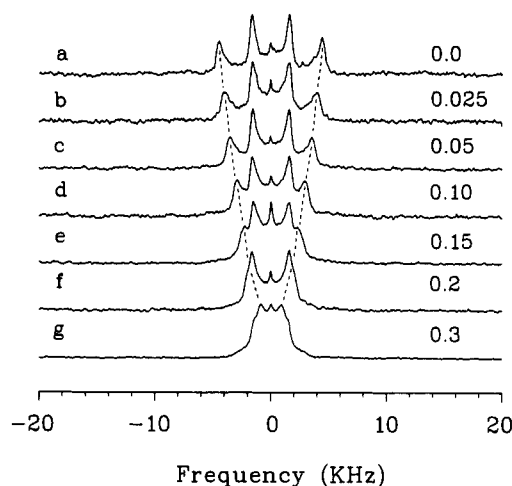[a] Lipid and peptide concentrations are expressed in molar ratio.

FIGURE 4: ^{2}H NMR spectra at 46 MHz of DMPS α-CD$_2$ recorded from DMPC/DMPS (5:1 M/M) bilayers in the presence of amphiphilic peptide K$_2$GL$_{20}$K$_2$A, with a molar lipid to peptide ratio of (a) 6:0, (b) 6:0.025, (c) 6:0.05, (d) 6:0.1, (e) 6:0.15, (f) 6:0.2, and (g) 6:0.3. Measuring temperature was 34 °C. A line broadening of 100 Hz was used.
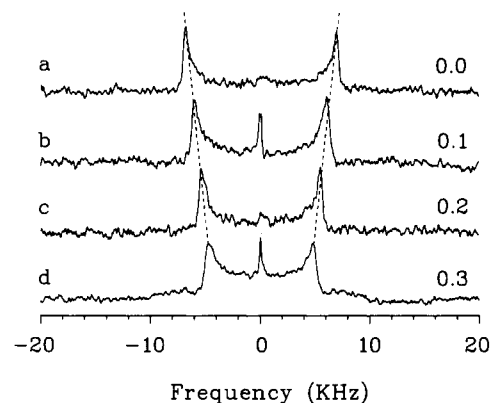

FIGURE 5: ^{2}H NMR spectra at 46 MHz of DMPS β-CD recorded from DMPC/DMPS (5:1 M/M) bilayers in the presence of amphiphilic peptide K$_2$GL$_{20}$K$_2$A, with a molar lipid to peptide ratio of (a), 6:0, (b) 6:0.1, (c) 6:0.2, and (d) 6:0.3. Measuring temperature was 34 °C. A line broadening of 100 Hz was used.

When the relative amounts of peptide K$_2$GL$_{20}$K$_2$A and DMPS are such that the net membrane charge is zero (DMPC/DMPS/peptide K$_2$GL$_{20}$K$_2$A, 5:1:0.2 M/M/M), ^{2}H NMR spectra (data not shown) are very close to those displayed by pure DMPC membranes. The values of the choline headgroup quadrupolar splittings measured from the NMR spectra of Figure 3 are summarized in Table I.

Headgroup-Deuteriated DMPS. Deuterium NMR spectra of headgroup-deuteriated phosphatidylserine in lipid bilayers show two distinct quadrupolar splittings for the α-CD$_2$ methylene (Figure 4a) due to the inequivalence of the two
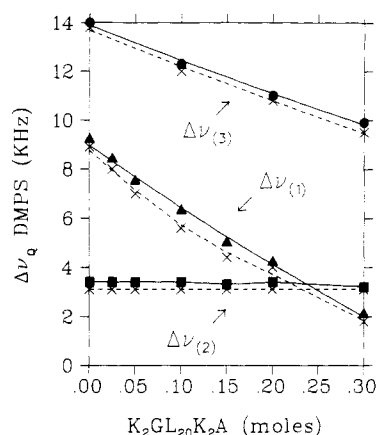
FIGURE 6: ^2H De-Paked quadrupolar splittings measured for the α-CD$_2$ group $\Delta\nu_{(1)}$ (▲) and $\Delta\nu_{(2)}$ (■) and the β-CD group $\Delta\nu_{(3)}$ (●) of the DMPS headgroup in DMPC/DMPS (5:1 M/M) membranes, as a function of the molar amount of amphiphilic peptide K$_2$GL$_{20}$K$_2$A incorporated in the lipid bilayers. Note the systematically smaller values measured from original and non-De-Paked spectra (dotted line (×).

Table II: Relaxation Times T_1 and T_{2e} for Headgroup-Deuteriated DMPS Measured in DMPC/DMPS (5:1 M/M) Membranes[a]

	T_1 (ms)		T_{2e} (ms)	
	α-CD$_2$	β-CD	α-CD$_2$	β-CD
DMPC/DMPS (5:1 M/M)	7.7	12.0	0.81	0.96
DMPC/DMPS/K$_2$GL$_{20}$K$_2$A (5:1:0.2 M/M)	8.8	9.0	0.87	0.82

[a] The experimental error is less than 10%.

deuterons (Browning & Seelig, 1980) and one splitting for the β-CD group (Figure 5a). All these splittings are smaller in mixtures of DMPS and DMPC than in pure DMPS membranes (Browning & Seelig, 1980; Roux & Neumann, 1986). Incorporation of increasing amounts of the amphiphilic peptide K$_2$GL$_{20}$K$_2$A in DMPC/DMPS (5:1) bilayers led to a gradual decrease of the outer α-CD$_2$ splitting (9.2 kHz in the absence of peptide), whereas the Pake doublet with the smaller splitting remained unaffected (3.4 kHz) (Figure 4, spectra b–g). For a PS/peptide molar ratio of 1:0.3, the splitting of 3.4 kHz was accompanied by a smaller one of 2.1 kHz, which may reasonably be attributed to the reduced initial "outer splitting". The quadrupolar splitting of the DMPS headgroup β-CD group was decreased from 14 to 9.9 kHz after 0.2 mol of amphiphilic peptide was added in the mixed DMPC/DMPC (5:1 M/M) membranes. The variations of the DMPS headgroup quadrupolar splittings for PC/PS membranes containing various amounts of the amphiphilic cationic peptide K$_2$GL$_{20}$K$_2$A are summarized in Figure 6, which show that these variations are quasi-linear.

Relaxation Experiments. T_1 and T_{2e} of the α- and β-deuterons of the DMPS headgroup were measured for DMPC/DMPS (5:1) bilayers in the absence and in the presence of peptide K$_2$GL$_{20}$K$_2$A. As may be seen from the T_1 and T_{2e} values in Table II, the introduction of peptide molecules had only a minor effect on the relaxation times.

QUALITATIVE DISCUSSION

Interactions of chain-perdeuteriated phospholipids with large intrinsic membrane proteins do not generally produce large changes of the average chain orientational order parameter (Bloom & Smith, 1985). Our results show that this is also true for interactions between the DMPC acyl chains and the integral peptide K$_2$GL$_{20}$K$_2$A. Space-filling models show that each molecule of peptide K$_2$GL$_{20}$K$_2$A should be surrounded
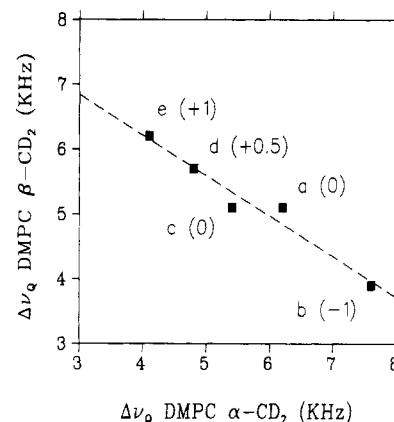

FIGURE 7: Quadrupolar splitting $\Delta\nu_{(\beta)}$ of the β-CD$_2$ group plotted as a function of the corresponding splitting $\Delta\nu_{(\alpha)}$ of the α-CD$_2$ group, measured from ^2H NMR spectra of various headgroup-deuteriated DMPC containing membranes. (a) DMPC; (b) DMPC/DMPS, 5:1; (c) DMPC/DMPS/K$_2$GL$_{20}$K$_2$A, 5:1:0.2; (d) DMPC/DMPS/K$_2$GL$_{20}$K$_2$A, 5:1:0.3; (e) DMPC/K$_2$GL$_{20}$K$_2$A, 6:0.2. The numbers refer to the bilayer net charge, calculated from the relative amount of anionic DMPS and cationic peptide. The straight line drawn through the data has no theoretical significance.

by about 16–18 molecules of phospholipid. In the absence of aggregation of the amphiphilic peptide at the lipid/peptide ratio used in our study (6:0.2 M/M), we would expect that approximately 60% of the total lipid chains are in contact with polyleucine α-helices. However, the quadrupolar splittings of the paraffinic chains do not change much upon incorporation of the amphiphilic peptide in the lipid bilayer. Previous deuterium NMR works of Davis et al. (1983), carried out with zwitterionic DPPC bilayers and the K$_2$GL$_{20}$K$_2$A analogue, have also shown that the lipid chain order was barely altered by this peptide.

In contrast with the results obtained with chain-deuteriated lipids, the data recorded from headgroup-deuteriated derivatives show that the choline and serine quadrupolar splittings are considerably affected by the presence of the peptide K$_2$GL$_{20}$K$_2$A in the lipid bilayers. The perturbations of the quadrupolar splitting of the choline α-CD$_2$ group induced by the incorporation of either DMPS or peptide K$_2$GL$_{20}$K$_2$A molecules in DMPC membranes are always in the opposite sense to those of the β-CD$_2$ deuterons. As pointed out by Seelig and his co-workers [see Seelig et al. (1987) and references cited therein], such quadrupolar splitting variations cannot be explained by a decrease or an increase of the average order of the choline moiety, which would give rise to reduced or increased quadrupolar splittings for both the α- and β-deuterons. Thus, the spectral changes induced by the DMPS or the amphiphilic peptide imply at least that the DMPC headgroup undergoes a conformational change corresponding to a change in its average orientation. Analogous results have been reported in deuterium NMR studies of headgroup-deuteriated phosphatidylcholine interactions with metallic cations (Akutsu & Seelig, 1981) or positively charged anesthetics (Boulanger et al., 1981). Figure 7 is an (α, β) plot of the ^2H NMR data obtained with the amphiphilic peptide, in which the β-CD$_2$ quadrupolar splitting of the DMPC headgroup is plotted against that of the α-CD$_2$. This type of graphical display of the ^2H NMR data was introduced by Akutsu and Seelig (1980) in an earlier NMR study of cation interactions with headgroup-deuteriated DPPC bilayers. These authors found that data obtained with various amounts of different mono-, di-, or trivalent ions fall on a unique straight line. They were then able to conclude that the ion-induced conformational changes of the choline headgroup were qualitatively similar

for all species investigated. The perturbation of the choline headgroup observed after incorporation of the peptide $K_2GL_{20}K_2A$ should be similar to this conformational change, since the data obtained with DMPC or DMPC/DMPS membranes fall, to a first approximation, on a straight line analogous to that obtained by Akutsu and Seelig (1980). It is interesting to note that an (α, β) plot of the data published by Boulanger et al. (1981), with headgroup-deuteriated EPC membranes and charged tetracaine, also leads to a similar straight line. Therefore, it seems that the headgroup perturbation induced by very different biological compounds such as metallic cations, anesthetics, or peptides could be basically explained in terms of electrostatic interactions between the choline dipole and the net positive surface charge of membrane containing these molecules. This interpretation is supported by the observation that the interaction between PC and neutral tetracaine in lipid bilayers leads to a completely different variation of the choline headgroup quadrupolar splittings (Boulanger et al., 1981), one which is closer to those obtained with other neutral molecules such as cholesterol or chloroform (Akutsu & Seelig, 1980).

The data of Figure 7 indicate that the quadrupolar splitting changes are to a certain extent correlated to the total charge of the membrane. In particular, the quadrupolar variations induced by the cationic peptide in pure DMPC membranes are partially canceled by the incorporation of the negative charges of the DMPS molecules. When these two compounds are incorporated simultaneously with a molar ratio leading to bilayer neutrality (considering that there is one negative charge per DMPS molecule and five positive charges per peptide molecule), a small change in the α-CD_2 quadrupolar splitting is detected, while the β splitting does not change at all. This indicates that the change in the quadrupolar splitting of the two methylenes of the choline headgroup upon incorporation of the peptide $K_2GL_{20}K_2A$ is due to the resulting increase in positive charge density at the membrane surface rather than to the formation of a particular PC/peptide complex.

Another interesting feature displayed by Figure 7 concerns the DMPC headgroup perturbation induced by the contribution of a net negative charge density provided by the DMPS molecules in the pure DMPC/DMPS (5:1) membranes. The ^{2}H NMR data recorded from these negatively charged membranes show that the quadrupolar splitting variations of both methylene groups have the opposite sign to those caused by positive bilayers. Sixl and Watts (1983) have obtained ^{2}H NMR data for mixtures of the same lipids (myristoyl derivatives) at various PC/PS ratios. An (α, β) plot of these data leads to a straight line with a slope having the same sign as those measured with PC-containing positively charged membranes, although the absolute value of the slope is higher.

Our earlier studies (Roux et al., 1988) of the same lipid systems in the presence of the cationic extrinsic pentalysine differ significantly from those obtained with the intrinsic peptide $K_2GL_{20}K_2A$, since pentalysine did not induce any detectable variations of the choline headgroup quadrupolar splittings. Yet, the intrinsic and extrinsic peptides have almost identical amounts of positive charges, and both interact electrostatically with the bilayer surface by means of their lysine side chains. Unlike intrinsic peptides, polylysines do not penetrate in the bilayer and interact only superficially, at the membrane surface. The positive charges of pentalysine are then probably less tightly and deeply anchored than those of the peptide $K_2GL_{20}K_2A$, accounting for the absence of significant effects of this extrinsic peptide on the choline quadrupolar splittings.

The main feature of the ^{2}H NMR data obtained from headgroup-deuteriated DMPS is that the incorporation of increasing amounts of amphiphilic peptide in the DMPC/DMPS membranes affects selectively one of the two quadrupolar splittings of the α-CD_2 spectrum, the other one being unmodified. Thus, as for the results obtained with the PC deuteriated headgroup discussed in the previous section, the changes in ^{2}H NMR splittings observed upon addition of peptide are related to changes of the average orientation of the serine headgroup rather than an increase of the amplitude of the angular fluctuations. The question then arises as to the nature of the molecular interactions which lead to these different conformational changes of the serine headgroup. As in the case of the PC headgroup studies, the observed effects may be related to the response of this headgroup to the presence of positive net charge density at the membrane surface. In this respect, it is noteworthy that the addition of structurally different compounds such as the lithium ion (Roux & Neumann, 1986) and the amphiphilic cationic peptide induce similar effects on the PS headgroup, namely, a decrease of the external α-CD_2 quadrupolar splitting with little change of the inner doublet.

The phosphatidylserine headgroup has a net negative charge and may thus interact more specifically with the cationic peptide than does phosphatidylcholine. If such interactions led to the formation of a DMPS/peptide complex, the associated perturbations of the serine headgroup might account for the observed spectral changes, although "bound" and "free" PS molecules should be in fast exchange on the NMR time scale since single-component spectrum are observed under all conditions. The relaxation data are consistent with this interpretation since the values of both T_1 and T_{2e} are not changed appreciably by the addition of the integral peptide. Then, the PS headgroup NMR data do not demonstrate the existence of a specific PS/peptide complex. As for the PC headgroup, the PS quadrupolar splitting variations induced by the amphiphilic peptide $K_2GL_{20}K_2A$ are very different from those recorded upon adsorption of pentalysine on the surface of the DMPC/DMPS bilayer, namely, a slight increase of the α-CD_2 outer splitting (Roux et al., 1987). As discussed previously, such differences could be correlated to the distinct way these two peptides interact with the lipid bilayer. In that sense, it is striking that changes in ^{2}H NMR spectra due to the binding of extrinsic cytochrome c to DMPC/DMPS bilayers (Devaux et al., 1986) are similar to those obtained with pentalysine. Thus, the differences in ^{2}H NMR quadrupolar splittings of the PS headgroup due to the interaction with extrinsic and intrinsic peptides would be related to various degrees of penetration of the lysine cationic side chains in the polar region of the bilayer.

MODEL FOR A QUANTITATIVE INTERPRETATION OF THE HEADGROUP QUADRUPOLAR SPLITTINGS

A Two-Step Model for Molecular Reorientation. We propose here a simple model for the parametrization and quantitative interpretation of quadrupolar splittings in polar headgroups. The model was stimulated by a particularly striking feature of the ^{2}H NMR results in DMPS but should be applicable to both the PC and the PS headgroups studied here. Indeed, our model is an extension of one originally proposed (Akutsu & Seelig, 1981; see Figure 9) to explain experimental results in [2]H-labeled PC headgroup interaction with metallic cations.

In PS headgroups, three quadrupolar splittings $\Delta\nu_{(i)}$ are observed. Two of them, $i = 1$ and 2, are associated with the ^{2}H nuclei of the α-methylene segment,[3] and the third, $i = 3$,

Table III: Allowed Values[a] of θ_i (deg) for the Quadrupolar Splittings $\Delta\nu_{(i)}$ (kHz) and Various Values of S_f

$\Delta\nu_{(i)}$	S_f	θ_i
14.0	0.112–1.0	0.0–50.3
9.2	0.112–1.0	28.6–51.8
3.4	0.112–1.0	45.3–53.6
9.2–2.1	1.0	51.8–54.1
9.2–2.1	0.112	28.6–48.8

[a] Only values of $\theta_i < \theta_m = 54.74°$ are shown. Additional allowed values of $\theta_i > \theta_m$ may be easily derived from eq 1.

is associated with the β-CD moiety. While $\Delta\nu_{(1)}$ and $\Delta\nu_{(3)}$ are very sensitive to the membrane surface charge density, $\Delta\nu_{(2)}$ hardly changes over a wide range of surface charge density from a variety of sources of charge as discussed in the previous section.

Following Akutsu and Seelig (1981), we assume that the polar headgroup has two types of motion so that the orientational order parameter $S^{(i)}$ may be expressed as the product of two order parameters, $S_g^{(i)}$ and S_f, characteristic of each type of motion:

$$|S^{(i)}| = \frac{\Delta\nu_{(i)}}{\Delta\nu_Q} = |S_g^{(i)}S_f| = \left|\frac{1}{2}(3\cos^2\theta_i - 1)S_f\right| \quad (1)$$

where $\Delta\nu_Q \approx 125$ kHz is the quadrupolar splitting associated with a C–D bond in the absence of motion. One of the motions, characterized by S_f, is the ensemble of fluctuations which are independent of the position, i, in the molecule. The other, "geometrical", order parameter $S_g^{(i)}$ is assumed to be sensitive to the average angle θ_i between the ith C–D bond and a symmetry axis for the motions. We emphasize that eq 1 should be interpreted simply as an empirical formula for the parametrization of the experimental data in terms of geometrical parameters. In reality, the most general conformational fluctuations could depend on position i and need not necessarily be axially symmetric, so that the number of parameters required to characterize the fluctuations could be quite large [see Skarjune and Oldfield (1979)]. However, we are led by the simplicity of the experimental ^{2}H NMR results to search for a specific geometrical mechanism for the influence of membrane surface charges on headgroup conformation, and eq 1 will enable us to make explicit quantitative statements concerning the nature of the mechanism.

Allowed Value of the Average Orientation. The measured values of $\Delta\nu_{(i)}$ in our DMPS studies were all less than 14 kHz so that $|S^{(i)}| \leq 14/125 = 0.112$ for all i and all membrane charge densities studied. Since both $S_g^{(i)}$ and S_f must lie in the range between −0.5 and 1.0, it follows that $-0.5 \leq S_f \leq -0.112$ or $0.112 \leq S_f \leq 1.0$. The corresponding range of allowed values of θ_i is shown in Table III. It is clear from Table III that for $S_f \approx 1$ the value of θ_i for all the ^{2}H sites under all the experimental conditions used here would lie close to the magic angle $\theta_m = \cos^{-1}(1/\sqrt{3}) = 54.74°$ corresponding to $P_2(\cos\theta_m) = 0$. For $S_f = 1$, the large change in $\Delta\nu_{(1)}$ from 9.2 to 2.1 kHz upon addition of the peptide $K_2GL_{20}K_2A$ would involve a change of θ_1 of about 2°. For S_f near the bottom of its range, however, addition of the peptide would be associated with a rotation as large as 20°. In view of the fact that $\Delta\nu_{(2)}$ would also be extremely sensitive to small rotations for $S_f \approx 1$ and does not change upon addition of the peptide, it seems likely that the value of S_f is not close to 1 and that the
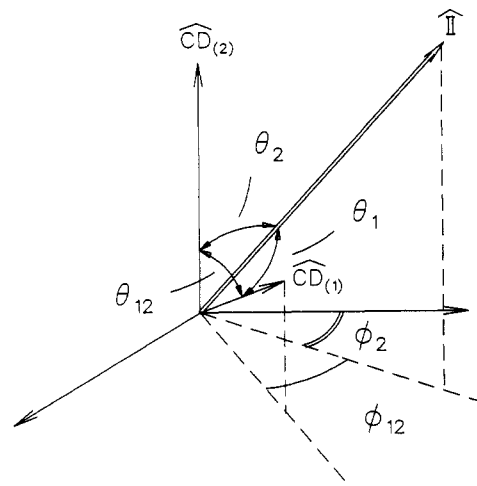
[3] Compelling arguments in favor of attributing $\Delta\nu_{(1)}$ and $\Delta\nu_{(2)}$ to the motional inequivalence of the ^{2}H nuclei in the α-methylene groups, rather than to two long-lived conformational states of the molecule, have been made by Browning and Seelig (1980).

FIGURE 8: Representation of the vectors C–D$^{(1)}$ and C–D$^{(2)}$ describing the CD bonds of the serine α-methylene group, in a coordinate system where the vector C–D$^{(2)}$ lies along the z axis. $\hat{I}$ is the symmetry axis for the headgroup motion.

effect of the peptide is to produce a rotation of the polar headgroup about an axis close to the C–D$^{(i)}$ bond direction for $i = 2$.

Rotation about the C–D$^{(2)}$ Bond Direction. Before examining the origin of the torque associated with the hypothetical rotation of the polar headgroup about the C–D$^{(2)}$ bond direction, we ask whether the rotation giving the desired change in $S_g^{(1)}$ is compatible with the geometry of the methylene group and the known value of $S_g^{(1)}/S_g^{(2)}$. We choose a coordinate system (Figure 8) with the z axis along the C–D$^{(2)}$ bond direction and denote the spherical polar angles of the symmetry axis for the headgroup motion by θ_2, ϕ_2 and those of the C–D$^{(1)}$ bond direction by θ_{12}, ϕ_{12}. The addition theorem for spherical harmonics gives (Rose, 1957)

$$P_2(\cos\theta_1) = P_2(\cos\theta_2)P_2(\cos\theta_{12}) +$$
$$3\sin\theta_2\cos\theta_2\sin\theta_{12}\cos\theta_{12}\cos\phi +$$
$$\frac{3}{4}\sin^2\theta_2\sin^2\theta_{12}\cos 2\phi \quad (2)$$

where $\phi = \phi_2 - \phi_{12}$. Changes in ϕ are produced by rotation about the C–D$^{(2)}$ bond direction. Using the angle $\theta_{12} = 109°$ for the tetrahedral angle and $S_g^{(i)} = P_2(\cos\theta_i)$, we obtain

$$S_g^{(1)} = -\frac{1}{3}S_g^{(2)} \pm 0.46\left[1 - \left(\frac{4S_g^{(2)} - 1}{3}\right)^2\right]^{1/2}\cos\phi +$$
$$0.45(1 - S_g^{(2)})\cos 2\phi \quad (3)$$

Since the values of S_f lie in the range $0.112 \leq S_f \leq 1.0$, the corresponding limits of $S_g^{(2)}$ are given for $\Delta\nu_{(2)} = 3.4$ kHz by $0.24 \geq |S_g^{(2)}| \geq 0.027$. As an example, for $S_g^{(2)} = 0.24$, the requirement that $S_g^{(1)} = 0.66$ for the DMPC/DMPS/ $K_2GL_{20}K_2A$ (5:1:0 M/M/M) sample ($\Delta\nu_{(1)} = 9.2$ kHz) is satisfied for $\phi = \phi_0 = 23°$ while the value of $S_g^{(1)} = 0.15$ which is obtained for $\Delta\nu_{(1)} = 2.1$ kHz with the DMPC/DMPS/ $K_2GL_{20}K_2A$ (5:1:0.3 M/M/M) sample is satisfied for $\phi = \phi_0 + \phi_r = 54.8°$. Thus for $S_f = 0.112$, the addition of $K_2GL_{20}K_2A$ peptide is predicted to produce a rotation of the polar headgroup of $\phi_r \geq 30°$. For larger values of S_f, values of ϕ_0 and ϕ_r satisfying eq 3 can also be found. As $S_f \to 1$, the change in ϕ upon addition of peptide is predicted to be progressively smaller leading to a value of $\phi_r \approx 3°$ for $S_f = 1$.

An interesting experiment suggested by these calculations is to look for such a rotation produced by the addition of
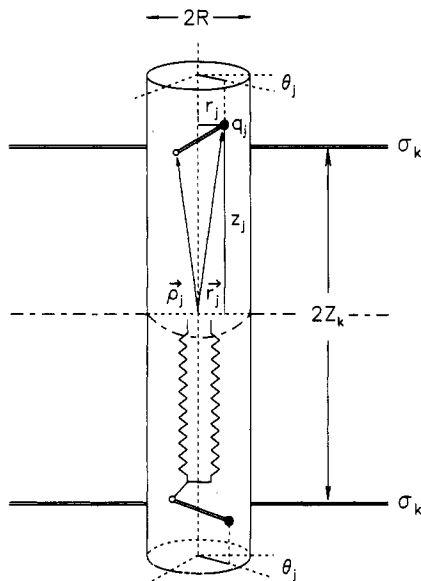
FIGURE 9: Schematic representation of phospholipid molecules in a bilayer. Parameters are described in the text.

peptide or ions such as Li⁺ with neutron diffraction experiments on ²H-labeled polar headgroups. A useful review of the manner in which neutron diffraction may be used along with other techniques to explore headgroup conformations has been given by Büldt and Wohlgemuth (1981).

It is obvious that the geometrical model used here can be used to interpret the influence of membrane surface charge on quadrupolar splittings of both PC and PS headgroups. We shall not go into the details of PC headgroup geometry here. Instead, we turn to a simple interaction model which is capable of explaining the origin of the torque required to produce the rotation discussed above.

CONTINUUM MODEL FOR THE INTERACTION BETWEEN CHARGED PARTICLES AND POLAR HEADGROUPS

Derivation of the Relationship between Torque and Rotation Angle. Most workers in this field have discussed the influence of charged particles on polar headgroup conformations in term of a specific binding mechanism. This is quite reasonable, since the Coulomb interaction is very strong as may be seen from the detailed theorical analysis of the serine phosphate–Na⁺ complex by Gresh and Pullman (1980). As these authors remark, however, in their conclusion section: "The present treatment did not incorporate the effects of the surroundings expected to prevail in biological or model membranes, namely the influence of solvation or of the vicinity of adjacent polar headgroups".

Looking at the summary of our experimental results given under Qualitative Discussion, it is evident that a tight binding approximation which would give rise to distinct ²H NMR quadrupolar splittings for each site i does not apply here, at least on the ²H NMR time scale ($\approx 10^{-5}$ s) (Seelig & Seelig, 1980; Devaux, 1983; Bloom & Smith, 1985). The simplicity of the dependence of the PC headgroup quadrupolar splittings on the concentrations of various charged molecules (see Qualitative Discussion) leads directly to our present proposal of a continuous, uniform membrane charge density. As shown in Figure 9, we assume a uniform[4] charge density σ_k of charged particles of species k at a height z_k in the membrane. A

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[4] A discussion of the validity of the use of a uniform smeared charge model to describe the electrostatic properties of lipid bilayer membrane can be found in Winiski et al. (1986).

specific lipid probe molecule, labeled with ²H in the headgroup, is assumed to be a cylinder of radius R. Suppose that a charge q_j in the polar headgroup is located at a position $\vec{r}_j = (r_j, \theta_j, z_j)$ in cylindrical coordinates and is affected by all the charges $k = 1, 2, ...$ outside the molecule containing the charge q_j via the electric field:

$$\vec{E}_j = \sum_k \vec{E}(r_j, z_j - z_k) \qquad (4)$$

For a membrane system in which the average surface area per lipid is A and the average charge density for species k is $\sigma_k = f_k e / A$ (with $f_k > 0$ for positive charges and $f_k < 0$ for negative charges), it is easy to show that the electric field along the axis of the cylinder is normal to the bilayer and given by

$$\vec{E}_{(axis)} = \frac{1}{2\epsilon_0} \sum_k \frac{\sigma_k (z - z_k)\hat{z}}{[R^2 + (z - z_k)^2]^{1/2}} \qquad (5)$$

in SI units, where $\epsilon_0 = 8.85 \times 10^{-12}$ and $\hat{z}$ is the unit vector parallel to the bilayer normal. The electric field due to species k changes sign at $z = z_k$. For $|z - z_k| \gg R$, it takes on the value $\sigma_k / 2\epsilon_0$ characteristic of an infinite plane of uniform charge density. The electric field has a radial component off the axis of the cylinder.

If the polar headgroup is approximated as a quasi-rigid body hinged to the glycerol backbone at a position $\vec{p}$, the total torque exerted on the headgroup about the hinge position due to the interaction with the continuous charge distribution is given by

$$\vec{\tau} = \sum_j q_j (\vec{r}_j - \vec{p}) \times \vec{E}_j \qquad (6)$$

For a harmonic torsional potential energy $U(\phi) = (1/2)K\phi^2$, where ϕ is the rotation angle about the direction of $\vec{\tau}$, the average rotation, ϕ_r, produced by $\vec{\tau}$ is given by $\phi_r = \tau / K$, for which the external torque is balanced by the countertorque of the hinge.

Order of Magnitude Calculation of PS Rotation. In order to examine the validity of the model described above, it is useful to estimate the order of magnitude of the rotation angle of the PS headgroup in terms of geometrical parameters which have some intuitive significance. To this end, we estimate the relationship between the torsional constant K and the value of $\phi_r = \phi_r(k)$ due to charge species k in terms of a parameter d_k which expresses the effective separation normal to the membrane surface between the charges q_j in the polar headgroup and the charge species k. Approximating the average value of $\vec{E}_j$ by $\vec{E}_{(axis)}$ in eq 5, the parameter d_k is defined by

$$e\vec{d}_k = \sum_j \frac{q_j(\vec{r}_j - \vec{p}) \times (z_j - z_k)\hat{z}}{[R^2 + (z_j - z_k)^2]^{1/2}} \qquad (7)$$

Note that since $R \leq 5$ Å, $|\vec{r}_j - \vec{p}| \approx R$, $|z_j - z_k| \leq R$, we anticipate values of $|d_k| \leq$ a few angstroms. The torque due to charge species k may now be written in the form

$$\vec{\tau}(k) = K\phi_r(k) = \frac{\sigma_k}{2\epsilon_0} e\vec{d}_k = \frac{e^2}{2\epsilon_0 A}(f_k \vec{d}_k) \qquad (8)$$

In our earlier discussion of the geometrical model, we found that upon adding peptide to change the DMPC:DMPS:K₂GL₂₀K₂A molar ratio from 5:1:0 to 5:1:0.3 (M/M/M) a rotation corresponding to ϕ_r in the range $3° \leq \phi_r \leq 30°$ was compatible with the geometrical model. Using $e = 1.6 \times 10^{-19}$ C, $A \approx 60$ Å² = 6×10^{-19} m², and $f_k = (5 \times 0.3)/6 = 0.25$ for 0.3 peptide, where we have taken into account the five charges per peptide and neglected the area occupied by the peptide molecule, eq 8 gives

$$K \approx 6 \times 10^{-20} \left(\frac{d_k(\text{Å})}{\phi_r} \right) \text{J} \qquad (9)$$

where $d_k(\text{Å})$ is $|d_k|$ measured in angstroms and ϕ_r is in radians.

A representative value of K for chain molecules is that of n-butane in the trans conformation which gives $K \approx 10^{-19}$ J (Abe et al., 1966). With eq 9 the value of ϕ_r in the allowed range $3° \leq \phi_r \leq 30°$ (see Rotation about the C–D[(2)] Bond Direction) corresponds to the range $1.7 \text{ Å} \geq d \geq 0.17 \text{ Å}$, which are reasonable values of the effective *average* vertical separation of the positive charges of the lysines in the peptide $K_2GL_{20}K_2A$ and the charges in the PS headgroups.

Interpretation of the Experimental Data. The order of magnitude calculation (see Order of Magnitude Calculation of PS Rotation) of the PS rotation due to membrane surface charge demonstrates that the two-step model of headgroup reorientational motions is capable of providing a quantitative interpretation of ²H NMR quadrupolar splittings in polar headgroups. A systematic analysis of the effects of surface charges of different origins should ultimately yield information on the distribution of the charges as a function of the depth in the membrane. In this section, we review the qualitative features of the experimental data which can be understood with the help of the model.

In the case of PC headgroups, Seelig and co-workers (Akutsu & Seelig, 1981; Seelig et al., 1987) have argued convincingly that the linear relationship between $\Delta\nu_{(\alpha)}$ and $\Delta\nu_{(\beta)}$ as the membrane surface charge is varied (see also Figure 7) is associated with a rotation of the polar headgroup. The continuum model under Derivation of the Relationship between Torque and Rotation Angle accounts for the opposite signs for *changes* in the quadrupolar splittings depending on whether positive or negative charges are added to the membrane. Equation 8 predicts a reversal of the torque with the reversal of sign of the charge on the membrane. The perturbation to quadrupole splittings of the PC headgroups reported in this paper can be understood in such terms, as can the observations that the addition of other negatively charged lipids such as phosphatidylglycerol, phosphatidic acid, or cardiolipin (Sixl & Watts, 1983; Seelig et al., 1987) perturbs the quadrupolar splittings of PC headgroup in the same way as do PS headgroups.

The model also enables us to understand the important conclusion of Seelig et al. (1987) that $\Delta\nu_{(\alpha)}$ and $\Delta\nu_{(\beta)}$ act as an electrometer for the amount of charge effectively *bound* to the membrane surface. The charges associated with molecular species such as DMPS and $K_2GL_{20}K_2A$ are anchored firmly to the membrane and are thus automatically bound to the surface. When a salt such as NaCl or CaCl$_2$ is added to the membrane suspension, however, only a fraction f_b of the cationic charge is bound to the membrane surface. In the spirit of our model, we can describe this fraction in terms of a δ-function at $z_k = z_k^{(0)}$ with the remainder being distributed continuously in the aqueous medium over a wide range of values $z_k > z_k^{(0)}$ as described by a normalized distribution function $g(z_k)$. This can be summarized by the distribution function $F(z_k)$ for the cation charge density:

$$F(z_k) = f_b\delta(z_k - z_k^{(0)}) + (1 - f_b)g(z_k) \qquad (10)$$

Altenbach and Seelig (1984) have obtained the values of f_b and the form of $g(z_k)$ for different cationic charge species using the Gouy–Chapman theory. From our model, we can say that if the "bound" charges are sufficiently deep in the polar headgroup that $z_j - z_k > 0$ for typical values of z_j in eq 4 and 7, then the contribution to the torque in eq 6 and 8 from the parts of the continuous distribution function $g(z_k)$ for $z_j > z_k$

will be partially canceled by contributions from the region in which $z_j < z_k$, so that the bound charge fraction f_b will tend to dominate the torque. Furthermore, contributions for $z_k - z_j$ greater than the Debye shielding distance parameter will be reduced due to electrostatic shielding effects.

Similar arguments can be made to explain the weakness of the influence of pentalysine (Roux et al., 1988) on ²H NMR splittings relative to that of $K_2GL_{20}K_2A$ peptides. Pentalysine is not anchored in the hydrophobic region of the membrane but is associated with the external part of the polar headgroup region.

In the case of PS headgroups, the influence of pentalysine and also cytochrome c, which is known to be bound to the periphery of negatively charged membranes via lysine residues (Devaux et al., 1987), is also much weaker than that of the integral membrane $K_2GL_{20}K_2A$ peptide, but is still measurable. The fact that the small observed changes in $\Delta\nu_{(1)}$ and $\Delta\nu_{(3)}$ are of opposite sign to those produced by $K_2GL_{20}K_2A$ is predicted by eq 7, since the sign of $z_j - z_k$ is opposite for peripheral charges to that of charges on the inner side of the polar headgroup.

The observation that Li$^+$ ions produce the same sign of changes in $\Delta\nu_{(1)}$ and $\Delta\nu_{(3)}$ as do $K_2GL_{20}K_2A$ peptides is consistent with our earlier interpretation of the influence of Li$^+$ ions on DMPC/DMPS membranes (Roux & Neumann, 1986). The fact that Na$^+$ ions produce little change in $\Delta\nu_{(1)}$ and $\Delta\nu_{(3)}$ relative to that produced by Li$^+$ ions indicates that $d_{\text{Na}^+} \ll d_{\text{Li}^+}$; i.e., Li$^+$ ions are bound to PC/PS membranes at a greater depth than are Na$^+$ ions. It would be of considerable interest to check the interpretation by X-ray diffraction techniques.

Reduction of the concentration of the negatively charged DMPS molecules in DMPC/DMPS mixtures is almost equivalent to the addition of positively charged $K_2GL_{20}K_2A$ peptide, in that it results in a decrease in $\Delta\nu_{(1)}$ and $\Delta\nu_{(3)}$. An important difference, however, is that change in DMPS concentration in zwitterionic DMPC membranes modifies $\Delta\nu_{(2)}$ as well (Roux & Neumann, 1986). For consistency with the model, this requires that the torque on PS headgroups due to the distribution of charge in neighboring PS headgroups not be parallel to the CD[(2)] bond direction (see Model for a Quantitative Interpretation of the Headgroup Quadrupolar Splittings). A possible origin of this difference is that the DMPS headgroup's net negative charge of one unit is made up of two negative charges associated with the PO$_4^-$ and COO$^-$ groups and one positive charge due to the NH$_3^+$ moiety. Since the motions of these three sources of charge are correlated, they may well give rise to an *average* electric field which has a different direction than that of a uniform charge density arising from a single charge. It will be interesting to see whether a detailed calculation of the torque on PS headgroups will explain the observed variation of $\Delta\nu_{(2)}$. This picture could also explain another experimental observation concerning the effect of PS charges on neighboring PS headgroups, which is that different variations of $\Delta\nu_{(1)}$ and $\Delta\nu_{(2)}$ are observed whether dilution of this negative lipid takes place in zwitterionic PE or PC membranes (Roux & Neumann, 1986).

Further NMR experiments, such as those concerning the influence of metallic cations on the lipid headgroup interactions with the peptide $K_2GL_{20}K_2A$, are now under way, in order to test the validity of the continuum model, especially in the case of the PS headgroup.

with the computer graphics software developed at TRIUMF (Vancouver, B.C.).

Registry No. DMPC, 13699-48-4; DMPS, 64023-32-1; Lys-Lys-Gly-Leu₂₀-Lys-Lys-Ala-CONH₂, 95576-41-3.

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