Electric Charge Effects on Phospholipid Headgroups. Phosphatidylcholine in Mixtures with Cationic and Anionic Amphiphiles†

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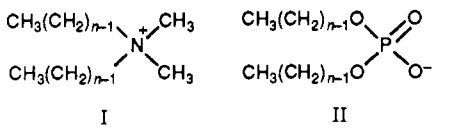
ABSTRACT: The influence of electric surface charges on the polar headgroups and the hydrocarbon region of phospholipid membranes was studied by mixing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with charged amphiphiles. A positive surface charge was generated with dialkyldimethylammonium salts and a negative surface charge with dialkyl phosphates. The POPC:amphiphile ratio and hence the surface charge density could be varied over a large range since stable liquid-crystalline bilayers were obtained even for the pure amphiphiles in water. POPC was selectively deuterated at both methylene segments of the choline moiety and at the cis double bond of the oleic acyl chain. Additional experiments were carried out with 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine labeled at the C-2 position of the glycerol backbone. Deuterium, phosphorus, and nitrogen-14 nuclear magnetic resonance (NMR) spectra were recorded for liquid-crystalline bilayers with varying concentrations of amphiphiles. Although the hydrocarbon region and the glycerol backbone were not significantly influenced by the addition of amphiphiles, very large perturbations of the phosphocholine headgroup were observed. Qualitatively, these results were similar to those observed previously with other cationic and anionic molecules and suggest that the electric surface charge is the essential driving force in changing the phospholipid headgroup orientation and conformation. While the P–N dipole is approximately parallel to the membrane surface in the pure phospholipid membrane, the addition of a positively charged amphiphile or the binding of cationic molecules moves the N^+ end of the dipole toward the water phase, changing the orientation of the phosphate segment by more than 30° at the highest amphiphile concentration. Negative charge at the membrane surface has the opposite effect, forcing the N^+ end toward the membrane interior. The NMR parameters measured for the POPC headgroup vary linearly with the amount of added amphiphile over a large concentration range. A quantitative comparison with data available on metal ions, local anesthetics, charged phospholipids, and charged peptides reveals the existence of just two types of conformational changes, one characteristic for positive surface charges and the other for negative surface charges.

Phospholipids are fundamental building blocks for biological membranes. Many structural and functional properties of membranes can be influenced, in principle, by the interaction of phospholipids with molecules present in the aqueous surroundings of the cell such as metal ions, hormones, proteins, local anesthetics, etc. [cf. Hauser and Phillips (1979)]. The role of phospholipid headgroups in these interactions is particularly intriguing since nature has provided a large variety of chemically different headgroups. In spite of intensive research, the specific functional roles of these headgroups are still, by and large, unknown.

The binding/absorption of metal ions and small inorganic ions to zwitterionic phosphatidylcholine membranes is one of the most thoroughly studied examples of lipid headgroup interactions [see Hauser and Phillips (1979) and Tatulian (1987) and references cited therein]. Using magnetic resonance techniques, in particular deuterium nuclear magnetic resonance (^{2}H NMR),[1] it could be demonstrated that the binding of these agents led to a conformational change of the phosphocholine moiety [see Hauser et al. (1976), Brown and Seelig (1977), and Seelig et al. (1987) and references cited therein]. In the present work, we provide evidence that the phosphocholine headgroup is sensitive to electric charges at the membrane surface and that probably most of the previous results can be explained by a rather simple electrostatic picture. To this purpose, we have mixed zwitterionic phosphatidylcholine membranes with positively (I) or negatively (II) charged amphiphiles and have followed the change of the headgroup conformation by ^{2}H, ^{31}P, and ^{14}N NMR. The variation of the NMR parameters upon addition of amphiphile I or II was by far the largest effects that have hitherto been observed for any liquid-crystalline phosphatidylcholine bilayer studied. From the large change in the phosphorus chemical shielding anisotropy, it was possible to determine the extent and direction of the phosphate segment rotation in the presence of electric surface charges. Two types of conformational changes, characteristic for positive and negative membrane surface charges, could be deduced from the so-called α–β plots of the choline headgroup segments.[2]



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[1] Abbreviations: NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine; $2C_{12}{}^+N2C_1Br^-$, didodecyldimethylammonium bromide; $2C_{18}{}^+N2C_1Br^-$, dioctadecyldimethylammonium bromide; $2C_{16}{}^+N2C_1Br^-$, dihexadecyldimethylammonium bromide; $C_{12}C_{16}{}^+N2C_1Br^-$, dodecylhexadecyldimethylammonium bromide; $2C_{12}PO_4{}^-Na^+$, sodium didodecyl phosphate.

[2] Part of this work has been reviewed previously (Seelig et al., 1987).

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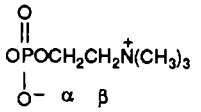
Materials and Methods

Materials. Didodecyldimethylammonium bromide ($2C_{12}{}^{+}N2C_1Br^{-}$) was purchased from Fluka (Switzerland); dioctadecyldimethylammonium bromide ($2C_{18}{}^{+}N2C_1Br^{-}$) and didodecylphosphoric acid ($2C_{12}PO_4H$) were from Sigma and Alfa Products, respectively. Nondeuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were obtained from Avanti Polar Lipids (Birmingham, AL). 1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine deuterated at the C-2 segment of the glycerol moiety was a gift of Prof. H. Hauser, Zürich. 1-Palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine ([9′,10′-$^2H_2$]POPC) was prepared according to Seelig and Waespe-Sarcevic (1978).

Synthesis of Amphiphiles and of Headgroup-Deuterated POPC. Dihexadecyldimethylammonium bromide ($2C_{16}{}^{+}N2C_1Br^{-}$) and hexadecyldodecyldimethylammonium bromide ($C_{12}C_{16}{}^{+}N2C_1Br^{-}$) were synthesized according to Okahata et al. (1979). The $2C_{16}{}^{+}N2C_1Br^{-}$ was prepared by reaction of dimethylamine and hexadecyl bromide in refluxing ethanol. $C_{12}C_{16}{}^{+}N2C_1Br^{-}$ was synthesized from *N*,*N*-dimethyldodecylamine and hexadecyl bromide. The purification was achieved by recrystallization from ethyl acetate. Headgroup-deuterated POPC was synthesized as described by Harbison and Griffin (1984). To simplify the discussion, the following headgroup nomenclature is introduced:



Preparation of Didodecyl Phosphate Sodium Salt. Sodium dialkyl phosphates ($2C_{12}PO_4{}^{-}Na^{+}$) were prepared by dissolving a weighed amount of dialkylphosphoric acid in methanol and adding an equimolar amount of 0.1 N methanolic sodium hydroxide. The solvent was evaporated, and the sodium salt was dried by high vacuum (under P_2O_5) for several hours. The sodium dialkyl phosphate was dissolved in dichloromethane/methanol (2:1), and undissolved particles were removed by filtration through a sintered glass funnel. The amphiphiles were checked by thin-layer chromatography (65:25:4 dichloromethane/methanol/water) and yielded single spots after exposure to iodine.

Sample Preparation for NMR and DSC Measurements. Mixtures of amphiphiles and POPC were prepared in NMR measuring tubes or in 10-mL test tubes (for DSC); 1–1.5 mL of a POPC stock solution (approximately 20 mg/mL) was pipetted into the sample tube, and the solvent was evaporated under nitrogen. The dry lipids (high vacuum under P_2O_5 for several hours) were weighed, and the appropriate volume of an amphiphile stock solution (according to the desired molar ratio) was added. After shaking to ensure complete solubilization of the two lipid components, the solvent was removed and the sample weighed again. The molar ratio, X_b, of amphiphile to total lipid (sum of POPC and amphiphile) was calculated from the dried lipids. The error in X_b was estimated as $\delta(X_b) \approx \pm 0.02$ for molar ratios of $X_b \leq 0.1$, but was smaller at higher molar ratios of amphiphile. Precautions were taken to avoid a phase separation in POPC membranes with intermediate concentrations of the anionic amphiphile: The well-mixed samples were brought into the magnet at elevated temperatures while still in the liquid-crystalline state.

Buffer. All measurements were carried out using the same buffer and the same ionic strength, except when indicated otherwise. The buffer was prepared with 20 mM HEPES (Sigma)/150 mM NaCl at pH 7.4 in deionized water. The lipid mixtures used for the ^2H NMR measurements were dispersed in buffer prepared with deuterium-depleted water in order to minimize the isotropic ^2H NMR signal due to natural abundance of deuterium in water.

Dispersion of the Lipid Mixtures. For all samples, a lipid:buffer ratio of 1:2 (wt %/vol %) was chosen. After addition of a few glass beads, the sample tube was sealed with a plastic plug, and the mixture was dispersed by extensive vortexing and freeze–thawing.

Variation of NaCl Concentration. The samples were prepared as described above. After the first ^2H NMR measurements, an appropriate volume of a concentrated solution of NaCl (4.5 M) was pipetted to the sample tube. Vortexing of the samples and several freeze–thaw cycles were performed in order to obtain a homogeneous distribution of NaCl in the samples.

DSC Measurements. The thermal transitions of the amphiphile/POPC/water mixtures were measured on a Perkin-Elmer DSC 7 differential scanning calorimeter. The scanning rate was 2 °C/min.

NMR Measurements. ^2H NMR measurements were recorded on a Bruker-Spectrospin CXP 300 spectrometer operating at 46.06 MHz. The quadrupole echo technique (Davis et al., 1976) was employed with full phase cycling by using 90° pulses of 2.5–4.5 μs, interpulse delays (τ) of 30–50 μs, recycle delays of 250–400 ms, a spectral width of 50 kHz, and a data size of 4K. ^{31}P NMR measurements were recorded at 121.48 MHz by employing a Hahn echo sequence with gated proton decoupling and phase cycling as described by Rance and Byrd (1983). The experimental conditions corresponded to 4.0–4.5 μs for 90° pulses, 40–50 μs for the echo spacing, 5 s for the recycle delay , 50 kHz for the spectral width, and 4K points for the data size. ^{14}N NMR measurements were made by using a broad-band probe head for solid-state measurements tuned to 21.68 MHz. The quadrupole echo technique was applied with phase cycling of the pulse pairs by using the experimental conditions as described by Tamm and Seelig (1983). The 90° pulses varied from 8 to 10 μs, and the echo spacing was 180 μs. Recycle delays, spectral width, and data size were as described for ^2H NMR measurements. Some of the ^2H and ^{14}N NMR data were recorded on a Bruker MSL 400 operating at 61.43 and 28.91 MHz, respectively, with similar experimental conditions.

The ^{31}P chemical shielding anisotropy ($\Delta\sigma$) was evaluated from the distance between the low-field and the high-field shoulder at half-height of the signal intensity. The error in $\Delta\sigma$ was estimated as ±2 ppm.

Results

Phase Behavior of Mixtures of POPC with Anionic and Cationic Amphiphiles. When dispersed in water or buffer, the phospholipid POPC as well as the synthetic dialkyldimethylammonium and dialkyl phosphate salts spontaneously form bilayer structures, as evidenced by different physical techniques (Kunieda, 1977; Kunitake & Okahata, 1977, 1978; Okahata et al., 1979; Mortara et al., 1978; Kajiyama et al., 1979; Fendler, 1980). These membranes are further characterized by well-defined gel-to-liquid-crystal phase transitions due to a melting of the hydrocarbon chains. The transition temperatures are, however, widely different and range from −5 °C for POPC (de Kruijff et al., 1973) to 50 °C for sodium didodecyl phosphate ($2C_{12}PO_4{}^{-}Na^{+}$) and to 55 °C for dioctadecyldimethylammonium bromide ($2C_{18}N2C_1{}^{+}Br^{-}$) (Okahata et al., 1981). Due to the large differences in the transition temperatures, mixtures of POPC with the synthetic amphiphiles can be expected to depart from an ideal mixing

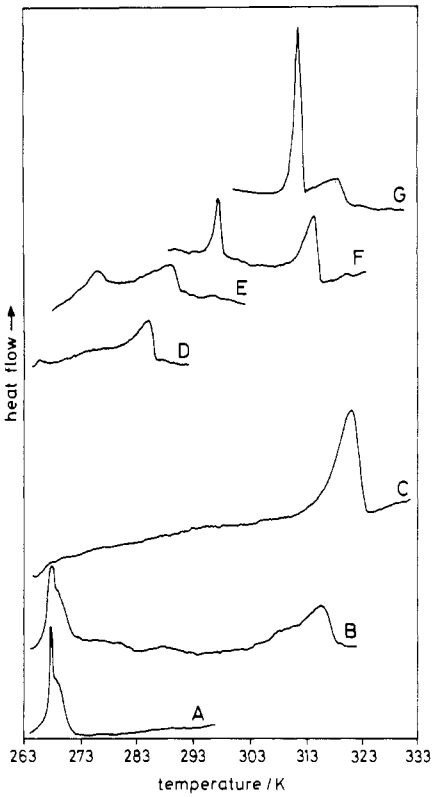


FIGURE 1: Differential scanning calorimetry of membranes composed of POPC in mixtures with various ratios of anionic sodium didodecyl phosphate (A–C) and cationic dialkyldimethylammonium bromide of varying chain lengths (D–G) dispersed in buffer. Molar mixing ratios X_b (mol of amphiphile/mol of total lipid): (A) $X_b = 0.17$, (B) $X_b = 0.45$, (C) $X_b = 0.79$, (D) $2C_{12}{}^+N2C_1Br^-$, $X_b = 0.74$, (E) $C_{12}C_{16}{}^+N2C_1Br^-$, $X_b = 0.73$, (F) $2C_{16}{}^+N2C_1Br^-$, $X_b = 0.73$, (G) $2C_{18}{}^+N2C_1Br^-$, $X_b = 0.74$. The scan rate was always 2 °C/min.

behavior. We have therefore characterized the samples used for the NMR experiments by differential scanning calorimetry (DSC). Figure 1A–C displays DSC traces of the POPC/$2C_{12}PO_4{}^-Na^+$ system at three different mixing ratios. At all three lipid compositions, a distinct endothermic transition is observed. At high percentage of POPC, the transition temperature is close to that of the pure POPC; at low percentage of POPC, it is close to that of didodecyl phosphate. At approximately equimolar mixing ratios, two separate transitions are seen (Figure 1B) with transition temperatures near those of the individual compounds.

Figure 1D–G contains DSC traces of mixtures of POPC with cationic amphiphiles. Here the percentage of added amphiphile was kept constant ($X_b \simeq 0.75$), but the chain length of the two hydrophobic chains of the quaternary ammonium salt was varied. All DSC scans exhibit two transition temperatures, indicating again a phase separation of amphiphile and POPC at low temperatures. The difference in transition temperatures between POPC and the respective amphiphile decreased with increasing chain length. At the same time, the transition temperature of the lower melting domain was gradually shifted from −5 °C (which is the transition temperature of pure POPC) to ~35 °C (Figure 1G).

NMR Studies of the Phospholipid Headgroup Region. Using the above information, we performed all NMR measurements at temperatures well above the highest phase transition temperature in order to guarantee a completely liquid-crystalline bilayer phase. Representative ^2H, ^{14}N, and ^{31}P NMR spectra of various POPC/amphiphile mixtures are summarized in Figure 2 for positively and negatively charged

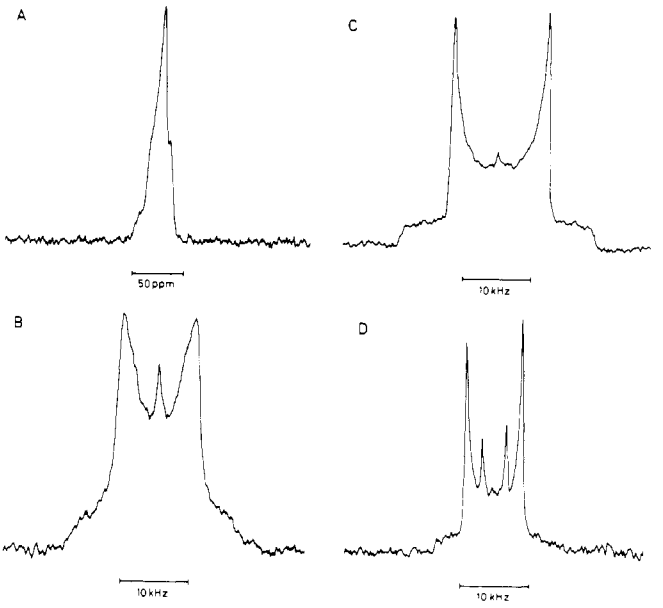


FIGURE 2: Representative ^{31}P NMR (A), ^2H NMR (B, C), and ^{14}N NMR (D) spectra of POPC in mixtures with cationic and anionic amphiphiles. (A) ^{31}P NMR; POPC/$2C_{12}PO_4{}^-Na^+$ membrane with $X_b = 0.5$ at 328 K; 256 scans. (B) ^2H NMR; $[(CH_3)_3{}^+NCH_2CD_2O]$POPC/$2C_{12}PO_4{}^-Na^+$ membrane with $X_b = 0.5$ at 328 K; 20 000 scans. (C) ^2H NMR; $[(CH_3)_3{}^+NCD_2CH_2O]$POPC/$2C_{16}{}^+N2C_1Br^-$ mixture with $X_b = 0.64$ at 318 K; 15 000 scans. (D) ^{14}N NMR; POPC/$2C_{16}{}^+N2C_1Br^-$ mixture with $X_b = 0.78$ at 333 K; 50 000 scans. Inner splitting: POPC. Outer splitting: $2C_{16}{}^+N2C_1Br^-$.

membranes. Taken together, the spectra are consistent with the organization of the lipids in liquid-crystalline bilayers. In particular, the ^2H NMR spectra of α-CD_2-POPC in mixtures with anionic (Figure 2B) or cationic (Figure 2C) amphiphiles are characterized by just a single quadrupole splitting, indicating a homogeneous environment for all POPC molecules. This result simplifies the interpretation of the ^{31}P and ^{14}N NMR spectra which both contain two signals. Since the bilayer phase itself is homogeneous, the two signals in the ^{31}P and ^{14}N NMR spectra must be assigned to different spectroscopic properties of the individual molecules. For the POPC–$2C_{12}PO_4{}^-Na^+$ bilayer, the ^{31}P NMR spectrum consists of two axially symmetric powder patterns with different chemical shielding anisotropies [cf. Seelig (1978)]. Assignment of the two superimposed spectra was based on a concentration series and on a comparison with bilayers composed of pure POPC or pure amphiphile alone. The ^{31}P NMR spectrum of Figure 2A can be interpreted as follows: the chemical shielding anisotropy of pure $2C_{12}PO_4{}^-Na^+$ membranes is about −19 ppm (Rupert et al., 1987) and decreases only slightly to −21 ppm in mixtures with 50 mol % POPC. In contrast, a much larger change is noted for the phosphate segment of the POPC molecules. Here, the chemical shielding tensor increases from −48 ppm for pure POPC [cf. Tamm and Seelig (1983)] to −37 ppm in the 1:1 mixture of POPC with $2C_{12}PO_4{}^-Na^+$.

The ^{14}N NMR spectra of mixtures of cationic quaternary ammonium salts with POPC also exhibit two signals. In Figure 2D, the assignment of the two signals can be made on the basis of the known lipid composition. The intense signal with a large splitting of 8.8 kHz originates from dihexadecyldimethylammonium bromide ($X_b = 0.78$) while the smaller splitting of 3.5 kHz is due to POPC.

Figure 3 displays the variation of the ^{31}P chemical shielding anisotropy of POPC, $\Delta\sigma$, upon addition of cationic amphiphile ($2C_{12}{}^+N2C_1Br^-$, right part of figure) or anionic amphiphile ($2C_{12}PO_4{}^-Na^+$, left part of figure). The latter compound

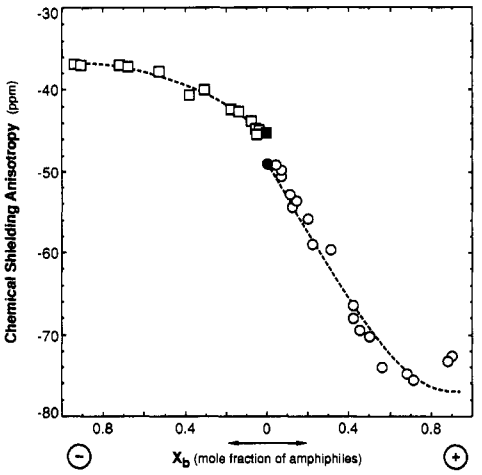


FIGURE 3: Variation of the ^{31}P chemical shielding anisotropy, $\Delta\sigma$, of the phosphocholine headgroup with the molar ratio of positively charged (○) $2C_{12}{}^{+}N2C_1Br^{-}$ (at 293 K) or negatively charged (□) $2C_{12}PO_4{}^{-}Na^{+}$ (at 328 K).

induces an approximately linear *increase* in $\Delta\sigma$ up to a molar ratio of $X_b \approx 0.3$ which can be approximated by

$$\Delta\sigma = -45.6 + 18.7X_b \text{ ppm} \quad (1)$$

($0 \leq X_b \leq 0.3$; correlation coefficient $R^2 \approx 0.97$). At very high ratios of amphiphile, the figure indicates a plateau value of $\Delta\sigma$ around −36 ppm. In contrast, the addition of positively charged amphiphile leads to a dramatic *decrease* of $\Delta\sigma$. Here, $\Delta\sigma$ varies linearly up to a molar ratio of $X_b \approx 0.6$ according to

$$\Delta\sigma = -47.6 - 46.2X_b \text{ ppm} \quad (2)$$

($0 \leq X_b \leq 0.6$, $R^2 = 0.985$). Hence, the positively charged amphiphile is almost 3 times as effective in changing the residual ^{31}P chemical shielding anisotropy as its negatively charged counterpart. At very high amphiphile concentrations, $\Delta\sigma$ appears to level off at about −75 ppm. Similar values were obtained with cationic amphiphiles containing even longer alkyl chains (Scherer, 1988). Note that the two membrane systems shown in Figure 3 were measured at different temperatures.

^2H NMR spectroscopy was used to visualize the effects of charged amphiphiles on the α- and β-segments of the POPC headgroup. Figure 4 summarizes the data of the various membrane mixtures investigated. In order to allow a simple comparison, the quadrupole splittings, $\Delta\nu_\alpha$ and $\Delta\nu_\beta$, of the different membranes were plotted according to

$$\delta_i = \Delta\nu_i(X_b) - \Delta\nu_i^\circ \text{ kHz} \quad (3)$$

where i stands for α or β, $\Delta\nu_i^\circ$ is the quadrupole splitting of the pure POPC membrane at a given temperature, and $\Delta\nu_i(X_b)$ is the quadrupole splitting of the mixture at the same temperature. Depending on the membrane transition temperature, the actual measuring temperatures varied between 20 and 55 °C (cf. legend to Figure 4). The abscissa of Figure 4 contains the mole fraction of cationic (anionic) amphiphile on the right (left) side. The quadrupole splittings of pure α-CD_2-POPC and pure β-CD_2-POPC membranes are both assumed to be positive since the sign of the quadrupole splitting is not accessible in conventional ^2H NMR. Figure 4 reveals a number of interesting features: (1) The α-splitting (closed symbols) *increases* with addition of anionic amphiphile and *decreases* with addition of cationic amphiphile. (2) The β-splitting (open symbols) shows the *opposite* behavior; i.e., it *decreases* with anionic amphiphile and *increases* with cationic amphiphile. (3) The variation of the quadrupole splittings is quite large.

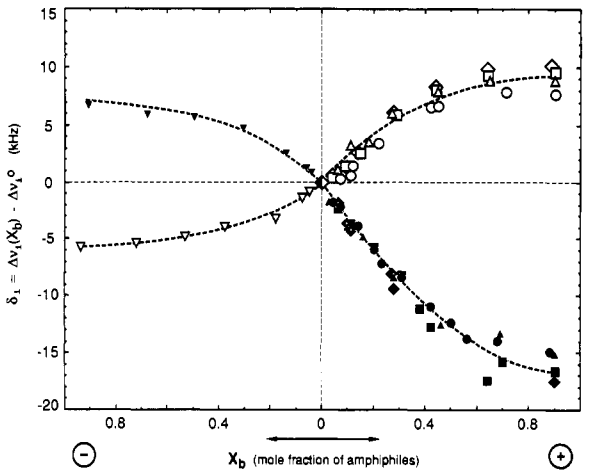


FIGURE 4: Variation of the deuterium quadrupole splittings of α-CD_2-POPC (closed symbols) and β-CD_2-POPC (open symbols) upon addition of cationic (right part of figure) and anionic (left part of figure) amphiphiles. δ_i represents the difference between the quadrupole splittings of the mixture and that of the pure POPC membrane at the same temperature. (●, ○) $2C_{12}{}^{+}N2C_1Br^{-}$ at 293 K; (▲, △) $C_{12}C_{16}{}^{+}N2C_1Br^{-}$ at 313 K; (■, □) $2C_{16}{}^{+}N2C_1Br^{-}$ at 313 K; (◆, ◇) $2C_{18}{}^{+}N2C_1Br^{-}$ at 323 K; (▼, ▽) $2C_{12}PO_4{}^{-}Na^{+}$ at 328 K. In the temperature interval investigated, the quadrupole splitting of α-CD_2-POPC (β-CD_2-POPC) changed from 6.4 kHz (5.9 kHz) at 293 K to 5.8 kHz (4.0 kHz) at 328 K.

Table I: Correlation of the Quadrupole Splitting ($\Delta\nu_i$) and the Chemical Shielding Anisotropy ($\Delta\sigma$) of POPC Membranes with the Mole Fraction of Added Cationic or Anionic Amphiphile[a]

amphiphile added	temp (K)	m_p (ppm)	m_α (kHz)	m_β (kHz)
positive electric charge				
$2C_{12}{}^{+}N2C_1Br^{-}$	293	−46.2	−27.4	+15.4
$C_{12}C_{16}{}^{+}N2C_1Br^{-}$	313		−29.9	+20.5
$2C_{16}{}^{+}N2C_1Br^{-}$	313		−30.9	+20.9
$2C_{18}{}^{+}N2C_1Br^{-}$	323		−31.4	+21.3
negative electric charge				
$2C_{12}PO_4{}^{-}Na^{+}$	328	+18.7	+15.1	−13.9

[a] The slopes m were evaluated according to $\Delta\nu_i = m_iX_b + \Delta\nu_i^\circ$ for the quadrupole splittings and $\Delta\sigma = m_pX_b + \Delta\sigma^\circ$ for the phosphorus chemical shielding anisotropy.

For the α-segment, the change from the anionic to the cationic membrane spans a range of about 24 kHz; for the β-segment, this range is 15 kHz. (4) Finally, the quadrupole splittings vary linearly with the mole fraction of the added amphiphile, X_b, up to $X_b \simeq 0.3$–0.6, depending on the type of amphiphile. In this concentration range, the data can be summarized according to

$$\Delta\nu_i(X_b) = \Delta\nu_i^\circ + mX_b \text{ kHz} \quad (4)$$

Table I lists the slopes m as derived from a linear regression analysis of the experimental data shown in Figure 4 together with those obtained by ^{31}P NMR. Inspection of Table I confirms the opposite effects of cationic and anionic amphiphiles on the choline headgroup and also the counterdirectional variation of the α- and β-segment quadrupole splitting. The latter result can only be explained by a *conformational change* of the choline headgroup. This follows from the fact that any change in the average ordering of the phospholipid headgroup, i.e., any increase or decrease of the angular fluctuations of the headgroup, as a whole would change the NMR parameters of the two segments in the *same* direction. The molecular nature of this conformational change will be discussed below.

The variations of the ^{14}N quadrupole splittings with membrane composition are shown in Figure 5. In membranes containing the anionic didodecyl phosphate (left part of upper panel), the ^{14}N quadrupole splitting of the choline moiety of

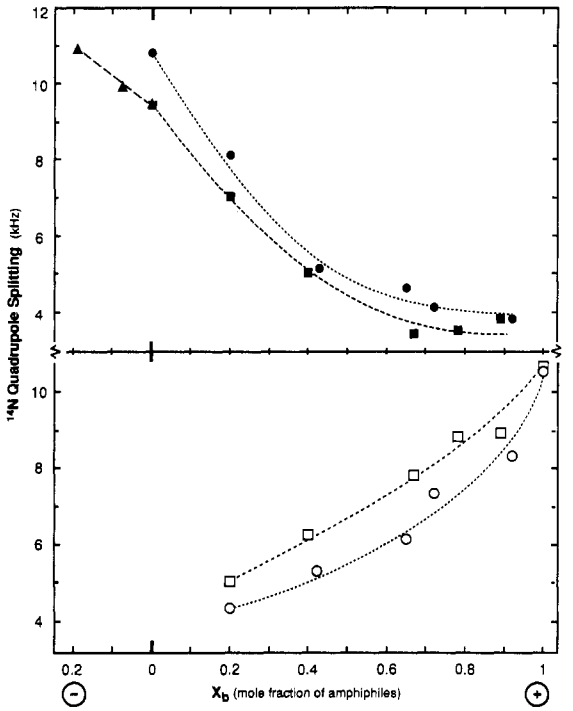


FIGURE 5: Membranes composed of POPC and charged amphiphiles. Variation of the ^{14}N quadrupole splitting with the molar ratio of charged amphiphile. Closed symbols refer to the nitrogen-14 quadrupole splittings of the phosphocholine headgroup; open symbols refer to those of the dialkyldimethylammonium salt. (●, ○) $2C_{12}{}^{+}N2C_1Br^{-}$ at 293 K; (■, □) $2C_{16}{}^{+}N2C_1Br^{-}$ at 333 K; (▲) $2C_{12}PO_4{}^{-}Na^{+}$ at 333 K.

POPC is the only signal contributing to the ^{14}N NMR spectrum. This quadrupole splitting *increases* with increasing anionic amphiphile. However, at concentrations of anionic amphiphile above $X_b \sim 0.2$, the ^{14}N quadrupole splitting of POPC completely disappears even though the ^{31}P NMR spectra still indicate a liquid-crystalline bilayer phase. This effect is probably due to a distortion of the tetrahedral symmetry around the choline moiety which increases the quadrupole splitting beyond the limit of detection (cf. below). On the other hand, two ^{14}N quadrupole splittings are detected in mixtures of POPC and cationic amphiphiles. Here, the choline ^{14}N quadrupole splitting *decreases* with increasing amount of positively charged amphiphile.

The ^{14}N quadrupole splitting of the positively charged amphiphile itself shows a different behavior (Figure 5, lower panel). At low mole fractions of amphiphile in POPC, this ^{14}N quadrupole splitting is small but increases with increasing amphiphile in the mixture. For the pure amphiphile membrane, the ^{14}N quadrupole splitting has practically the same value as for the pure POPC membrane (Scherer, 1988).

Two amphiphiles with different chain lengths (C_{12} and C_{16}) were investigated. As shown in Figure 5, the qualitative results are the same, but small quantitative differences of about 1 kHz are observed which can be explained, in part, by the different measuring temperatures.

As mentioned above, the ^{14}N quadrupole splitting is much more sensitive to the bonding pattern and bonding symmetry than the phosphate chemical shielding tensor or the deuterium quadrupole coupling constant. For a perfect tetrahedral symmetry such as found in $NH_4{}^{+}$ or $^{+}N(CH_3)_4$, the static ^{14}N quadrupole splitting is zero while in a highly asymmetric environment it may reach values of up to 4 MHz (Lehn & Kintzinger, 1973). Small distortions from a perfect tetrahedral bonding pattern are therefore sufficient to cause significant changes in the ^{14}N quadrupole coupling constant. Hence, the

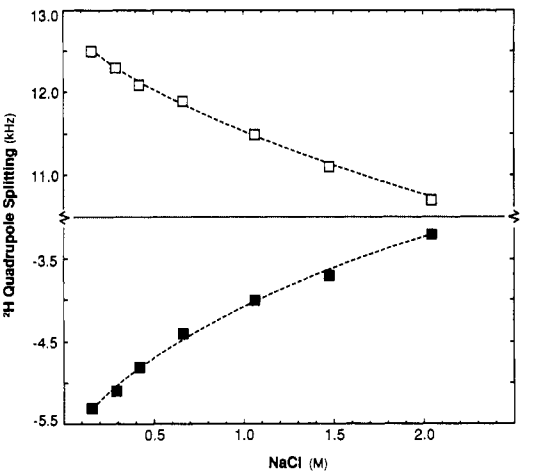


FIGURE 6: Screening of membrane surface charge with NaCl. α-CD_2-POPC (■) and β-CD_2-POPC (□) were mixed with $2C_{16}{}^{+}N2C_1Br^{-}$ at a molar ratio $X_b = 0.42$. Measuring temperature 313 K.

variation of the nitrogen quadrupole splittings could arise not only from a change in the headgroup conformation but also from distortions of the tetrahedral bonding pattern. For the choline trimethylammonium group, the C_β–N bond is an axis of rotational symmetry, and the static ^{14}N quadrupole coupling constant must be rotationally symmetric around the C_β–N bond. No such symmetry axis exists for the dialkyldimethylammonium salts, and the quadrupole splittings of the two compounds may thus have different signs. The value of ^{14}N NMR for a conformational analysis is thus rather limited.

The opposite effects of cationic and anionic amphiphiles on the phosphocholine headgroup NMR parameters suggested that the main driving force for changing the lipid conformation was the electric charge at the membrane surface. We have therefore investigated the influence of NaCl on $\Delta\nu_Q$ since increasing NaCl concentrations should lead to a partial screening of the electric surface charges. POPC was mixed with cationic $2C_{16}{}^{+}N2C_1Br^{-}$ at a molar ratio of $X_b = 0.42$, and the NaCl concentration was varied in the range of $0 \leq C_{NaCl} \leq 2$ M. The variation of the quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ with the concentration of NaCl is shown in Figure 6. With increasing NaCl concentration, $\Delta\nu_\alpha$ increases whereas $\Delta\nu_\beta$ decreases. This behavior is qualitatively consistent with a partial neutralization of the positive membrane surface charge which can be explained by an accumulation of Cl^- ions near the membrane surface. Addition of NaCl to pure phospholipid membranes has only a very small effect (Akutsu & Seelig, 1981).

NMR Studies of the Glycerol Backbone and the Hydrocarbon Chain Region. Since cationic and anionic amphiphiles exert large effects on the phospholipid headgroups, the question arises if these conformational changes are propagated into the glycerol backbone region or even into the hydrophobic interior of the lipid bilayer. We have investigated this question with 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine deuterated at the C-2 position of the glycerol backbone (GC-2-DPPC) and with POPC deuterated at the cis double bond of the oleic acyl chain ([9′,10′-$^{2}H_2$]POPC). Both lipids were mixed with cationic and anionic amphiphiles as well as with electrically neutral 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE). POPE has no effect on the phosphocholine headgroup (Scherer & Seelig, 1987) and served as a control only.

Membranes composed of pure GC-2-DPPC, at temperatures above the phase transition of 41 °C, give rise to just one quadrupole splitting of 26.1 kHz. This large splitting is an

Table II: Deuterium Quadrupole Splittings of POPC Deuterated at the cis Double Bond and DPPC Deuterated at C-2 of the Glycerol Backbone in Mixtures with Neutral Phospholipid and Cationic and Anionic Amphiphiles

membrane composition	mol fraction of amphiphile	^{2}H quadrupole splittings (kHz) at C-9′	C-2	C-10′	temp (K)
[9′,10′-$^2H_2$]POPC					
pure POPC[a]	0	13.4		2.5	300
pure POPC[a]	0	12.0		2.3	319
POPC/POPE	0.58	15.3		4.0	313
POPC/$2C_{16}{}^+N2C_1Br^-$	0.61	14.3		2.4	318
POPC/$2C_{12}PO_4{}^-Na^+$	0.63	13.5		1.3	328
GC-2-DPPC					
pure DPPC	0		26.1[b]		318
POPC/POPE	0.64		25.8		318
POPC/POPE	0.64		24.7		328
POPC/$2C_{16}{}^+N2C_1Br^-$	0.65		31.5		318
POPC/$2C_{12}PO_4{}^-Na^+$	0.66		24.5		328

[a] Data from Seelig and Waespe-Sarcevic (1978). [b] E. Leung, H. Hauser, and J. Seelig (unpublished results).

indicator of the limited flexibility and the high ordering of the glycerol backbone as has been discussed previously for naturally occurring lipids (Gally et al., 1981). Upon addition of $2C_{12}PO_4{}^-Na^+$ ($2C_{16}{}^+N2C_1Br^-$) at a mole fraction of X_b = 0.65, this quadrupole splitting decreased (increased) to 24.5 kHz (31.5 kHz). Addition of POPE had no effect, however (cf. Table II). The relative change of $\Delta\nu_Q$ at the glycerol C-2 segment was thus only 20% and was distinctly smaller than that of the phosphocholine group. Nevertheless, the glycerol backbone was sensitive to the presence of amphiphiles and reacted in opposite directions for positive and negative surface charge.

POPC deuterated at the cis double bond gives rise to two widely different quadrupole splittings of about 13 and 2 kHz at ambient temperature which can be assigned to the C-9′ and the C-10′ deuteron, respectively (Seelig & Waespe-Sarcevic, 1978). Table II summarizes the results for pure POPC bilayers at two different temperatures and for POPC in mixtures with more than 50 mol % POPE, $2C_{16}{}^+N2C_1Br^-$, or $2C_{12}PO_4{}^-Na^+$. Under all conditions, we observed only small changes in the quadrupole splittings of the double bond, i.e., changes on the order of less than 2 kHz. Neutral and charged lipids gave rise to similar results. It can be concluded, therefore, that the addition of cationic and anionic amphiphiles to POPC bilayers has very little influence on the ordering of the hydrocarbon chains, at least as measured by the deuterated cis double bond.

Discussion

The phosphocholine headgroup has a large dipole moment of about 19 D (Shepherd & Büldt, 1978), and structural, dielectric, and spectroscopic measurements have shown that the $^-P–N^+$ dipole lies almost parallel to the plane of the membrane [for a review, see Seelig et al. (1987)]. NMR methods have further demonstrated that the orientation and conformation of the choline headgroup are not constant but can be influenced by the binding or adsorption of metal ions (Hauser et al., 1976; Brown & Seelig, 1977; Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Siminovitch et al., 1984; Macdonald & Seelig, 1987a,b), hydrophobic ions (Altenbach & Seelig, 1985), charged local anesthetics (Boulanger et al., 1981; Browning & Akutsu, 1982; Seelig et al., 1988), chaotropic anions (Macdonald & Seelig, 1988), and charged peptides (Sixl & Watts, 1985; Dempsey & Watts, 1987; Roux et al., 1989; Kuchinka & Seelig, 1989) as well as by mixing phosphatidylcholine with negatively charged lipids (Sixl & Watts, 1983; Scherer & Seelig, 1987).

The present data, then, provide evidence that the main driving force for the conformational change of the phospholipid headgroup is an electrostatic attraction/repulsion mechanism, irrespective of the chemical nature of the compounds involved. The phosphocholine headgroup apparently behaves like a "molecular electrometer", responding quite accurately to the electric charge at the membrane surface. Moreover, the data also shed light on some molecular details of the $^-P–N^+$ dipole reorientation.

Advantages of Amphiphiles in Studying Electric Charge Effects. Compared to metal ions or small organic ions, the amphiphiles used in this study have a number of advantages. (1) The amphiphiles themselves are bilayer-forming compounds. They can be mixed with POPC at any ratio, and the membrane composition is determined simply by the mixing ratio. Hence, the electric charge density at the membrane surface can be varied over a very wide range, i.e., from an electrically neutral POPC bilayer to a pure anionic or cationic mesophase. In contrast, the binding of inorganic and organic ions to zwitterionic lipids is rather weak, and only a small amount of electric charge can be brought onto the membrane surface. It should also be noted that the dialkyldimethylammonium salts are probably the simplest molecules which conveniently form positively charged bilayers. Charged phospholipids which can be isolated from biological materials almost exclusively carry a negative electric charge. (2) The packing density of phosphatidylcholine molecules in a fluidlike lipid bilayer is determined by the cross-sectional area of the two hydrocarbon chains and not by steric constraints at the headgroup level (Büldt et al., 1978, 1979). The area requirements of negatively charged dialkyl phosphates and positively charged dialkyldimethylammonium salts are not much smaller than those of POPC since they also carry two long hydrocarbon chains. A specific complex formation between the different lipids in the mixed membrane is thus unlikely since the distance between the headgroups is too large. (3) Very large changes in the NMR headgroup parameters were observed upon addition of charged amphiphiles, much larger than detected with any of the agents studied previously. This result, which can be explained by the large surface charge produced by the addition of charged amphiphiles, distinctly simplifies the quantitative interpretation, at least for phosphorus 31 NMR.

Influence of Electric Charge on the Orientation of the Phosphate Segment and Phosphocholine Dipole. Let us first discuss the orientation of the phosphate segment as revealed by the phosphorus chemical shielding anisotropy, $\Delta\sigma$.

The quantitative analysis of ^{31}P NMR spectra of phospholipid membranes is hampered by the fact that the residual chemical shielding anisotropy is determined by two independent order parameters (Niederberger & Seelig, 1976; Seelig, 1978). Knowledge of $\Delta\sigma$ alone is usually not sufficient to derive the average orientation of the phosphate group. However, in the special case where $\Delta\sigma$ assumes rather large negative values, as observed experimentally for POPC in mixtures with dialkyldimethylammonium salts, a unique and unambiguous molecular interpretation of $\Delta\sigma$ can be given. In the following, we shall demonstrate that for chemical shielding anisotropies of $\Delta\sigma < -75$ ppm the σ_{11} axis of the phosphate tensor must be almost exactly parallel to the bilayer normal.

The analysis starts with the *static* chemical shielding tensor of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, the principal components of which are $\sigma_{11} = -81$ ppm, $\sigma_{22} = -21$ ppm, and $\sigma_{33} = +108$ ppm (Herzfeld et al., 1978; Griffin, 1981) and

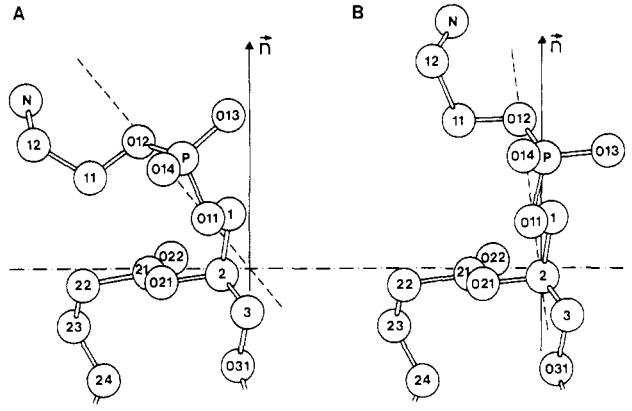


FIGURE 7: Approximate orientation of the phosphocholine headgroup in the absence (A) and in the presence (B) of positive electric charge. (A) corresponds to the crystal structure of phosphatidylethanolamine (Hitchcock et al., 1974). (B) Only the orientation of the phosphate segment can be determined from ^{31}P NMR. The choline part was not changed compared to (A).

are assumed to also hold true for POPC. In the liquid-crystalline bilayer, the phosphate group is executing an axially symmetric rotation around the bilayer normal which averages the static chemical shielding anisotropies [cf. Seelig (1978)]. Considering an extreme situation where the σ_{11} principal axis (x axis) is the rotation axis and is thus exactly parallel to the bilayer normal n, we calculate an averaged chemical shielding anisotropy of $\Delta\sigma = -124$ ppm (Herzfeld et al., 1978). For the σ_{22} (σ_{33}) principal axis parallel to n, the same calculation yields $\Delta\sigma = -34$ ppm (+150 ppm).

Next we take into account the wobbling motion of the phosphate group. The molecules constituting a fluidlike lipid bilayers are not aligned perfectly parallel to the bilayer normal n but execute angular oscillations perpendicular to this axis (in addition to their rotational motion around the long molecular axis). These angular fluctuations reduce the averaged magnetic anisotropies calculated above even further. The phosphate segment is linked to the rest of the lipid molecule via the C(3)–C(2) axis of the glycerol backbone for which an order parameter of $S = 0.66$ has been evaluated (Gally et al., 1975). This order parameter does not change very much upon addition of charged amphiphiles since the C-2 quadrupole splitting varies by not more than 20% upon addition of amphiphiles (Table II). Adopting a wobbling parameter of $S = 0.66$ also for the phosphate group in the presence of amphiphiles, the chemical shielding anisotropies are reduced to −82 ppm [x-axis rotation; cf. Herzfeld et al. (1978) and Griffin et al. (1978)], −23 ppm (y-axis rotation), and +99 ppm (z-axis rotation), respectively. No smaller chemical shielding anisotropy than $\Delta\sigma = -82$ ppm is thus possible under these conditions. The experimental result of $\Delta\sigma = -75$ ppm for POPC in mixtures with a high percentage of cationic amphiphile is very close to this extreme value, and it thus follows that the x axis of the phosphate tensor must be oriented almost exactly parallel to the bilayer normal n. As the membrane content of dialkyldimethylammonium is reduced, the chemical shielding anisotropy is reduced to smaller absolute values ($\Delta\sigma = -48$ ppm for pure POPC), and the x axis of the phosphate chemical shielding tensor moves away from the bilayer normal n. A change in the angular orientation by at least 30° is required[3] to produce a $\Delta\sigma = -48$ ppm.

How can we visualize these data in terms of a molecular model? Figure 7A reproduces a part of the crystal structure of 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (Hitchcock et al., 1974), and the $^{-}P–N^{+}$ dipole is seen to be approximately parallel to the plane of the crystalline bilayer. The orientation of the phosphate chemical shielding tensor with respect to this molecular frame can be inferred from two different model compounds (Herzfeld et al., 1978; Hauser et al., 1988). These data demonstrate that the σ_{11} axis of the σ-tensor coincides with the vector connecting the two esterified oxygens (O_{11}–O_{12} direction) which is indicated by the dashed line in Figure 7A. Upon addition of a high percentage of dialkyldimethylammonium bromide, this axis must be aligned almost parallel to the bilayer normal, and this situation is depicted schematically in Figure 7B. In the latter figure, all other torsion angles have been kept constant with the final result that the phosphocholine dipole moves out of the bilayer plane. On the other hand, addition of negative surface charge decreases the size of the chemical shielding anisotropy, and we conclude that the σ_{11} axis and hence the phosphocholine dipole will perform the opposite rotation; i.e., the addition of negative charge will pull the $^{-}P–N^{+}$ dipole backward toward the bilayer interior.[4]

From an electrostatic point of view, these results are easy to understand. Both the cationic and anionic amphiphiles are characterized by very small headgroups, and, in all likelihood, the plane of these headgroups is below that of the phosphocholine moiety since the hydrocarbon chains of the amphiphiles align with those of POPC. Hence, the positive end of the phosphocholine dipole will be pushed out into the aqueous medium by a sublayer of positive electric charge but will be pulled down toward the hydrocarbon region by a sublayer of negative charge. A more quantitative analysis based on an electrostatic model has recently been attempted (Roux et al., 1989).

General Features of the Phosphocholine Headgroup Conformation under the Influence of Electric Fields. Dialkyl phosphates and dialkyldimethylammonium salts have opposite effects on the orientation of the phosphocholine headgroup (and also on that of the phosphoethanolamine headgroup; E. Leung and J. Seelig, unpublished results). Since a complex formation appears to be unlikely, we attribute this result to the difference in charge of the two amphiphiles. In fact, this simple electrostatic model also explains the results of a variety of other binding studies which have been reviewed recently (Seelig et al., 1987) and to which additional data have been added since then (cf. references above). In brief, all positively charged compounds which interact with the POPC membrane surface such as metal ions, hydrophobic ions, and positively charged peptides parallel the effect of positively charged dialkyldimethylammonium salts. In contrast, negatively charged ions such as I^{-}, SCN^{-}, or tetraphenyl borate [Macdonald & Seelig, 1988; Malthaner and Seelig, unpublished results; cf. also Figure 4 of Seelig et al. (1987)] as well as negatively charged lipids induce the same effects as negatively charged dialkyl phosphates. Some of these agents, such as the metal ions or the small inorganic ions, are adsorbed to the membrane surface; others, like hydrophobic ions and local anesthetics, will penetrate deeper into the glycerol backbone region; the lipids and amphiphiles, finally, are an integral part of the lipid bilayer. Nevertheless, independent of the chemical nature of the compounds involved or their actual location in the lipid bilayer, they all exert qualitatively the same effect on the

[3] ^{31}P NMR spectra of oriented DPPC multilayers in the gel state have shown that the angle between the bilayer normal and the σ_{11} axis is about 50° (Griffin et al., 1978).

[4] These conclusions are further supported by a detailed analysis of the deuterium quadrupole splittings (J. P. Caniparoli and J. Seelig, unpublished results).

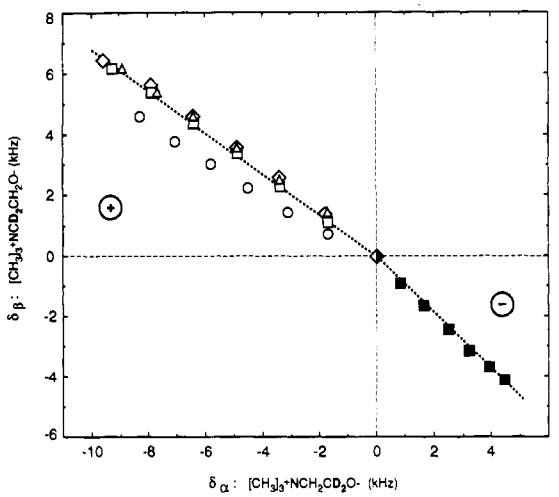


FIGURE 8: α–β plot for POPC mixed with cationic and anionic amphiphiles (data are interpolated from Figure 4). The quadrupole splitting of the β-choline segment is plotted versus that of the corresponding α-segment measured under identical conditions. Closed symbols: negative surface charge. Open symbols: positive surface charge. (○) $2C_{12}{}^{+}N2C_1Br^{-}$; (□) $2C_{16}{}^{+}N2C_1Br^{-}$; (◇) $2C_{18}{}^{+}N2C_1Br^{-}$; (△) $C_{12}C_{16}{}^{+}N2C_1Br^{-}$; (■) $2C_{12}PO_4{}^{-}Na^{+}$.

phosphocholine headgroup as long as their electric charge has the same sign. On the other hand, neutral molecules which carry neither a net electric charge nor a large electric dipole have no or comparatively small effects on the phosphocholine headgroup (Scherer & Seelig, 1987; Bechinger et al., 1988).

The influence of the electric charge is essentially limited to the headgroup region. When the deuterium label is moved to the glycerol backbone of the phospholipids or into the hydrocarbon chains, the influence of the electric charge is very small (cf. Table II).

At first sight, a *quantitative* comparison of different molecules appears to be difficult. The actual change in the NMR parameters depends not only on the sign of the electric charge but also on the valency of the molecules involved, on their extent of binding, and on the distance of closest approach to the positive or negative end of the $^{-}P–N^{+}$ dipole. Nevertheless, the large amount of the available data can be conveniently reduced and compared by using a so-called α–β plot (Akutsu & Seelig, 1981; Browning & Akutsu, 1982; Altenbach & Seelig, 1984; Seelig et al., 1988; Roux et al., 1989). In this representation, the quadrupole splitting of the β-segment, $\Delta\nu_\beta$, is plotted versus the corresponding splitting of the α-segment, $\Delta\nu_\alpha$, measured under the same conditions. In essence, such a plot reveals the differential effects of the adsorbed or bound molecules on the α- and β-segments. For example, if both quadrupole splittings are affected to equal extent over the whole concentration range, the α–β plot will yield a straight line with slope $m = 1$.

In Figure 8, such a plot is shown for the experimental data of Figure 4. A strictly linear relationship between the two splittings is observed over the whole concentration range, extending even into the region where the quadrupole splittings are not longer linearly dependent on the mole fraction of added amphiphile. The slopes in these α–β plots are always negative, reflecting counterdirectional change of the α- and the β-segment with membrane surface charge. However, the *steepness* of the slope is characteristic of the *sign* of the electric charge. For the dialkyl phosphate–POPC membrane, the slope of the α–β plot is −0.92. Very similar values have been observed for negatively charged phospholipids mixed with POPC, with slopes ranging between −0.87 for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid to −1.13 for phosphatidylinositol (Scherer & Seelig, 1987). Likewise, the binding of SCN^{-} to POPC is characterized by a slope −1.1 in the α–β plot (Macdonald & Seelig, 1988). On the other hand, the positively charged dialkyldimethylammonium salts yield slopes in the range of −0.56 to −0.69. This result is consistent with the binding of di- and trivalent metal ions, positively charged local anesthetics, or melittin (a peptide with five positive charges), all yielding slopes in the range of −0.43 to −0.53 (Akutsu & Seelig, 1981; Browning & Akutsu, 1982; Altenbach & Seelig, 1984; Seelig et al., 1988; Roux et al., 1989; Kuchinka & Seelig, 1989). The existence of different slopes therefore suggests that the phosphocholine headgroup undergoes only two types of conformational change, one characteristic for positive charge and the other characteristic for negative charge, regardless of the quantitative efficiency of a given compound.

The quantitative nature of this conformational change is at present under investigation. Qualitatively, the data are consistent with a optimum charge neutralization by a close lateral approach of opposite charges. Molecules with positive charge will position their charge in the vicinity of the negative end of the $^{-}P–N^{+}$ dipole; hence, the neighboring α-segment is affected more than the distant β-segment. Conversely, negatively charged molecules are preferentially located near the positive end of the $^{-}P–N^{+}$ dipole, increasing the conformational change at the β-segment.

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Characterization of the *Escherichia coli* Transcription Factor σ^{70}: Localization of a Region Involved in the Interaction with Core RNA Polymerase†

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ABSTRACT: A set of internal deletions and frame-shift mutations was made in the structural gene for the major σ factor of *Escherichia coli* RNA polymerase (σ^{70}). The truncated proteins from these various mutants were examined to determine if they retained the ability to bind core RNA polymerase. Two assays were used to determine core-binding activity. Gel filtration was used to separate free σ^{70} from σ^{70} bound to core polymerase. Immunoprecipitation of polymerase using an anti-α-subunit monoclonal antibody was also used to determine if the various truncated proteins were bound to core. Results from these experiments indicate core-binding activity is retained when large portions of the σ^{70} protein are deleted. Deletion of a region in the central portion of the protein caused a large decrease in core-binding activity. The results suggest that the region spanning amino acids 361-390 is important for efficient core-binding activity. Sequence comparison of various σ factors shows highly conserved amino acids in this region. A synthetic peptide having the sequence of amino acids 361-390 was synthesized and examined for the ability to bind core RNA polymerase.

Specific initiation of transcription in *Escherichia coli* is dependent upon the σ subunit of RNA polymerase. The σ subunit binds to the core RNA polymerse ($\alpha_2\beta\beta'\omega$) to form the holoenzyme ($\alpha_2\beta\beta'\omega\sigma$). The holoenzyme recognizes and binds to a promoter permitting transcription initiation from a specific site (Burgess et al., 1969; Reznikoff et al., 1985). The core polymerase is unable to recognize promoter sequences, so it is the addition of the σ factor which specifies the location of transcription initiation. There are multiple σ factors in bacteria which recognize different sequences as

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