Response of the Headgroup of Phosphatidylglycerol to Membrane Surface Charge As Studied by Deuterium and Phosphorus-31 Nuclear Magnetic Resonance†

Francesca M. Marassi and Peter M. Macdonald*

Department of Chemistry and Erindale College, University of Toronto, 80 St. George Street,
Toronto, Ontario, Canada M5S 1A1

Received March 14, 1991; Revised Manuscript Received July 9, 1991

ABSTRACT: The response to membrane surface charge of the glycerol headgroup of dimyristoylphosphatidylglycerol (DMPG) was investigated via deuterium and phosphorus-31 nuclear magnetic resonance spectroscopy. The membrane surface charge was manipulated by adding various amounts of neutral dimyristoylphosphatidylcholine (DMPC) and/or positively charged didodecyldimethylammonium bromide (DDAB) to the negatively charged DMPG, selectively deuterated at the α and β segments of its glycerol headgroup. The deuterium and phosphorus-31 nuclear magnetic resonance spectra were all characteristic of random dispersions of liquid-crystalline lipids in a bilayer configuration. Differential scanning calorimetry showed that all mixtures investigated exhibited gel to liquid-crystalline phase transitions below 35 °C. Measurements of the deuterium quadrupole splitting and of the phosphorus-31 chemical shift anisotropy lead to the following observations. (1) Dilution of the negative surface charge density by the addition of DMPC had little effect on the quadrupole splitting from either α- or β-deuterated DMPG. (2) Direct cancellation of the negative surface charge density by addition of DDAB led to a progressive decrease in the quadrupole splitting measured from α-deuterated DMPG, while the quadrupole splitting measured from β-deuterated DMPG increased. For α-deuterated DMPG addition of 0.3 mole fraction of DDAB resulted in the appearance of two distinct quadrupole splittings. No such effect was observed for β-deuterated DMPG. The addition of mole fractions of DDAB greater than 0.3 resulted in the formation of nonbilayer lipid phases. (3) Ternary mixtures of 0.5 mole fraction of DMPC plus X_a mole fraction of DMPG and X_b mole fraction of DDAB (where $X_a + X_b = 0.5$) yielded bilayer-like phosphorus-31 line shapes for all values of X_a and X_b. Progressively increasing the mole fraction of DDAB in such ternary mixtures again caused the quadrupole splitting from α- (β-) deuterated DMPG to decrease (increase). At neutral surface charge density ($X_a = X_b = 0.25$) two quadrupole splittings were resolved for the α-labeled headgroup, while a single quadrupole splitting was observed for the β-labeled headgroup. For positive surface charge densities ($X_a < X_b$) both the quadrupole splittings from the deuterons at the α position continued to decrease while the single splitting from the deuteron at the β position increased. (4) Increasing the mole fraction of DDAB in the ternary mixtures brought about a slight decrease in the chemical shielding anisotropy of DMPG and a larger decrease in the chemical shielding anisotropy of DMPC. No evidence was found for any long-lived complex between negatively charged and positively charged species. We conclude that, while the phosphoglycerol headgroup of DMPG is less sensitive to membrane surface charge than the phosphocholine headgroup, nevertheless, it does undergo a unique conformational change under the influence of modifications of surface charge. Our semiempirical molecular orbital calculations comparing the atomic charge distributions in the phosphoglycerol and phosphocholine headgroups indicate that this conformational change is possibly the result of the local headgroup dipoles (i.e., OH or CO) seeking to align themselves with the direction of the electrical field at the surface.

The membrane surface electrical potential is known to regulate many important biological processes such as membrane protein binding and transmembrane ion transport, to name but a few (Jain, 1988). In order to understand these processes, it is of primary importance to obtain a clear picture of the effect of surface charge on the structure of the membrane and of the individual membrane components. Phospholipids are major building blocks of biological membranes, and the charge carried by the phospholipid headgroups is a major factor responsible for the regulation of the membrane electrical properties. It is well established, from deuterium nuclear magnetic resonance (^{2}H NMR)[1] studies, that the headgroup of phosphatidylcholine undergoes a conformational change in response to surface charge [Seelig et al. (1987) and references therein]. The ^{2}H NMR results are consistent with a "choline tilt" model in which the entire headgroup tilts with respect to the plane of the membrane surface as the phosphorus–nitrogen dipole reorients under the influence of membrane surface charge (Scherer & Seelig, 1989; Roux et al., 1989; Macdonald et al., 1991). Roux et al. (1989) have observed a similar effect for the headgroup of phosphatidylserine, but the response of

---

† This work was supported by the National Science and Engineering Research Council (NSERC) of Canada.

* Author to whom correspondence should be addressed.

[1] Abbreviations: NMR, nuclear magnetic resonance; $\Delta\sigma$, chemical shielding anisotropy; $\Delta\nu_i$, quadrupole splitting; DSC, differential scanning calorimetry; TLC; thin-layer chromatography; MNDO, modified neglect of diatomic overlap; LCAO SCF, linear combination of atomic orbitals with self-consistent field; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DDAB, didodecyldimethylammonium bromide; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPS, 2,3,5-triisopropylbenzenesulfonyl chloride.

0006-2960/91/0430-10558$02.50/0

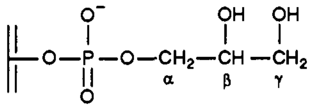
other phospholipid polar groups to surface charge remains unknown.

In this report we present evidence that the phosphoglycerol headgroup of dimyristoylphosphatidylglycerol (DMPG) in liquid-crystalline bilayer membranes is sensitive to electric charges at the membrane surface. Phosphatidylglycerol, by virtue of its negatively charged headgroup, is believed to play a major role in the interaction of membranes with ions and proteins. We have employed ^2H NMR and ^{31}P NMR measurements of DMPG, selectively deuterated at the phosphoglycerol headgroup, to probe the response to membrane surface charge. In order to manipulate the membrane surface charge, we have resorted to two tactics: dilution of the negative surface charge by admixture of zwitterionic dimyristoylphosphatidylcholine (DMPC) and direct cancellation of the negative surface charge by admixture of positively charged didodecyldimethylammonium bromide (DDAB).

In addition, using the MNDO (modified neglect of diatomic overlap) semiempirical molecular orbital method (Dewar & Thiel, 1977a,b; Dewar et al., 1978), we have calculated the atomic charge distributions and dipole moments of the phosphocholine and phosphoglycerol headgroups. These calculations support the theory that the response of phospholipid headgroups to membrane surface charge occurs via an alignment of the internal dipole moments of the headgroups with the electrical field at the surface.

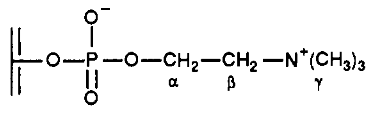
MATERIALS AND METHODS

Synthesis of Headgroup-Deuterated Lipids. The structures of the glycerol (I) and choline (II) headgroups and of didodecyldimethylammonium bromide (III) are shown below. The α, β, γ nomenclature employed for the deuterium-labeled segments of the choline and glycerol headgroups is also indicated



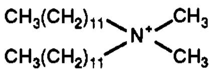
$-O-\overset{O^-}{\underset{O}{P}}-O-\underset{\alpha}{CH_2}-\overset{OH}{\underset{\beta}{CH}}-\overset{OH}{\underset{\gamma}{CH_2}}$

I



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

II



III

Nondeuterated lipids were purchased from Avanti (Alabaster, AL). The synthesis of DMPG was achieved by coupling 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA) with isopropylideneglycerol (Aldrich) using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) as the condensing agent (Aneja et al., 1970), followed by treatment with acetic acid to remove the protecting group (Harlos & Eibl, 1980). Coupling of 2,3-*O*-isopropylidene-*sn*-[1,1-$^2H_2$]glycerol, prepared by the method of Lok et al. (1976), with DMPA yielded DMPG-α,α-d_2 with the naturally occurring L,D configuration. Coupling of *rac*-isopropylidene[2-^2H]glycerol, prepared by the method of Renoll and Newman (1955), with DMPA gave racemic DMPG-β-d, which is a mixture of 3,3′-DMPG-β-d and 3,1′-DMPG-β-d.

DMPC was selectively deuterated at the β segment of the choline headgroup, to obtain DMPC-β,β-d_2, by a combination of the methods described by Harbisson and Griffin (1984) and Aloy and Rabaut (1913). All the products were further purified by chromatography on CM-52 as described by Comfurius and Zwaal (1977), and their purity was checked by TLC, DSC, and ^1H NMR.

Sample Preparation. Samples for NMR studies were prepared as follows. Five to ten milligrams of deuterated DMPG, plus various amounts of DMPC and/or DDAB, all in chloroform/methanol solution, were mixed, and the solvents were removed under a stream of nitrogen followed by high vacuum. The dried lipids were dispersed in excess deuterium-depleted buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) by repeated warming to 40 °C and vortexing. The dispersions were then centrifuged at 13000*g* for 30 min, and the pellets were taken for NMR measurement.

NMR Measurements. ^2H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz. The quadrupole echo technique (Davis et al., 1976) was employed, using quadrature detection with complete phase cycling of the pulse pairs (Griffin, 1981), and a 90° pulse length of 2.0 μs, an interpulse delay of 40 μs, a recycle delay of 250 ms, a spectral width of 100 kHz, and a data size of 2K.

^{31}P NMR spectra were acquired at 121.25 MHz using a Hahn echo sequence (Rance & Byrd, 1983) with proton decoupling. The following experimental conditions were employed: a 90° pulse length of 4.25 μs, an echo spacing of 40 μs, a recycle delay of 1 s, a spectral width of 100 kHz, and a data size of 2K.

Differential Scanning Calorimetry. Gel to liquid-crystalline phase transition temperatures were measured using a Du Pont 910 differential scanning calorimeter operating at a scan rate of 5 °C/min.

Semiempirical Calculations. The atomic charge distributions and dipole moments of the phosphoglycerol and phosphocholine headgroups were calculated using the MNDO approximation to LCAO SCF molecular orbital theory (Dewar & Thiel, 1977a,b; Dewar et al., 1978). All the geometrical parameters, consisting of bond lengths and angles, were optimized except for the torsion angles which were taken from the crystallographic X-ray conformations of DMPG (Pascher et al., 1987) and DMPC (Pearson & Pascher, 1979). The phosphate O(11) atom [atom numbering from Sundaralingam (1972)] was terminated by a hydrogen atom. The PC Model molecular modeling software (Serena Software, Bloomington, IN) was used to construct and display the lipid structures. All calculations were done on the Macintosh II.

RESULTS

Differential Scanning Calorimetry. Previous studies have shown that DMPG, DMPC, and DDAB aggregate spontaneously into bilayers, when dispersed in aqueous buffer, and that these bilayers exhibit distinct gel to liquid-crystalline phase transitions. The transition temperatures for these lipids all fall within close range of one another: 24 °C for DMPG and DMPC and 16 °C for DDAB (Jain, 1988; Blume, 1983; Kunitake & Okahata, 1977; Kajiyama et al., 1979). Representative DSC scans of pure DMPG and a ternary mixture of DMPG, DDAB, and DMPC are shown in Figure 1. All cases exhibited well-defined endothermic phase transitions. Pure α- and β-deuterated DMPG exhibited a transition temperature of 24 °C. Increasing the mole fraction of DDAB led to an increase in the transition temperature to 29 °C and to a progressive broadening of the DSC peaks with the appearance of a shoulder. For all mixtures the phase transition was completed below 35 °C.

^2H NMR Spectroscopy. The ^2H NMR spectra at 35 °C of DMPG deuterated at the α and β segments of the glycerol headgroup are shown in Figure 2, parts a and c. The spectra are typical of random dispersions of liquid-crystalline lipids in a bilayer arrangement (Seelig, 1977) as expected from the DSC results. The size of the quadrupole splittings, corre-

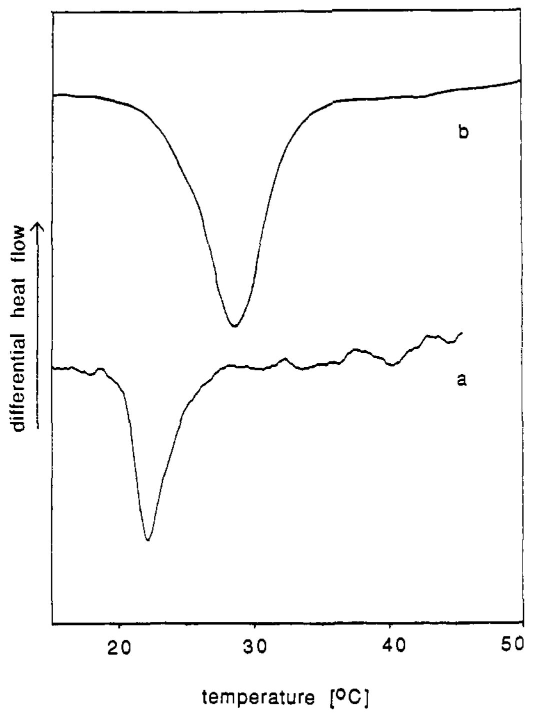


FIGURE 1: Representative differential scanning calorimetry traces of membranes composed of (a) pure DMPG and (b) DMPG/DDAB/DMPC in the molar ratio 20/30/50.

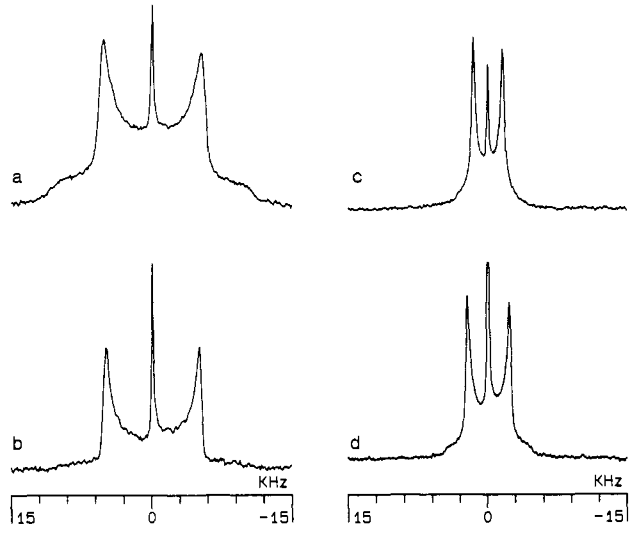


FIGURE 2: ^{2}H NMR spectra from α and β headgroup deuterated DMPG in membranes composed of different mole fractions (X) of DMPG and DMPC. (a) Pure DMPG-α,α-d_2; (b) DMPG-α,α-d_2/DMPC mixture with $X_{\rm DMPG}$ = 0.20; (c) pure DMPG-β-d; (d) DMPG-β-d/DMPC mixture with $X_{\rm DMPG}$ = 0.20.

sponding to the separation in hertz between the two maxima of the spectrum, is 10.7 and 3.2 kHz for DMPG-α,α-d_2 and DMPG-β-d, respectively, in excellent agreement with previously reported results for headgroup-deuterated DPPG (Wohlgemuth et al., 1980). Increasing the temperature from 24 to 60 °C caused a slight decrease (increase) in the quadrupole splitting from the α (β) deuterons. At temperatures of 24 °C and lower a distinct broadening of the ^{2}H NMR spectrum occurred as expected upon transition from the liquid-crystalline to the gel phase.

Addition of as much as 80 mol % DMPC to DMPG had only a small effect on the ^{2}H NMR spectra from both the α and β headgroup deuterated DMPG, causing the quadrupole splitting from the α segment to decrease and that from the β segment to increase (Figure 2, parts b and d). However,

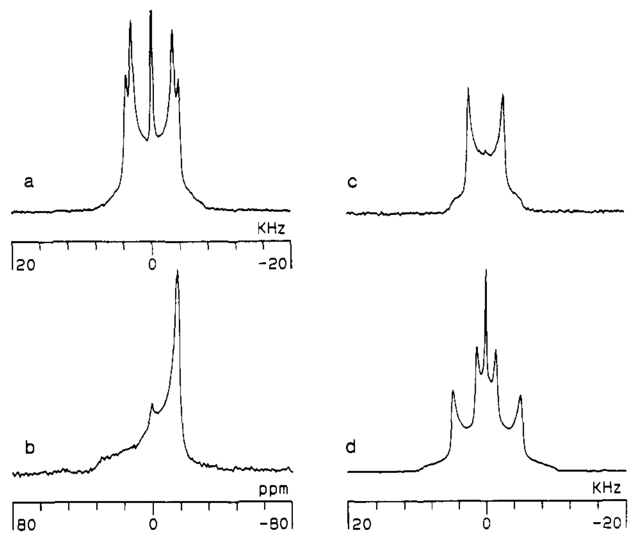


FIGURE 3: Representative ^{2}H NMR (a, c, d) and ^{31}P NMR (b) spectra showing the effect of adding cationic DDAB to membranes of DMPG and of DMPG/DMPC. (a and b) DMPG-α,α-d_2/DDAB mixture in the molar ratio 70/30; (c) pure DMPC-β,β-d_2; (d) DMPG-α,α-d_2/DDAB/DMPC-β,β-d_2 mixture in the molar ratio 63/12/25 (inner splitting, DMPC-β,β-d_2; outer splitting, DMPG-α,α-d_2.

addition of positively charged DDAB to DMPG-α,α-d_2 bilayers caused a far greater decrease in the quadrupole splitting from the α deuterons. Furthermore, progressively increasing the concentration of DDAB in these binary mixtures led to the gradual appearance of two quadrupole splittings for DMPG-α,α-d_2, this effect being particularly marked at 30 mol % DDAB as displayed in Figure 3a.

The ^{31}P NMR spectrum of this composition is shown in Figure 3b and is consistent with a bilayer arrangement of the lipids in the liquid-crystalline phase. Only a single component is observed in the ^{31}P NMR spectrum, indicating that, on the time scale of the ^{31}P NMR experiment, there exists a homogeneous environment for all the DMPG molecules in the membrane. Binary DMPG and DDAB mixtures with greater than 30 mol % DDAB did not form bilayer phases and yielded ^{31}P NMR spectra having predominantly isotropic and/or cubic characteristics. In pure DMPG and pure DDAB membranes the negative or positive surface charges help to stabilize the lipid packing arrangement into a bilayer phase via electrostatic repulsions between adjacent lipids. Upon mixing, mutual charge neutralization occurs and is probably the cause of the collapse of the bilayer structure. In order to continue the studies at higher positive surface charge densities, we investigated ternary mixtures of DMPG, DDAB, and DMPC, where the latter acts as a spacer and has the effect of stabilizing the bilayer phase.

Figure 3d displays the ^{2}H NMR spectrum of a ternary lipid mixture containing 63, 12, and 25 mol % DMPG, DDAB, and DMPC, respectively. In this instance α-deuterated DMPG was used together with DMPC deuterated at the β segment of the choline headgroup. The spectrum is characterized by two overlapping quadrupole splittings, at 9.9 and 2.8 kHz. We assign the outer splitting to DMPG-α,α-d_2 and the inner splitting to DMPC-β,β-d_2 on the following basis. Since this ternary mixture should retain an overall negative surface charge, the molecular voltmeter concept dictates that the quadrupole splitting from the β deuterons of DMPC should decrease. That this has indeed occurred can be ascertained by comparison with Figure 3c, which shows the ^{2}H NMR spectrum of pure DMPC-β,β-d_2 where the quadrupole splitting equals 5.8 kHz. In contrast, the splitting from DMPG-α,α-d_2 is only slightly changed, from 10.7 kHz, in pure DMPG, to

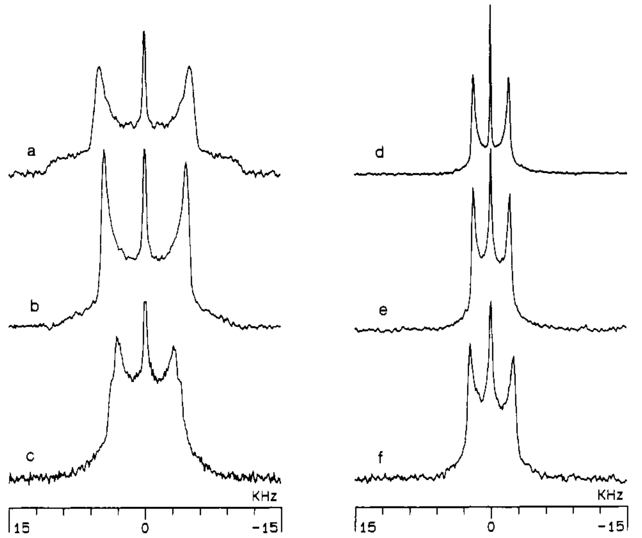


FIGURE 4: ^2H NMR spectra of the α (a–c) and β (d–f) deuterons of headgroup-deuterated DMPG in membranes composed of 50 mol % DMPC and different mole fractions (X) of DMPG and DDAB. (a) $X_{DDAB} = 0.0$; (b) $X_{DDAB} = 0.15$; (c) $X_{DDAB} = 0.30$; (d) $X_{DDAB} = 0.0$; (e) $X_{DDAB} = 0.15$; (f) $X_{DDAB} = 0.30$.

9.9 kHz here, in parallel with the behavior of the DMPG headgroup in binary mixtures. Both lipids appear to experience the combined surface charge effects of the cationic DDAB, the anionic DMPG, and the neutral DMPC, indicating that all three lipid components are homogeneously distributed throughout the membrane.

The changes that occur in the ^2H NMR spectra of headgroup-deuterated DMPG with increasing amounts of positively charged DDAB are illustrated in Figure 4. These spectra were acquired from ternary lipid mixtures consisting of 50 mol % DMPC plus varying amounts of DMPG and DDAB. Addition of 50 mol % DMPC to DMPG has a negligible effect on the quadrupole splitting of either α or β headgroup deuterated DMPG (Figure 4, parts a and d). Figure 4a–c displays the ^2H NMR spectra of DMPG-α,α-d_2 mixtures. Increasing the amount of DDAB led to a progressive decrease in the quadrupole splitting and to the gradual appearance of two quadrupole splittings for the α deuterons, similar to those observed with the binary mixtures. The effect of positive charge on the ^2H NMR spectrum of DMPG-β-d is shown in Figure 4d–f. The quadrupole splitting of the β deuteron increases at higher concentrations of DDAB. However, the β deuteron is less sensitive to surface charge than the α deuterons as evidenced by the smaller change in the DMPG-β-d quadrupole splitting.

Figure 5 provides a summary of the effect of membrane surface charge on the ^2H NMR quadrupole splittings of the DMPG headgroup. Here the quadrupole splittings from the α and β headgroup deuterons have been plotted against the mole fraction of DDAB. The progressive decrease in the quadrupole splittings from both deuterons of α-deuterated DMPG is not linear but approaches a limiting value with increasingly positive surface charge. By contrast, the increase observed in the quadrupole splitting from the β deuteron is too small to warrant any such conclusion.

^{31}P NMR Spectroscopy. The ^{31}P chemical shielding anisotropy ($\Delta\sigma$) was measured in order to investigate the effect of surface charge on the phosphate group of DMPG. The ^{31}P NMR spectra of pure DMPG, pure DMPC, mixed DMPG/DMPC (50/50), and mixed DMPG/DDAB/DMPC (25/25/50) are shown in Figure 6. Their shapes are each characteristic of liquid-crystalline bilayers. The $\Delta\sigma$'s of pure DMPG and DMPC (Figure 6, parts a and b) were measured to be −36 and −47 ppm, respectively, in close agreement with previously reported data for DPPG and POPC (Wohlgemuth et al., 1980; Tamm & Seelig, 1983). In the 50/50 mixture of DMPG/DMPC (Figure 6c) the negative surface charge, imposed on the membrane by DMPG, has the effect of reducing the magnitude of $\Delta\sigma$ ($|\Delta\sigma|$) for DMPC from 48 to 39 ppm, whereas dilution of the negative charge by the addition

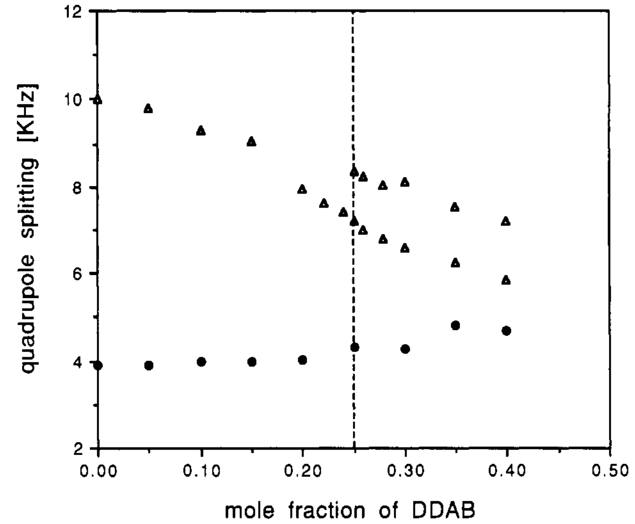


FIGURE 5: Effect of increasing the positive surface charge density on the ^2H quadrupole splittings from headgroup-deuterated DMPG. The membranes were composed of DMPG/DDAB/DMPC mixtures where the mole fraction of DMPC was kept constant at 0.50 and the mole fractions of DMPG and DDAB were varied. (▲) DMPG-α,α-d_2; (●) DMPG-β-d.

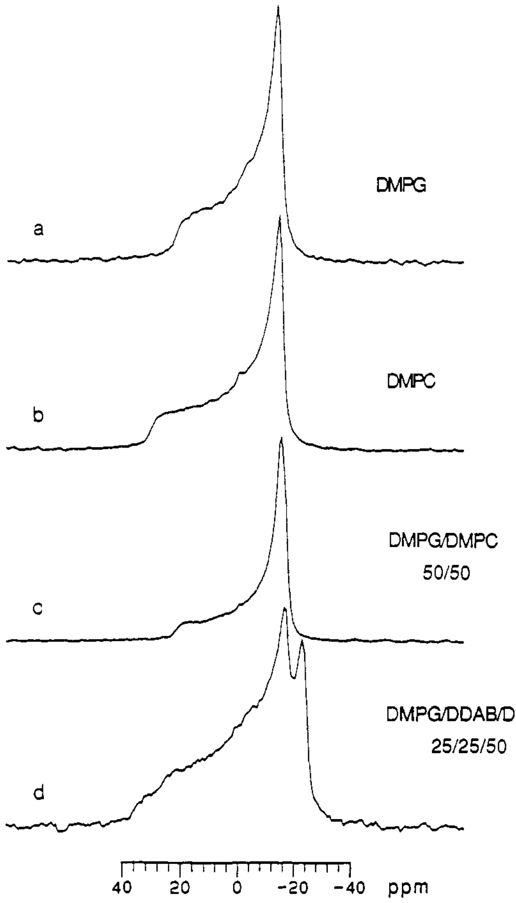


FIGURE 6: ^{31}P NMR spectra of membranes composed of (a) pure DMPG; (b) pure DMPC; (c) DMPG/DMPC mixture in the molar ratio 50/50; and (d) DMPG/DDAB/DMPC mixture in the molar ratio 25/25/50 (inner spectrum, DMPG; outer spectrum, DMPC).

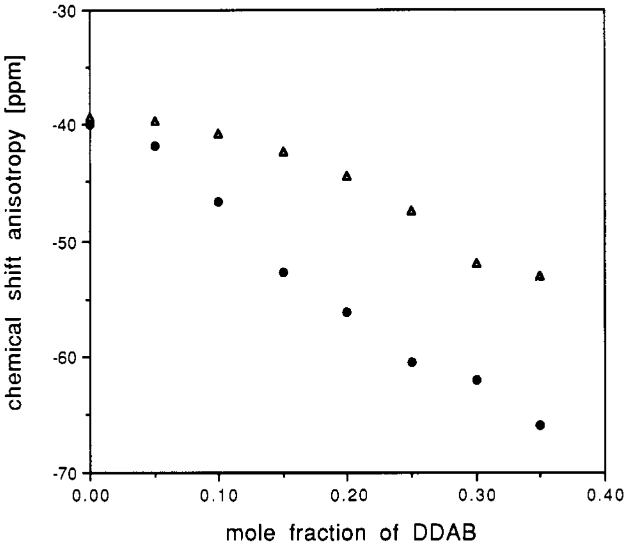


FIGURE 7: Variation of the ^{31}P chemical shielding anisotropy of DMPG and DMPC under the effect of increasing the positive surface charge density. The membranes were composed of DMPG/DDAB/DMPC mixtures where the mole fraction of DMPC was kept constant at 0.50 and the mole fractions of DMPG and DDAB were varied. (▲) DMPG; (●) DMPC.

of DMPC has almost no effect on the $\Delta\sigma$ of DMPG. Hence, the ^{31}P NMR powder spectra from DMPC and DMPG overlap completely. By contrast, increasing the positive surface charge density through admixture of DDAB has the effect of progressively increasing the magnitude of $\Delta\sigma$ of both DMPG and DMPC. At high positive surface charge densities the ^{31}P NMR spectra clearly consist of two superimposed, axially symmetric powder patterns with distinct $\Delta\sigma$'s, as illustrated in Figure 6d. Since the ^{2}H NMR data suggest that the bilayer phase is homogeneous throughout the concentration series in the ternary lipid mixtures, the overlapping powder spectra cannot be due to two separate phases with distinct lipid compositions. Figure 7 shows how the $\Delta\sigma$'s of these two overlapping powder spectra evolve as a function of the mole fraction of DDAB. A comparison of these results with the ^{31}P NMR data obtained by Scherer and Seelig (1989) for binary mixtures of POPC with cationic quaternary ammonium amphiphiles reveals that, for comparable net surface charge densities, the change in $\Delta\sigma$ of POPC is close to that observed here for the spectral component with the $\Delta\sigma$ of largest magnitude. On this basis we attribute this spectral component to DMPC. By elimination, the spectral component with the $\Delta\sigma$ of lesser magnitude is attributed to DMPG. As expected on the basis of such an assignment, the intensities of the two overlapping ^{31}P NMR spectra vary directly with the proportion of DMPC to DMPG in the ternary mixtures. We conclude that the response of the phosphate segment of DMPC to changes in surface charge is greater than that of DMPG.

DISCUSSION

The response of phosphatidylcholine to the presence of electrical charge at the surface of the phospholipid bilayer occurs via a conformational change in the choline headgroup (Seelig et al., 1987). This effect, which is referred to as the "molecular voltmeter", has proved to be operative in all cases involving the deposition of charges at the surface of a membrane. The origin of these charges may be external to the membrane, as is the case for the binding of surface ligands like divalent cations or peptides, or internal to the membrane, as is the case for the mixing of charged phospholipids and amphiphiles [for a recent review see Seelig et al. (1987)]. The data indicate that the P^{-}–N^{+} dipole moment of the choline headgroup is the critical property responsible for the sensitivity to changes in membrane surface charge. A conformational change is observed when this dipole realigns itself in a positive or negative field. Thus, positive charge repels (attracts) the N^{+} positive (PO_4^{-} negative) end of the choline headgroup, while negative charge has the opposite effect. Recently, Roux et al. (1989) have shown that the serine headgroup of DMPS also undergoes a conformational perturbation directly related to surface charge and have presented a model that describes the influence of membrane surface charge on headgroup conformation. This model is based on the premise that surface charge exerts a torque on the headgroup dipole causing it to tilt with respect to the membrane surface, thereby inducing the changes in the ^{2}H NMR quadrupole splittings. Application of this model to data for the choline headgroup has been shown to reproduce the essential features of the relationship between the ^{2}H NMR quadrupole splittings of choline and membrane surface charge (Macdonald et al., 1991). This model is also expected to hold well for other zwitterionic headgroups, such as that of phosphatidylethanolamine, where the size of the headgroup dipole is probably substantial, so that headgroup tilts can be treated as dipole tilts.

In the studies mentioned above, charged, bilayer-forming lipids, such as DDAB and DMPG, were used to manipulate the membrane surface charge while preserving other aspects of the bilayer structure. The ^{31}P spectra (Figures 3b and 6) and the DSC results show that, for all the mixtures investigated in this work, the lipids are in a liquid-crystalline bilayer arrangement. Moreover, it is unlikely that the addition of DDAB and DMPC to DMPG bilayers should induce appreciable changes in intermolecular interactions, such as hydrogen bonding. For hydrated lipid bilayers hydrogen bonds between polar headgroups constitute only a minor stabilizing factor, since they are easily replaced by hydrogen bonds to water (Gennis, 1989; Boggs, 1987). X-ray diffraction and ^{2}H NMR studies of phosphoglycerol, phosphocholine, and phosphoethanolamine, in the gel and in the liquid-crystalline phases, have shown that, in these bilayers, there is a primary hydration shell in rapid exchange with bulk water (Watts et al., 1981; Borle & Seelig, 1983). Thus, the opportunity for DMPG to hydrogen bond should remain virtually unchanged as a consequence of adding DDAB or DMPC. We conclude that the observed changes in the quadrupole splittings from headgroup-deuterated DMPG are due to changes in the surface charge rather than to changes in the intermolecular interactions between neighboring headgroups.

A further advantage of using the charged lipids, DDAB and DMPG, as a means of manipulating the membrane surface charge is that there is no ambiguity regarding the location of the charges, i.e., all the charged groups remain localized at the bilayer surface. For the case of homogeneously mixed, liquid-crystalline bilayers we can consider the charge as being smeared over the entire surface, and we can estimate the magnitude of the surface charge from the lipid mixing ratio.

^{2}H NMR. The addition of DDAB and/or DMPC leads to a counterdirectional shift in the quadrupole splittings from the α and β headgroup deuterons of DMPG, suggesting that the glycerol headgroup is responding via some specific conformational change. A nonspecific response, such as an increase (decrease) in headgroup flexibility, would lead to a concurrent decrease (increase) of the quadrupole splittings from both the α and β deuterons (Seelig et al., 1987).

The magnitude of the conformational change undergone by phosphoglycerol with increasingly positive surface charge can be estimated using

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

Equation 1 is simply an empirical formula which can be used for the parametrization of the experimental data. It relates the size of the observed quadrupole splitting, $\Delta\nu_i$, to the angle, θ, between the C–D bond vector and the main axis of motional averaging, assumed to lie parallel to the lipid long molecular axis. S_f is an order parameter representing the ensemble of fluctuations which are independent of the α and β positions in the headgroup, and encompassing various modes of flexibility. $\Delta\nu_Q \approx 125$ kHz and represents the size of the quadrupole splitting in the absence of motional averaging. In order to employ eq 1 to obtain the angle through which the C–D bond must rotate in order to give a certain, observed change in $\Delta\nu_i$, it is only necessary to assume that S_f is constant for a given headgroup segment. This allows us to make a useful comparison between the angular excursions spanned by the phosphocholine and phosphoglycerol headgroups. S_f has not been measured experimentally for the phosphoglycerol group, but it is likely to be less than 0.66, which is the order parameter for the C(1) position of the glycerol backbone of DPPC (Gally et al., 1975). Since the largest quadrupole splitting measured here is 10.65 kHz, eq 1 constrains S_f to be greater than 0.084. This value is somewhat low and closer to that expected for a dynamically isotropic system. As a first guess we set a value of $S_f = 0.10$. Increasing the positive surface charge density, in the ternary mixtures, from $X_{DDAB} = 0.00$ to $X_{DDAB} = 0.40$ causes the $\Delta\nu_i$'s from the two α deuterons to decrease from 10.02 kHz to either 5.84 kHz or 7.20 kHz. This corresponds to respective angular excursions of the α C–D bond vectors of 15.3° and 10.8°. For the case of $S_f = 0.20$, the same changes in $\Delta\nu_i$ yield angular excursions of 6.4° and 4.4°, respectively. These conformational changes are much smaller in magnitude than those measured for the headgroup of phosphatidylcholine (Scherer & Seelig, 1989), where comparable densities of surface charge lead to headgroup rotations, causing changes in θ of about 49° for $S_f = 0.10$. For phosphoglycerol, the fact that θ changes by an approximately equal amount for both the α deuterons suggests that the conformational change of the polar headgroup involves a rotation about an axis around which both deuterons are symmetrical.

Further insight into the precise nature of the conformational change experienced by the phosphoglycerol headgroup is provided by a consideration of the origin of the two quadrupole splittings observed at the α positions. With increasing amounts of positively charged DDAB, the two deuterons of the α-glycerol segment give rise to two overlapping quadrupole splittings which can be resolved at a mole fraction of DDAB ≥ 0.25. Most CD_2 segments of phospholipids give rise to a single quadrupole splitting because the molecular fluctuations about the director axis average out the differences between the two deuterons (Seelig, 1977). There are four possible explanations for the observation of two quadrupole splittings: (1) DMPG and DDAB could undergo specific, long-lived, pairwise complex formation; (2) DMPG could be partitioned between DDAB-rich and DDAB-poor lipid domains; (3) a decrease in flexibility could result in incomplete motional averaging of the two deuterons; and (4) the two α deuterons could become motionally inequivalent through a conformational change.

If the two quadrupole splittings from the α deuterons were due to the presence of two types of DMPG, one type free and the other in complex with DDAB, the relative intensities of the two signals should change with the proportion of DDAB, while their quadrupole splittings ($\Delta\nu_i$) should remain constant. This was not observed to occur. Instead, the relative intensities of the two splittings remain constant with increasing mole fraction of DDAB, while both quadrupole splittings continue to decrease with increasing positive charge. Also, in the event of specific complex formation, one would expect the ^{31}P NMR spectra to reflect the presence of two populations. Figure 3b shows that the ^{31}P NMR detects a single, homogeneous environment for DMPG. The time scales for ^{2}H NMR and ^{31}P NMR are so similar as to make it unlikely that an event which is slow on the ^{2}H NMR time scale could be fast on the ^{31}P NMR time scale. Similar arguments can be invoked to suggest that the second possibility, that DMPG is partitioning between DDAB-rich and DDAB-poor domains, is also unlikely. In addition, we note that the ^{2}H NMR spectrum from β-deuterated DMPC, in ternary mixtures with DMPG and DDAB (Figure 3d), reports only a single charge environment. In ^{2}H NMR studies with α-deuterated DMPC, both in binary mixtures with DDAB and ternary mixtures with DDAB and DMPG, only a single quadrupole splitting is observed (Marassi and Macdonald, unpublished results).

A decrease in molecular flexibility would cause the quadrupole splittings from both the α and β headgroup deuterons to increase. This is contrary to our observation; therefore, possibility 3 can be excluded. It seems most likely, then, that the increasingly positive surface charge causes the two α deuterons to become motionally inequivalent as a result of a conformational change in the phosphoglycerol headgroup. Motional inequivalence also explains the presence of two quadrupole splittings in the ^{2}H NMR spectrum of the α-dideuterated glycerol headgroup in bilayers of pure DPPG, having the L configuration in both the glycerol backbone and glycerol headgroup (Wohlgemuth et al., 1980), and in the ^{2}H NMR spectrum of α-dideuterated DMPS bilayers (Roux et al., 1989). Stereospecific deuteration of the glycerol α segment should provide unambiguous proof for this interpretation. As will be discussed below, the onset of motional inequivalence for the two α deuterons of the DMPG headgroup can be explained in terms of changes in the internal torsion angles of glycerol, undergone by the headgroup in response to surface charge effects.

^{31}P NMR. The ^{31}P NMR studies show that the phosphate segment of DMPG is also sensitive to membrane surface charge. For both DMPG and DMPC, $\Delta\sigma$ decreases with increasing positive surface charge density. From an analysis of the changes expected in the ^{31}P NMR chemical shift anisotropies of phosphocholine for different orientations of the phosphate group with respect to the axis of motional averaging, Scherer and Seelig (1989) have concluded that, with positive surface charge, the entire phosphate segment reorients, leaving the σ_{11} component of the chemical shielding tensor aligned perpendicular to the axis of motional averaging. This reorientation most likely involves a rotation about the C(1)–O(11) glycerophosphate bond. Such a quantitative analysis of the ^{31}P NMR results was only possible because $\Delta\sigma$ approached a limiting value expected for a particular alignment of the chemical shielding tensors. Since $\Delta\sigma$ is determined by two independent order parameters, the average orientation of the phosphate usually cannot be determined from $\Delta\sigma$ alone (Niederberger & Seelig, 1976; Seelig, 1978). In our studies for DMPG, at high positive surface charge, the lowest $\Delta\sigma$ observed is −53 ppm, a value that is still too far from any limiting value to allow an unambiguous conformational interpretation of the ^{31}P NMR data. However, the fact that, for DMPG, the changes in $\Delta\sigma$ with positive surface charge follow the same trend observed for phosphocholine suggests

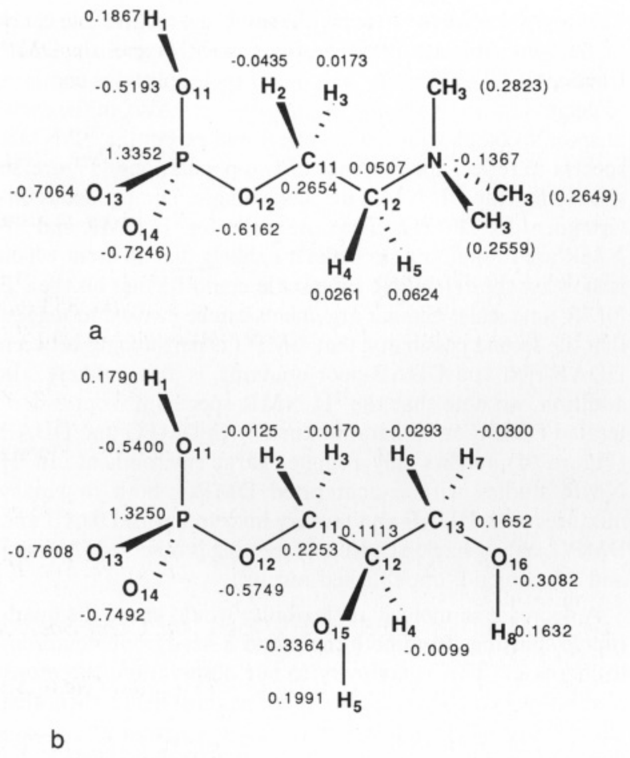


FIGURE 8: Net atomic charges calculated for the phosphocholine (a) and phosphoglycerol (b) headgroups using the MNDO semiempirical molecular orbital method. For these calculations the phosphate O(11) atom was terminated by a hydrogen.

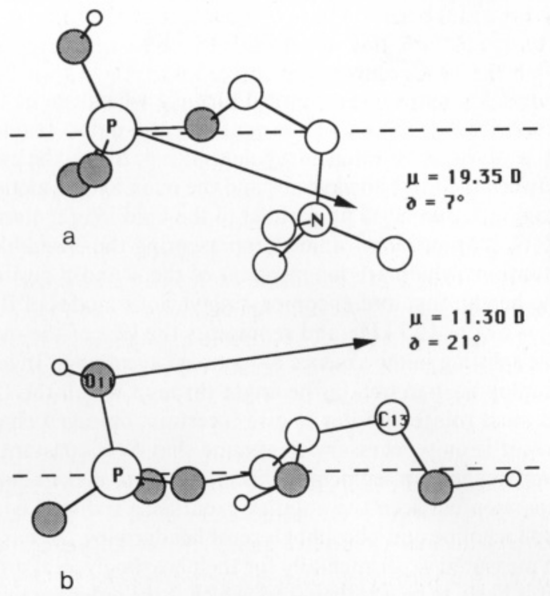


FIGURE 9: Headgroup dipole moments (μ) calculated for the phosphocholine (a) and phosphoglycerol (b) headgroups using the MNDO semiempirical molecular orbital method. For these calculations the phosphate O(11) atom was terminated by a hydrogen. ∂ is the angle between the dipole moment vector and the P–N and P–C(13) headgroup vectors of phosphocholine and phosphoglycerol, respectively. The arrow represents the dipole moment vector, and the dashed line represents the bilayer plane.

that a similar conformational change involving rotation about the C(1)–O(11) bond could also be occurring in the phosphoglycerol headgroup.

Nature of the Conformational Change of the DMPG Headgroup. The ^{2}H and ^{31}P NMR results together indicate that all segments of the phosphoglycerol headgroup undergo a conformational change in response to changes in membrane surface charge. Quantitatively, phosphoglycerol appears to be a less sensitive probe of membrane surface electrostatics by a factor of about 4 relative to phosphocholine. Qualitatively, the response of phosphocholine to surface charge is believed to involve a concerted tilt of the entire headgroup, without requiring any changes in internal torsion angles. The force causing this tilt is the torque experienced by the P–N zwitterionic dipole of phosphocholine in the presence of an electrical field. In order to gain insight into the possible conformational changes undergone by the phosphoglycerol group, we have calculated its atomic charge distribution and its headgroup dipole moment using the MNDO method, and we have compared the results with those we obtained for phosphocholine. The results of MNDO semiempirical calculations have been found to be as accurate as those of minimum basis set ab initio and other semiempirical methods, when compared to experimental data. The net atomic charges calculated by MNDO are usually similar to the ones calculated by ab initio methods (STO-3G basis set) (Dewar & Thiel, 1977a,b; Dewar & Ford, 1979). Furthermore, owing to the computational advantages of MNDO over minimum basis set, and particularly over more sophisticated, ab initio methods, MNDO seems to be the method of choice for the computation of large molecular structures such as those of phospholipid headgroups.

Figure 8 shows the MNDO atomic charge distributions for phosphocholine and phosphoglycerol. The zwitterionic character of phosphocholine and the anionic character of phosphoglycerol are predicted correctly by MNDO. Although MNDO gives a net negative atomic charge for the choline nitrogen, adding the charges of the four interacting groups around the quaternary nitrogen, specifically the β-methylene and the three γ-methyl groups, yields a net cationic charge of +0.8056, localized at the nitrogen, as expected. A similar treatment of the phosphate groups, including the terminal hydrogen atom bonded to O(11) and the P, O(11), O(12), O(13), and O(14) atoms, gives total charges of −1.0446 and −1.1209 for the phosphocholine and phosphoglycerol phosphates, respectively.

The dipole moment (μ) of the phosphocholine headgroup of DMPC (Figure 9a) was determined with respect to a coordinate system having the P atom as the origin. This was chosen to allow a direct comparison between μ and the P–N headgroup vector, since the net atomic charges of Figure 8 indicate that the negative charge of the phosphocholine phosphate group is approximately centered around the P atom. Note that the dipole moment of an electrically neutral, zwitterionic species is independent on the choice of coordinate system. For phosphocholine we obtained a value of μ = 19.35 D, in close agreement with the experimental value of 19 D obtained by Shepherd and Buldt (1978). We found that the direction of the dipole vector is parallel (within 7°) to the phosphocholine P–N vector. The size of the torque experienced by a dipole in an electric field varies as a function of the sine of the angle between the dipole and the field vectors. Furthermore, membrane surface charges produce an electrical field emanating outward from the plane of the membrane surface, and the X-ray data indicate that the phosphocholine headgroup lies nearly parallel to the plane of the membrane surface (Pearson & Pascher, 1979). It is evident then that the phosphocholine headgroup will experience the full measure of any torque exerted by an electrical field on its dipole moment.

The dipole moment of the phosphoglycerol headgroup of DMPG was calculated in a coordinate system having as its origin the O(11) atom, which links the headgroup to the glycerol backbone. Within the context of the torque–countertorque picture, this corresponds to the hinge position about which the headgroup might rotate under the influence of a torque due to an electrical field (Roux et al., 1989). This choice of origin allows us to evaluate the extent of the torque, if any, experienced by the phosphoglycerol headgroup for a given surface electrical field. For phosphoglycerol we obtained a value of $\mu = 11.30$ D, oriented at approximately 21° to the O(11)–C(13) headgroup vector. This result suggests that a concerted tilting of the entire headgroup is not the major mechanism through which phosphoglycerol responds to surface charge. First, the size of the dipole moment is considerably smaller than that of phosphocholine, indicating a much reduced interaction with the electrical field. This perhaps explains the low sensitivity of phosphoglycerol to changes in the membrane surface charge. Second, the dipole moment vector and the phosphoglycerol headgroup are not colinear, so that, unlike the phosphocholine case, a reorientation of the dipole moment need not involve a reorientation of the entire headgroup.

More specifically, a close inspection of Figure 8b reveals that the nonesterified phosphate oxygens of phosphoglycerol, O(13) and O(14), carry the largest portion of the negative anionic charge and that the hydroxyl oxygens, O(15) and O(16), also have relatively large negative charges. Therefore, it is possible for the P–O(13) and P–O(14) dipoles as well as the α and β C–O bond dipoles to align themselves with the direction of an electrical field through changes in the corresponding internal torsion angles. It is generally held that phospholipid headgroups are best described as preferring certain quasiconformations in which the torsion angles are constrained to fall within more or less distinct ranges, rather than being characterized by either complete motional freedom or hindrance (Skarjune & Oldfield, 1979). In the context of such a picture, we view the effects of surface electrical fields as influencing the average position about which the torsion angles are distributed, without necessarily affecting the distribution about the average. Such changes in the average torsion angles could explain not only the changes in the ^{2}H quadrupole splittings and the ^{31}P chemical shift anisotropy but also the onset of motional inequivalence of the two α deuterons of phosphoglycerol. In contrast, for the phosphocholine group, the negative and positive charge distributions are symmetric about the phosphate and amine centers, respectively, implying that, in the absence of steric hindrance, changing the internal torsion angles of the headgroup will do little to relieve the torque felt by the headgroup in the presence of a surface electrical field. Instead, the tension is released by tilting the entire phosphocholine group.

Finally, we note that some caution must be exercised in viewing the changes in quadrupole splittings of phospholipid headgroups induced by charged species as being purely a function of uniform surface electrostatic fields. For example, the quadrupole splittings measured for the mixtures 0.20/0.80 DMPG/DMPC and 0.35/0.15/0.50 DMPG/DDAB/DMPC differ by approximately 1 kHz although, nominally, the two mixtures have identical surface charge densities. Our ^{2}H NMR studies of α and β headgroup deuterated DMPC in similar binary and ternary mixtures with DDAB and DMPG (Marassi and Macdonald, unpublished results) indicate that the observed quadrupole splitting is the net result of specific, pairwise, electrostatic interactions between the lipids, weighted according to the mole fraction of charged species present in the mixture. It is important to realize that different charged species vary in the extent to which they change the quadrupole splitting. Different mixtures can yield different quadrupole splittings despite their having the same nominal surface charge. The same situation likely pertains to the case of the DMPG headgroup.

REFERENCES

Aloy, M. M., & Rabaut, C. (1913) *Bull. Soc. Chim. Fr. 13*, 457–460.

Aneja, R., Chada, J. S., & Davies, A. P. (1970) *Biochim. Biophys. Acta 218*, 102–111.

Blume, A. (1983) *Biochemistry 22*, 5436–5442.

Boggs, J. M. (1987) *Biochim. Biophys. Acta 906*, 353–404.

Borle, F., & Seelig, J. (1983) *Biochim. Biophys. Acta 735*, 131–136.

Cevc, G., & Marsh, D. (1987) *Phospholipid Bilayers: Physical Principles and Models*, Wiley, New York.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.

Dewar, M. J. S., & Thiel, W. (1977a) *J. Am. Chem. Soc. 100*, 4899–4907.

Dewar, M. J. S., & Thiel, W. (1977b) *J. Am. Chem. Soc. 100*, 4907–4917.

Dewar, M. J. S., & Ford, G. P. (1979) *J. Am. Chem. Soc. 101*, 5558–5561.

Dewar, M. J. S., McKee, M. L., & Rzepa, H. S. (1978) *J. Am. Chem. Soc. 100*, 3607.

Gally, H. U., Niederberger, W., & Seelig, J. (1975) *Biochemistry 14*, 3647–3653.

Gennis, R. B. (1989) *Biomembranes: Molecular Structure and Function*, Springer Verlag, New York.

Griffin, R. G. (1981) *Methods Enzymol. 72*, 108–174.

Harbisson, G. S., & Griffin, R. G. (1984) *J. Lipid Res. 25*, 1140–1142.

Harlos, K., & Eibl, H. (1980) *Biochemistry 19*, 895–899.

Jain, M. K. (1988) *Introduction to Biological Membranes*, 2nd ed., Wiley, New York.

Kajiyama, T., Kumano, A., Takayanagi, M., Okahata, Y., & Kunitake, T. (1979) *Chem. Lett.*, 645–648.

Kunitake, T., & Okahata, Y. (1977) *J. Am. Chem. Soc. 99*, 3860–3861.

Lok, C. M., Ward, J. P., & van Dorp, D. A. (1976) *Chem. Phys. Lipids 16*, 115–122.

Macdonald, P. M., Leisen, J., & Marassi, F. M. (1991) *Biochemistry 30*, 3558–3566.

Niederberger, W., & Seelig, J. (1976) *J. Am. Chem. Soc. 98*, 3704–3706.

Pascher, I., Sundell, S., Harlos, K., & Eibl, H. (1987) *Biochim. Biophys. Acta 896*, 77–88.

Pearson, R. H., & Pascher, I. (1979) *Nature 281*, 499.

Rance, M., & Byrd, R. A. (1983) *J. Magn. Reson. 52*, 221–240.

Renoll, M., & Newman, M. S. (1955) *Organic Syntheses*, Collect. Vol. 3, pp 502–504, Wiley, New York.

Roux, M., Neumann, J. M., Hodges, R. S., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313–2321.

Scherer, P. G., & Seelig, J. (1989) *Biochemistry 28*, 7720–7728.

Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.

Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–141.

Seelig, J., Macdonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541.

Shepherd, J. C. W., & Buldt, G. (1978) *Biochim. Biophys. Acta 514*, 83–94.

Skarjune, R., & Oldfield, E. (1979) *Biochemistry 26*, 5903–5909.

Sundaralingam, M. (1972) *Ann. N.Y. Acad. Sci. 195*, 324-355.

Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474-1483.

Watts, A., Harlos, K., & Marsh, D. (1981) *Biochim. Biophys. Acta 654*, 91-96.

Wohlgemuth, R., Waespe-Sarcevic, N., & Seelig, J. (1980) *Biochemistry 19*, 3315-3321.

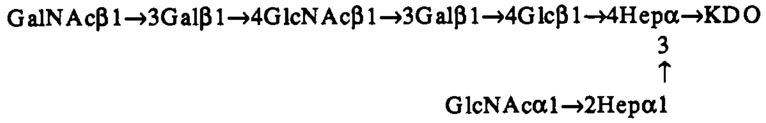
Structural Determination of Oligosaccharides Derived from Lipooligosaccharide of *Neisseria gonorrhoeae* F62 by Chemical, Enzymatic, and Two-Dimensional NMR Methods[†]

Ryohei Yamasaki,[*,‡,§] Bradley E. Bacon,[§] Wade Nasholds,[‡,§] Herman Schneider,[‖] and J. M. Griffiss[‡,§]

Center for Immunochemistry and Department of Laboratory Medicine, University of California, San Francisco, and Veterans Administration Medical Center (113A), San Francisco, California 94121, and Walter Reed Research Institute, Washington, D.C. 20307

Received May 10, 1991; Revised Manuscript Received July 30, 1991

ABSTRACT: F62 LOS of *Neisseria gonorrhoeae* consists of two major LOS components; the higher and smaller molecular weight (MW) components were recognized by MAbs 1-1-M and 3F11 respectively. Base-line separation of the two major oligosaccharide (OS) components from F62 LOS was achieved by Bio-Gel P-4 chromatography after dephosphorylation of the OS mixture. The structures of the two major OSs were studied by chemical, enzymatic, and 2D NMR methods [double quantum filtered COSY (DQF-COSY), delayed COSY (D-COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), pure-absorption 2D NOE NMR] as well as methylation followed by GC/MS analysis. The OS component derived from the MAb 1-1-M defined LOS component was determined to have a V^3-(β-*N*-acetylgalactosaminyl)neolactotetraose structure (GalNAc is β1→3-linked to a neolactotetraose) at one of its nonreducing termini as shown below. The above pentaose is linked to a branched diheptose-KDO core in which a GlcNAc is α-linked. The OS component derived from the MAb 3F11 defined LOS component did not have a GalNAc residue. The rest of its structure was identical to that of the OS-1, and a neolactotetraose is exposed at its nonreducing terminus.



$$\begin{array}{rl} \text{GalNAc}\beta1\rightarrow3\text{Gal}\beta1\rightarrow4\text{GlcNAc}\beta1\rightarrow3\text{Gal}\beta1\rightarrow4\text{Glc}\beta1\rightarrow4 & \text{Hep}\alpha\rightarrow\text{KDO}\\ & \quad 3 \\ & \quad \uparrow \\ \text{GlcNAc}\alpha1\rightarrow2 & \text{Hep}\alpha1 \end{array}$$

Lipooligosaccharides (LOSs)[1] are important antigenic and pathogenic components of the outer membrane of *Neisseria gonorrhoeae*. Gonococcal LOSs cause severe damage to fallopian tubes (Gregg et al., 1981) and are also target molecules for bactericidal activity by normal human sera (Schoolnik et al., 1976). The heterogeneity of gonococcal LOSs in relation to their antigenicities has been extensively investigated. Schneider et al. (1984) showed that gonococcal LOSs consist of several components with molecular weights ranging from 3200 to 7100. Immunochemical analysis of gonococcal LOSs using several murine monoclonal antibodies (MAbs) has shown that gonococcal LOSs consist of discrete individual LOS components, which indicates that the antigenic heterogeneity is due to structural heterogeneity of LOS components (Schneider et al., 1984; Mandrell et al., 1986). Out of several murine MAbs studied, 1-1-M, 3F11, and O6B4 (all IgM) were found to bind to almost all gonococcal LOSs.

Recent immunochemical characterization of human blood group antigen precursors by MAbs 3F11 and O6B4 revealed that the antigenic similarity exists between gonococcal LOSs and glycosphingolipids (Mandrell et al., 1988). Mandrell et al. showed that the two MAbs bind to neutral glycosphingolipids carrying *N*-acetyllactosamine (Galβ1→4GlcNAc) at their nonreducing termini. Further, Stromberg et al. (1988) identified a gonococcal surface component, distinct from pilin and protein II, that binds to epithelial surface glycolipids. This surface component was further investigated and suggested to

---

[†] This work was supported by NIH grants (AI 22998 and AI 22160). This is Report No. 44 from the Center for Immunochemistry, University of California, San Francisco.

[*] Address correspondence to this author at UCSF/VAMC 113A, 4150 Clement St., San Francisco, CA 94121.

[‡] University of California.

[§] VA Medical Center.

[‖] Walter Reed Research Institute.

[1] Abbreviations: 2D, two dimensional; COSY, two-dimensional correlation spectroscopy; DQF-COSY, double quantum filtered COSY; D-COSY, delayed COSY; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GC, gas chromatography; GlcNAc, *N*-acetylglucosamine; Hep, heptose; HF, hydrofluoric acid; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; KDO, 2-keto-3-deoxy-manno-octulosonic acid; LOS, lipooligosaccharide; MAb, monoclonal antibody; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; OS, oligosaccharide; TFA, trifluoroacetic acid.

0006-2960/91/0430-10566$02.50/0