Hexham, J. M., Totty, N. F., Waterfield, M. D., & Crompton, M. J. (1986) *Biochem. Biophys. Res. Commun. 134*, 248–254.
Hogaboom, G. K., Cook, M., Newton, J. F., Varrichio, A., Shorr, R. G. L., Sarau, H. M., & Crooke, S. T. (1986) *Mol. Pharmacol. 30*, 510–519.
Jakschik, B. A., & Lee, L. H. (1980) *Nature (London) 287*, 51–52.
Jakschik, B. A., Falkenhein, S., & Parker, C. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4577–4581.
Jakschik, B. A., Lee, L. H., Shuffer, G., & Parker, C. W. (1978) *Prostaglandins 16*, 733–748.
Jakschik, B. A., Sun, F. F., & Steinhoff, M. M. (1980) *Biochem. Biophys. Res. Commun. 95*, 103–110.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Maas, R. L., Ingram, C. D., Taker, D. F., Oates, J. A., & Brash, A. R. (1982) *J. Biol. Chem. 257*, 13515–13519.
Matsumoto, T., Funk, C. D., Radmark, O., Hoog, J.-O., Jornvall, H., & Samuelsson, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 26–30.
Maycock, A. L., Anderson, M. S., Desousa, D. M., & Kuehl, F. A., Jr. (1982) *J. Biol. Chem. 257*, 13911–13914.
Ochi, K., Yoshimoto, T., & Yamamoto, S. (1983) *J. Biol. Chem. 258*, 5754–5758.
Orning, L., Hammarström, S., & Samuelsson, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2014–2017.
Panossian, A., Hamberg, M., & Samuelsson, B. (1982) *FEBS Lett. 150*, 511–513.
Parker, C. W., Falkenhein, S. F., & Huber, M. M. (1980) *Prostaglandins 20*, 863–886.
Radmark, O., Malmsten, C., & Samuelsson, B. (1980a) *Biochem. Biophys. Res. Commun. 92*, 954–961.
Radmark, O., Malmsten, C., Samuelsson, B., Goto, G., Marfat, A., & Corey, E. J. (1980b) *J. Biol. Chem. 255*, 11828–11831.
Rouzer, C. A., & Samuelsson, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7393–7397.
Samuelsson, B. (1983) *Science (Washington, D.C.) 220*, 568–575.
Sudhof, T. C., Walker, J. H., & Fritsche, U. (1985) *J. Neurochem. 44*, 1302–1307.
Tripp, C. S., Mohoney, M., & Needleman, P. (1985) *J. Biol. Chem. 260*, 5895–5898.

Anion Binding to Neutral and Positively Charged Lipid Membranes†

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ABSTRACT: Aqueous anion binding to bilayer membranes consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated by using deuterium and phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy. Only those anions that exhibit chaotropic properties showed significant binding to POPC membranes. A detailed investigation of thiocyanate binding to neutral POPC and to positively charged mixed POPC/dihexadecyldimethylammonium bromide (DHDMAB) (8:2 mol/mol) membranes revealed changes in the ^{2}H NMR quadrupole splittings from POPC specifically deuteriated at either the α-segment or the β-segment of the choline head group which were consistent with a progressive accumulation of excess negative charge at the membrane surface with increasing SCN^- concentration. Both the ^{2}H and ^{31}P NMR spectra indicated the presence of fluid lipids in a bilayer configuration up to at least 1.0 M NaSCN with no indication of any phase separation of lipid domains. Calibration of the relationship between the change in the ^{2}H NMR quadrupole splitting and the amount of SCN^- binding provided thiocyanate binding isotherms. At a given SCN^- concentration the positively charged membranes bound levels of SCN^- 3 times that of the neutral membranes. The binding isotherms were analyzed by considering both the electrostatic and the chemical equilibrium contributions to SCN^- binding. Electrostatic considerations were accounted for by using the Gouy–Chapman theory. For 100% POPC membranes as well as for mixed POPC/DHDMAB (8:2 mol/mol) membranes the thiocyanate binding up to concentrations of 100 mM was characterized by a partition equilibrium with an association constant of $K \approx 1.4 \pm 0.3$ M^{-1}. Hence, the greater levels of thiocyanate binding occurring in the presence of positively charged membranes were primarily the result of electrostatic effects. The ^{2}H NMR results further indicated that the anions were binding in the plane of the POPC choline head group quaternary nitrogen. The factor critical to the ability of an aqueous anion to bind to lipid membrane surfaces appeared to be the ease with which its hydration shell water molecules could be removed.

Lipid bilayers bind a variety of aqueous cations [cf. McLaughlin (1977)], anions (Tatulian, 1983), hydrophobic ions [cf. Honig et al. (1986)], polyamines (Chung et al., 1985b), and proteins (Deveaux & Seigneuret, 1985). Most of these studies have been performed by measuring the electrophoretic mobility of phospholipid vesicles from which it was possible to calculate the so-called ζ potential, i.e., the average electrostatic potential at the plane of shear. The ζ potential could then be related to the electric surface charge density, in terms of the Gouy–Chapman theory, and, in turn, also to

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the binding equilibrium [cf. McLaughlin (1977)].

In order to demonstrate the surface binding of aqueous cations we have suggested recently a different approach involving deuterium magnetic resonance (^{2}H NMR)[1] spectroscopy (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984, 1985; Macdonald & Seelig, 1987a,b). It was found that the conformation of the choline head group is influenced by the charge density at the membrane surface. Its response is reflected in the quadrupole splitting from ^{2}H NMR spectra of head group deuteriated phosphatidylcholines. Thus, the choline head group behaves as a "molecular electrometer", sensing and reporting the charge state of the membrane surface (Seelig et al., 1987).

Employing this response, we show here that only those aqueous anions classified as "water-structure breakers" (Collings & Washabough, 1987) influence the head-group structure to any significant extent. They do so without disrupting the long-range organization of the macroscopic lipid ensemble. Furthermore, the response of the ^{2}H NMR quadrupole splittings of head group deuteriated phosphatidylcholines to the binding of aqueous anions conforms to the predictions of the molecular electrometer concept, being symmetric and opposite in sense to the effects of cation binding. Moreover, we demonstrate that aqueous anion binding is enhanced by the presence of positively charged lipids in a manner analogous to the increased binding of aqueous cations observed in the presence of negatively charged lipids (Macdonald & Seelig, 1987a,b). Finally, these ^{2}H NMR data are interpreted in terms of aqueous anion binding isotherms. By considering both electrostatic and chemical equilibrium contributions to the observed binding, we have obtained the association constants for aqueous anion binding to both neutral and positively charged lipid membranes.

Materials and Methods

The following nomenclature is employed for phosphatidylcholine containing deuterons at the indicated positions:

$$^{-}O_3POCH_2\overset{\alpha}{C}H_2\overset{\beta}{C}H_2N(CH_3)_3^{+}$$

1-Palmitoyl-2-oleoyl-*sn*-glycerol 3-phosphate (POPA) was purchased from Avanti Polar Lipids (Birmingham, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) selectively deuteriated at the α-methylene segment (α-CD_2-POPC) or the β-methylene segment (β-CD_2-POPC) was synthesized by starting from POPA as described by Tamm and Seelig (1983). Dihexadecyldimethylammonium bromide (DHDMAB) was purchased from Merck (Switzerland). All salts were of the highest grade available commercially.

^{2}H NMR spectra were recorded on a Bruker CXP-300 spectrometer operating at 46.1 MHz by employing the quadrupole echo technique (Davis et al., 1976), complete phase cycling of the pulse pairs (Griffin, 1981), and other experimental conditions as described previously (Seelig et al., 1981; Tamm & Seelig, 1983). Particulars regarding 90° pulse lengths (2.5 μs), interpulse delays (50 μs), recycling delays (250 ms), spectral widths (50 kHz), data size (2K), and number of acquisitions (15 000) are noted in parentheses.

^{31}P NMR spectra were recorded at 121.48 MHz by employing a Hahn echo sequence with proton decoupling and phase cyclizing of the pulses as described by Rance and Byrd (1983) and other experimental conditions as described previously (Seelig et al., 1981). Particulars regarding 90° pulse lengths (3.5 μs), interpulse delays (50 μs), recycling delays (1 s), spectral widths (50 kHz), data size (2K), and number of acquisitions (5000) are noted in parentheses.

The method of sample preparation and the assay of ion binding followed a procedure similar to that described previously (Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b). Typically, a volume of dichloromethane containing 20 μmol of POPC was dried under a stream of nitrogen, and any residual solvent was removed under high vacuum. The lipid was dispersed in 400 μL of aqueous solution containing 10 mM Tris-HCl, pH 7.4, plus the desired concentration of NaSCN. A homogeneous suspension was obtained via repeated warming to 45 °C and vortexing. Equilibrium was achieved through repeated cycles of freeze–thaw, maintenance for 48 h at 4 °C, and finally intermittant vortexing over a 6 h period at 25 °C. The suspension was centrifuged for 30 min at 20000*g* and the clear supernatant removed. The thiocyanate concentration in the supernatant was assayed colorimetrically in the concentration range up to 100 mM by employing a reaction with Fe^{3+} (Müller, 1953).

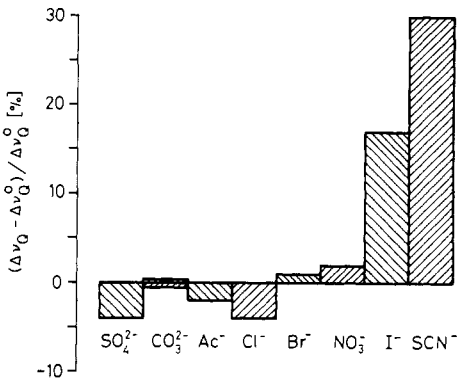

FIGURE 1: Comparison of the effects of various aqueous anions on the ^{2}H NMR quadrupole splitting of α-CD_2-POPC lipid membranes. These anions represent a Hofmeister series and are arranged, from left to right, in order of their increasing tendency to act as water-structure breakers (chaotropes). All anions were present as their Na^+ salts (0.20 M, plus 0.01 M Tris-HCl, pH 7.4, 25 °C). The results are expressed as the percent change in $\Delta\nu_Q$ relative to the value measured in the presence of buffer alone ($\Delta\nu_Q^0$).

Results

Our fundamental indicator of the occurrence of charged-ligand binding at a membrane surface is the change induced in the ^{2}H NMR quadrupole splitting, $\Delta\nu_Q$, measured from the ^{2}H NMR spectrum of choline head group deuteriated phospholipid membranes (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b). This change in $\Delta\nu_Q$ is a result of the excess surface charge that accumulates upon binding charged ligands (Seelig et al., 1987).

A series of aqueous anions were investigated for their ability to influence the conformation of the lipid head group. Figure 1 shows a comparison of the relative change of the quadrupole splitting of α-CD_2-POPC membranes induced by different anions. Only those ions known to exhibit strong water structure breaking (chaotropic) properties significantly altered $\Delta\nu_Q$ relative to its value in buffer alone. The anions in Figure 1 represent a Hofmeister series and are arranged here, from left to right, in order of their increasing tendency to disrupt water structure (Jarvis & Scheiman, 1968). The water-structure makers (kosmotropes), those anions toward the left in the figure, caused either no change or a small decrease in

[1] Abbreviations: NMR, nuclear magnetic resonance; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycerol 3-phosphate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHDMAB, dihexadecyldimethylammonium bromide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

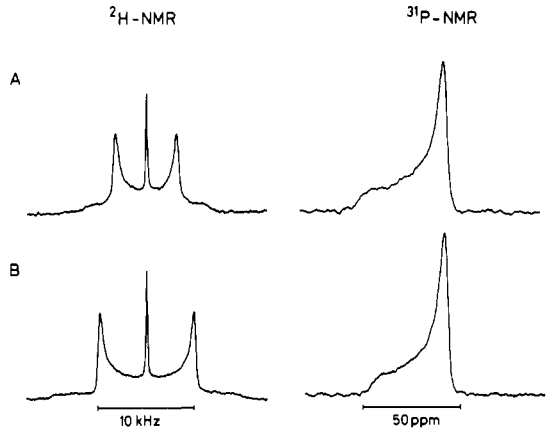

FIGURE 2: Influence of thiocyanate on the ^{2}H NMR (left) and ^{31}P NMR (right) spectra of 100% POPC membranes: (A) α-CD_2-POPC in the absence of NaSCN; (B) α-CD_2-POPC in the presence of 1.0 M NaSCN. All spectra were acquired at 25 °C. All solutions contained 0.01 M Tris-HCl, pH 7.4.

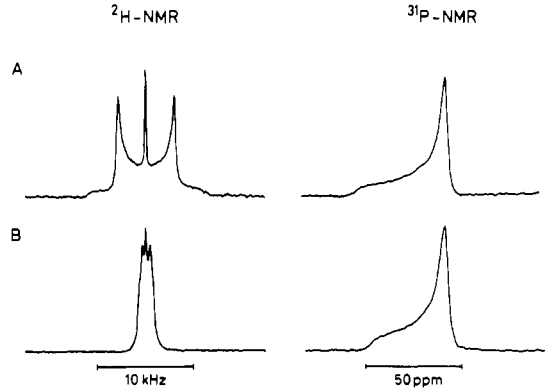

FIGURE 3: Influence of thiocyanate on the ^{2}H NMR (left) and ^{31}P NMR (right) spectra of 100% β-CD_2-POPC membranes: (A) pure β-CD_2-POPC membranes in buffer; (B) β-CD_2-POPC membranes in the presence of 1.0 M NaSCN.

$\Delta\nu_Q$. A detailed interpretation of these small effects is complicated by the probable binding of Na^+, which, although weak (Lau et al., 1981; Macdonald & Seelig, 1987a), is sufficient to account for such changes.

For SCN^-, the aqueous anion showing the largest influence on $\Delta\nu_Q$, a detailed investigation was performed. Figures 2 and 3 show ^{2}H NMR spectra and the corresponding ^{31}P NMR spectra for lipid membranes composed of either α-CD_2-POPC or β-CD_2-POPC, respectively, and hydrated in buffer with or without 1.0 M NaSCN. Both the ^{2}H and ^{31}P NMR spectra were in all cases typical of a random dispersion of fluid lipids in a bilayer configuration (Seelig, 1977, 1978). The size of the quadrupolar splitting, $\Delta\nu_Q$ (corresponding to the separation between the two maxima in the deuterium NMR spectrum), increased in the presence of 1.0 M NaSCN for α-CD_2-POPC (Figure 2) and decreased for β-CD_2-POPC (Figure 3).[2] This counterdirectional change in the size of the quadrupolar splittings for the α- versus β-segment is attributed to a concerted conformational change experienced by the entire choline head group in response to the presence of charges at the membrane surface. Any species influencing the membrane surface charge density has such an effect. The direction of the change observed in the presence of NaSCN ($\Delta\nu_\alpha$ increases, $\Delta\nu_\beta$ decreases) (Table I) is opposite that of metal ions or positively charged amphiphiles (Seelig et al., 1987; cf. below) and indicates the accumulation of excess negative charge at the surface of the POPC membrane. This excess negative charge does not disrupt the long-range order of the membrane, as is evidenced by the ^{31}P NMR spectra that, although showing an approximate 10% decrease in the absolute value of the chemical shift anisotropy in the presence of 1.0 M NaSCN (from −42 to −38 ppm), retain a line shape consistent with an overall bilayer configuration for the lipids. Neither the ^{2}H nor the ^{31}P NMR spectra showed any evidence of lateral phase separation or the presence of separate "free" and "ion-bound" lipid populations. Therefore, the thiocyanate exchange rate must be fast on the NMR time scale; i.e., the residence time of an SCN^- ion at an individual phospholipid head group is less than 10^{-6} s.

The variation of the quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ with the SCN^- concentration is shown in Figure 4A. The abscissa refers to the nominal SCN^- concentration in the buffer, which deviates from the SCN^- equilibrium concentration, C_{eq}, by about 5% (low SCN^- concentration) to 0.5% (high SCN^- concentrations). C_{eq} is smaller than the nominal concentration because of SCN^- binding to the membrane surface. Figure 4 demonstrates quite significant changes in the quadrupole

Table I: ^{2}H NMR Quadrupole Splittings from Neutral and Positively Charged Membranes for Various Thiocyanate Concentrations[a]

[NaSCN] (M)	100 mol % POPC		80 mol % POPC + 20 mol % DHDMAB	
	$\Delta\nu_\alpha$ (kHz)	$\Delta\nu_\beta$ (kHz)	$\Delta\nu_\alpha$ (kHz)	$\Delta\nu_\beta$ (kHz)
0	6.3	5.8	0	10.1
0.01	6.5	5.3	2.0	8.2
0.05	7.3	4.2	4.4	6.6
0.1	7.8	3.9	5.3	5.7
0.2	8.2			4.5
0.5	9.3	2.0	8.0	2.7
1.0	9.8	0.8	9.0	1.3

[a] Plus 0.01 M Tris-HCl, pH 7.4, 25 °C.

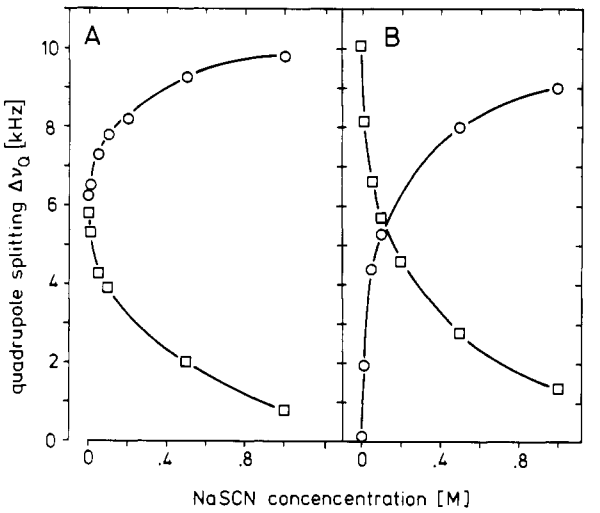

FIGURE 4: ^{2}H NMR quadrupole splittings of neutral and positively charged lipid membranes as a function of the thiocyanate concentration: (A) 100% POPC; (B) 80 mol % POPC + 20 mol % DHDMAB; (circles) α-CD_2-POPC labeled; (squares) β-CD_2-POPC labeled.

[2] The formation of highly curved bilayer structures as well as a general disordering of the lipid packing would reduce the NMR anisotropy and hence the quadrupole splittings at *all* lipid segments. The experimental observation of a *counterdirectional change* at the α- and β-methylene segments excludes this possibility. Measurements of the hydrocarbon chain order parameter for various bound agents also indicated only small variations in the hydrocarbon region. No measurements were made with lipid vesicles since the rapid tumbling of the vesicles averages out the quadrupole splittings.

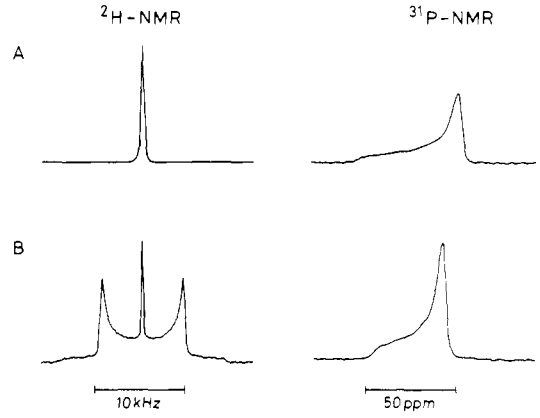

FIGURE 5: Influence of thiocyanate on the ^{2}H NMR (left) and ^{31}P NMR (right) spectra of 80 mol % α-CD_2-POPC + 20 mol % DHDMAB lipid membranes: (A) without NaSCN; (B) with 1.0 M NaSCN. All spectra were acquired at 25 °C. All solutions contained 0.01 M Tris-HCl, pH 7.4.

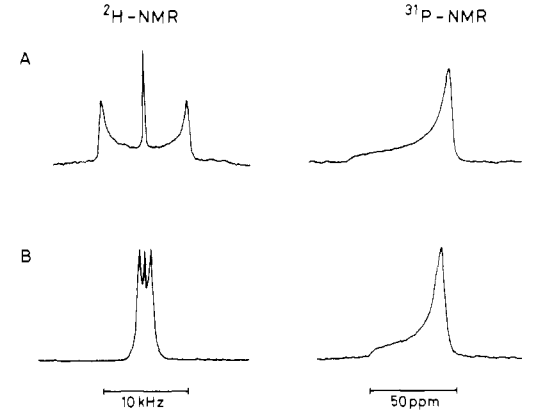

FIGURE 6: Influence of thiocyanate on the ^{2}H NMR and ^{31}P NMR spectra of 80 mol % β-CD_2-POPC + 20 mol % DHDMAB lipid membranes: (A) without NaSCN; (B) with 1.0 M NaSCN. All spectra were acquired at 25 °C. All solutions contained 0.01 Tris-HCl, pH 7.4.

splittings, i.e., changes of the order of 5 kHz (accuracy of the measurement ±50 Hz).

Electrostatic considerations predict that the binding of aqueous anions should be enhanced by the presence of a positively charged membrane surface. Such a condition may be created by mixing neutral POPC with synthetic cationic amphiphiles (Winiski et al., 1986; Seelig and Scherer, unpublished results). Provided their hydrocarbon chains are sufficiently long, such amphiphiles spontaneously aggregate into bilayers (Kunitake, 1979; Fendler, 1980; Kunitake et al., 1986). Figures 5 and 6 show ^{2}H NMR spectra and the corresponding ^{31}P NMR spectra for lipid membranes composed of 20 mol % dihexadecyldimethylammonium bromide (DHDMAB) plus 80 mol % either α-CD_2- or β-CD_2-POPC, respectively, and hydrated in buffer with or without 1.0 M NaSCN. Consistent with the predictions of the molecular electrometer concept, mixing POPC with the positively charged amphiphile caused $\Delta\nu_\alpha$ to decrease (Figure 5) and $\Delta\nu_\beta$ to increase (Figure 6) relative to 100% POPC membrane; i.e., the changes are exactly opposite those observed for the negatively charged SCN^-. In Figure 5A the concentration of the positively charged amphiphile was deliberately chosen in order to collapse the quadrupole splitting of α-CD_2-POPC (Figure 5A). Now the addition of 1.0 M NaSCN caused a dramatic increase in $\Delta\nu_\alpha$ (Figure 5B) and decrease in $\Delta\nu_\beta$ (Figure 6B), indicating that the membrane surface charge has swung radically toward negative values.

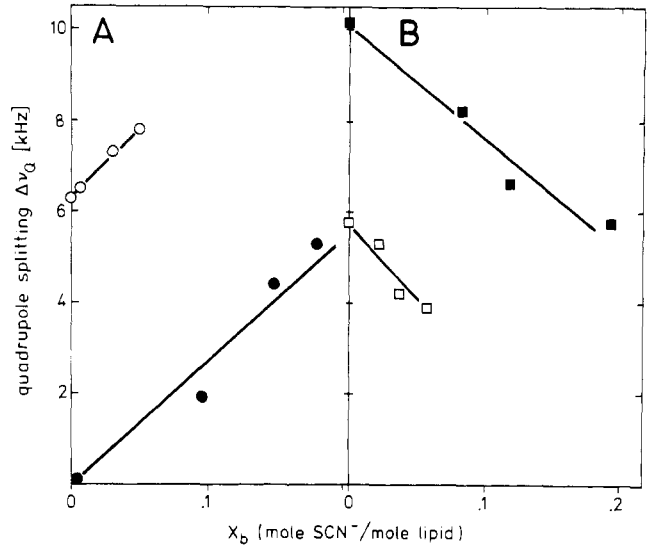

FIGURE 7: Calibration of the relationship between $\Delta\nu_Q$, the ^{2}H NMR quadrupole splitting, and X_b, the number of moles of thiocyanate bound per mole of total lipids: (open symbols) 100% POPC membranes; (filled symbols) 80 mol % POPC + 20 mol % DHDMAB membranes; (A) α-CD_2-POPC labeled; (B) β-CD_2-POPC labeled.

For these membranes, regardless of the changes in $\Delta\nu_Q$ resulting from surface charge effects, the ^{31}P NMR spectra indicated that the lipids remain organized in a bilayer configuration. This is not the case for all such mixtures under all conditions. For example, 1:1 mixtures of DHDMAB:POPC plus 0.2 M NaSCN gave ^{2}H and ^{31}P NMR spectra indicative of overall isotropic motional averaging (spectra not shown). Evidently the macroscopic phase preference of DHDMAB/POPC mixtures is modulated by SCN^- binding in a manner analogous to that observed for cardiolipin/POPC mixtures in the presence of Ca^{2+} [see Seelig and Macdonald (1987b) and references cited therein].

The ^{31}P NMR chemical shift anisotropy measured for POPC in the presence of 20 mol % DHDMAB increased in absolute value relative to that of POPC alone by 30% (from −42 to −55 ppm). For the 80 mol % POPC plus 20 mol % DHDMAB mixture the addition of 1.0 M NaSCN induced an decrease in the absolute value of the ^{31}P NMR chemical shift anisotropy of 22% (from −55 to −43 ppm); i.e., the latter value is again typical of an electrically neutral membrane.

For the positively charged membranes the variation of $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ upon addition of SCN^- was dramatically larger than that measured with neutral membranes, as is shown in Figure 4B. The changes in $\Delta\nu_Q$ with increasing SCN^- concentration were once again progressive with increasing SCN^- concentration and counterdirectional for the α- versus β-segment of the choline head group. It is is assumed that the change in $\Delta\nu_Q$ for a given amount of bound SCN^- ions is proportionately the same for both positively charged and neutral membranes, then it follows that more SCN^- ions are bound to the positively charged membranes.

In order to test the latter hypothesis and to generate ion-binding isotherms, we calibrated the relationship between the quadrupole splitting, $\Delta\nu_Q$, and the number of moles of SCN^- bound per mole of lipid, X_b, using an independent assay of the extent of ion binding (cf. Materials and Methods). We employed in the present case a colorimetric determination of ion concentrations based on the reaction of SCN^- with Fe^{3+} (Müller, 1953). From the difference between the nominal concentration in buffer and the actually measured SCN^- concentration in the presence of lipid, the amount of membrane-bound SCN^-, X_b, could be determined. The results of

Table II: Calibrated Relationship between the ^2H NMR Quadrupole Splitting and the Level of Thiocyanate Binding for Neutral and Positively Charged Membranes[a]

lipid composition	slope m (kHz/mol)	intercept $\Delta\nu_0$ (kHz)
100% α-CD_2-POPC	30.5	6.3
100% β-CD_2-POPC	−36.1	5.85
80% α-CD_2-POPC + 20% DHDMAB	29.0	0.15
80% β-CD_2-POPC + 20% DHDMAB	−23.8	10.0

[a] Slope and intercept in the relationship $\Delta\nu_Q = \Delta\nu_0 + mX_b$, where $\Delta\nu_Q$ is the quadrupole splitting at a particular SCN^- concentration, $\Delta\nu_0$ is the quadrupole splitting in the absence of SCN^- (both in kilohertz) and X_b is the number of SCN^- ions bound per mole of total lipids at a particular SCN^- concentration (molar).

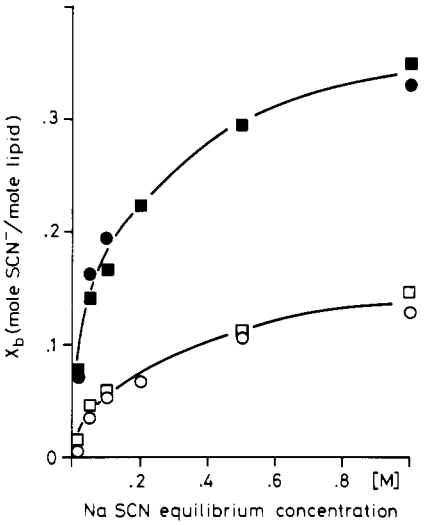

FIGURE 8: Thiocyanate binding isotherms for 100% POPC membranes (open symbols) and 80 mol % POPC + 20 mol % membranes (filled symbols): (circles) α-CD_2-POPC labeled; (squares) β-CD_2-POPC labeled. X_b was obtained from $\Delta\nu_Q$ as described in the text.

this calibration are summarized in Figure 7, where part A shows the variation of $\Delta\nu_\alpha$ (α-CD_2-POPC) with X_b (open circles refer to pure POPC and solid symbols to POPC + 20% DHDMAB) and part B the corresponding plots for $\Delta\nu_\beta$ (β-CD_2-POPC). It can be seen that in all cases a linear relationship exists between $\Delta\nu_Q$ and X_b of the form

$$\Delta\nu_Q = \Delta\nu_0 + mX_b \quad (1)$$

where $\Delta\nu_0$ is the quadrupole splitting measured in the absence of SCN^-. For identical deuteron positions $\Delta\nu_Q$ from neutral and positively charged membranes changed in parallel with increasing SCN^- binding. For deuterons at different positions (α versus β) the change in $\Delta\nu_Q$ with increasing SCN^- binding was always counterdirectional (slopes and intercepts are given in Table II). The limitations inherent to the present assay restrict accurate determinations of X_b to ion concentrations below 100 mM.

Nevertheless, a reasonable estimate of X_b at higher ion concentrations may be obtained by extrapolating the linear relationship between X_b and $\Delta\nu_Q$ to include all measured values of $\Delta\nu_Q$.

The thiocyanate-binding isotherms obtained from $\Delta\nu_Q$ data as described above are shown in Figure 8 for the two membranes (100% POPC; 80 mol % POPC + 20 mol % DHDMAB). Both $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ values were employed to obtain the X_b data tabulated in Table III. The initially positively charged membranes clearly bound far higher amounts of SCN^- than the initially neutral membranes. In order to extract SCN^- association constants, we analyze the SCN^--binding isotherms as described in the next section.

Table III: Thiocyanate Binding to Neutral and Positively Charged Membranes (0.01 M Tris-HCl, pH 7.4, 25 °C)

C_{eq}[a] (mM)	X_b[b] (mmol/mol)	σ[c] (mC/m²)	ψ_0[d] (mV)	C_M[e] (mM)	X_b/C_M (M^{-1})
		(A) 100% POPC			
0	0	0	0	0	
9.4 ± 0.4	10.7 ± 3	−2.5	−7.9	6.9	1.5
48.0 ± 0.6	39.1 ± 7	−9.2	−16.4	25.3	1.5
97.3 ± 0.6	50.4 ± 3	−12.1	−15.9	52.4	1.0
197 (est)	62	−14.5	−13.9	115	0.5
495 (est)	102 ± 4	−24.0	−14.6	280	0.4
994 (est)	125 ± 15	−29.8	−12.9	603	0.2
		(B) 80% POPC + 20% DHDMAB			
0	0	47.0	107.1	0	
6.2 ± 0.4	75.6 ± 1.3	29.2	72.6	104.9	0.7
42.4 ± 0.6	150.5 ± 6	11.6	21.3	97.2	1.5
90.7 ± 0.6	185.0 ± 3	3.5	4.8	109.4	1.7
188 (est)	232	−7.5	−7.4	140	1.6
485 (est)	294 ± 13	−22.1	−13.6	285	1.0
982 (est)	341 ± 26	−33.1	−14.4	560	0.6

[a] Measured colorimetrically, average of determinations on α-CD_2- and β-CD_2-POPC. [b] Calculated by using eq 1 with the parameters in Table II and averaged between α-CD_2- and β-CD_2-POPC. [c] Calculated by using eq 2. [d] Calculated by using eq 3. [e] Calculated by using eq 4.

Discussion

Any ligand that upon binding to a lipid membrane alters the size or the sign of the surface electrical charge will influence the conformation of the choline head group of phosphatidylcholine. The response of the choline head group to the surface charge is reflected in the ^2H NMR quadrupole splittings of the deuteriated head group segments. Here we have exploited this effect to describe the membrane surface binding properties of aqueous anions. As an important advantage of this approach, we note that the amount of bound anion, X_b, is obtained directly from the deuterium quadrupole splitting, $\Delta\nu_Q$.

In the case of charged ligands, a quantitative description of binding requires consideration of both electrostatic and chemical equilibrium contributions. The simplest model of surface electrostatics is the Gouy–Chapman theory, which has been described in detail elsewhere (Aveyard & Haydon, 1973; McLaughlin, 1977). In brief, we first calculate an effective surface charge density, σ, which is the sum of contributions from all charged species (lipids and/or ions) present at the surface according to

$$\sigma = (e_0/S)(X_A - X_b) \quad (2)$$

where X_A is the mole fraction of positively charged DHDMAB, X_b is the mole fraction of bound SCN^- (X_b = moles of ion bound/moles of total lipid), e_0 is the unit charge on the electron, and S is the cross-sectional area occupied per lipid molecule at the surface. Implicit in the form of this expression are the following assumptions: (i) Only one type of ion is binding to the surface; (ii) neutral and charged lipid have the same cross-sectional area (S = 68 Å²); and (iii) the cross-sectional areas are independent of the degree of ion binding. The values of X_b and σ calculated for SCN^- binding to both the neutral and positively charged lipid membranes are given in Table III.

From the surface charge density one may calculate the membrane surface potential, ψ_0, according to

$$\sigma^2 = 2000\epsilon_0\epsilon_R RT\sum_i C_{i,eq}(e^{-z_i F\psi_0/RT} - 1) \quad (3)$$

where ϵ_0 is the permittivity of free space, ϵ_R is the dielectric constant of water, R is the gas constant, T is the absolute temperature, $C_{i,eq}$ is the equilibrium concentration of ion i with

valency z_i in the bulk aqueous phase, and F_0 is the Faraday constant. The summation is over all ions i in solution, i.e., both cations and anions. Our calculated values of ψ_0 are listed in Table III.

The membrane surface potential is then used to calculate the concentration, C_M, of SCN^- ions at the plane of ion binding, i.e., in the solution adjacent to the membrane surface, by invoking the Boltzmann equation.

$$C_M = C_{eq} \exp(F_0\psi_0/RT) \quad (4)$$

The values of C_M calculated in this fashion are given in Table III.

Having accounted for the electrostatic influences, the chemical binding equilibrium may now be evaluated. This takes the general form

$$K_A C_M = X_b/X_f \quad (5)$$

where K_A is the association constant for SCN^- and C_M and X_b have been defined above. X_f is the mole fraction of free binding sites, each site being composed of n lipids.

The statistical evaluation of X_f is simple only if one assumes that the binding anion complexes with a single lipid (lipid/ion stoichiometry $n = 1$). Under these conditions $X_f = 1 - X_b$, and eq 5 takes the form

$$X_b/C_M = K(1 - X_b) \quad (6)$$

Limiting the discussison to SCN^- concentrations below 100 mM, the Langmuir adsorption isotherm degenerates even further into a simple partition equilibrium:

$$K = X_b/C_M \quad (7)$$

Inspection of Table III demonstrates that for low SCN^- concentrations we observe constant X_b/C_M ratios. For membranes consisting of 100% POPC, SCN^- binds with an association constant of 1.3 ± 0.3 M^{-1}; for membranes consisting of 80 mol % POPC + 20 mol % DHDMAB SCN^- binds with practically the same association constant of $K = 1.4 \pm 0.4$ M^{-1}. However, a different picture emerges if the high concentration data of Table III are also included in the evaluation. At $C_{SCN^-} > 100$ mM the ratio X_b/C_M decreases continuously. An approximate fit to the whole data set is provided by a more generalized adsorption isotherm of the form

$$X_b/C_M = (K^*/n)(1 - nX_b) \quad (8)$$

The statistical interpretation of this equation usually assumes independent ligand binding sites on the membranes, each site being composed of n lipids. Such an interpretation is not correct, as has been discussed by Stankowski (1983a,b, 1984). Hence we use eq 8 as a purely empirical two-parameter fit in order to allow a comparison with electrophoretic mobility measurements. Evaluation of Table III according to eq 8 yields $(K^*/n) = 1.65$ M^{-1}, $n = 7.5$, and $K^* = 12.6$ M^{-1} for pure POPC membranes.

The influence of various anions on the electrophoretic mobility of phospholipid vesicles has been investigated in a number of studies (McLaughlin et al., 1975; Hauser et al., 1977; Barsukov et al., 1977; Tatulian, 1983). In particular, Tatulian (1983) has analyzed the binding of SCN^- to membranes composed of dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) in terms of the adsorption isotherm

$$\sigma_{ads} = K^*(\sigma_{max} - \sigma_{ads})C_M \quad (9)$$

with $K^* = 10 \pm 1$ M^{-1} and $\sigma_{max} = 5.5$ $\mu C/cm^2$ in the concentration range 1 mM $< C_{SCN^-} <$ 1 M. Here σ_{ads} is the density of surface charge induced by anion adsorption, and σ_{max} is the maximum charge density at saturation. Since $\sigma_{max} = (e_0/A_L n)$ and $\sigma_{ads} = (e_0/A_L)X_b$, eq 9 is directly comparable to the generalized adsorption isotherm (8). We note a broad agreement between the deuterium NMR results ($K^* = 12.6$ M^{-1}, $n = 7.5$) and the electrophoretic measurements [$K^* = 10$ M^{-1}, $n = 4.9$ (using $A_L = 59$ Å^2 for DMPC)].

The significance of the above analysis is threefold. First, SCN^- binding to lipid membranes and the measured association constants provide an added perspective on the surface binding of small aqueous ions. Such ions are virtually lipid impermeable. They are excluded from the membrane lipid phase as a result of the unfavorable Born electrostatic energy barrier that arises from the low dielectric strength of the membrane interior relative to the bulk aqueous phase (Honig et al., 1986). Small aqueous cations do bind, however, to lipid membrane surfaces. Their association constants increase dramatically with valence charge, i.e., $Na^+ < Ca^{2+} < La^{3+}$ (Akutsu & Seelig, 1981), indicating this to be the decisive factor influencing aqueous cation binding. Such is not the case for aqueous anions, where divalent species such as SO_4^{2-} and CO_3^{2-} showed as little effect on the POPC head group as did monovalent species such as Cl^-. Instead, the characteristic property critical to the ability of an aqueous anion to bind to a membrane surface and to change the head group conformation appears related to its influence on water structure. Those aqueous anions showing significant surface binding possess hydration shells in which individual water moleucles are "disorganized", i.e., mobile, relative to bulk water (hence the term water-structure breakers) [e.g., Collins and Washabough (1985)]. A major consequence is that the hydration shell surrounding chaotropic ions is far more readily removed than that surrounding kosmotropic ions. Surface binding of ions, whether cations or anions, implies at least partial removal of that ion's hydration shell. This step appears to be the critical one for anions since (i) in general their hydration spheres are more tightly held than those of cations (Conway, 1978) and (ii) release from this stricture leads to extensive anion surface binding.

Regardless of whether we are concerned with the surface binding of aqueous cations or anions, their levels of binding are enhanced by introducing into the membrane mixture lipid species of opposite charge. The increased SCN^- binding observed here upon mixing POPC with positively charged DHDMAB parallels the increased Ca^{2+} binding that is observed upon mixing POPC with negatively charged phosphatidylglycerol (Macdonald & Seelig, 1987a) or cardiolipin (Macdonald & Seelig, 1987b). In both cases the increased level of binding represents primarily the effects of electrostatics since the ion association constants were not much altered by the presence of charged lipids.

The second area of significance to which these results relate concerns the molecular electrometer concept as delineated by Seelig et al. (1987). Its fundamental precept is that the choline head group of phosphatidylcholine responds to and, via the ^2H NMR quadrupole splitting, reports on the charge state of the membrane surface. The present findings confirm and extend the applicability of this interpretation of ^2H NMR results. The unifying features of this concept are exemplified by the range of surface electrostatic phenomena that it serves to explain. These include the effects of charged lipids, both negative (Macdonald & Seelig, 1987a,b; Scherer & Seelig, 1987) and positive (present study; Scherer and Seelig, unpublished results), hydrophobic ions, both negative (Malthaner and Seelig, unpublished results) and positive (Altenbach & Seelig, 1985), and aqueous cations (Akutsu & Seelig, 1981, 1984; Macdonald & Seelig, 1987a,b). The new results presented here demonstrate that the effects of binding aqueous anions to either neutral of positively charged membranes

conform to and, thereby, confirm the predictions of the molecular electrometer concept. Although a biologically artificial situation (there are no naturally occurring positively charged lipids), anion binding to positively charged membranes is a logical extension of previous studies, being electrostatically symmetric to the case of cation binding to negatively charged membranes.

Moreover, from the interdependence between $\Delta\nu_\alpha$ and $\Delta\nu_\beta$, ^{2}H NMR permits one to discern the binding location for a particular ligand. For example, it follows from eq 1 and Table I that $\Delta\nu_\beta$ and $\Delta\nu_\alpha$ are linearly related to each other such that for 100% POPC membranes

$$\Delta\nu_\beta = -1.18\Delta\nu_\alpha + 13.6 \text{ (kHz)} \quad (10)$$

while for 80 mol % POPC + 20 mol % DHDMAB membranes in the range $0 < C_{NaSCN} < 0.1$ M

$$\Delta\nu_\beta = -0.82\Delta\nu_\alpha + 9.9 \text{ (kHz)} \quad (11)$$

Compare these expressions with that obtained by Altenbach and Seelig (1984) for Ca^{2+} binding to POPC membranes, where it was found that

$$\Delta\nu_\beta = -0.49\Delta\nu_\alpha + 7.6 \text{ (kHz)} \quad (12)$$

From the slope in this latter expression it is apparent that, for a given level of Ca^{2+} binding, the choline α-segment experiences twice the change in $\Delta\nu_Q$ undergone by the β-segment. In contrast, for a given level of SCN^- binding, the change in $\Delta\nu_Q$ experienced by the choline α-segment is about equal to that of the β-segment. This fits with our intuition that would have Ca^{2+} ions binding in the plane of the lipid phosphate groups (and, therefore, closer to the choline α-segment) while SCN^- ion binding occurs in the plane of the choline quarternary nitrogen (and, hence, closer to the choline β-segment). The conformational change undergone by the choline head group with alterations in the membrane surface charge is understood by no means in its entirety and will require further investigations.

A third area of significance to which the present findings relate concerns the biological relevance of SCN^- binding to membrane lipids. Isothiocyanates, e.g., phenyl thiocyanate, have been reported to inhibit the chemical carcinogenesis associated with nitrosamine compounds (Chung et al., 1984, 1985a). Although the mechanistic details remain unclear, the inhibition is proposed to involve the interaction of isothiocyanates with microsomal membranes and the cytochrome P450 contained therein. Thiocyanate ion itself was ineffective as an inhibitor. The results reported here would indicate that, for electrostatic reasons, microsomal membranes which contain 10–20% negatively charged lipids should bind only negligible levels of SCN^-. Furthermore, it is to be expected that any enhancement of thiocyanate binding accompanying the introduction of hydrophobic substituents (e.g., a phenyl group) would result in an altered plane of membrane binding akin to that experienced by hydrophobic ions. As such, the isothiocyanates would provide an informative and relevant subject for membrane binding studies using the ^{2}H NMR method.

Registry No. POPC, 26853-31-6; DHDMAB, 70755-47-4; SO_4^{2-}, 14808-79-8; CO_3^{2-}, 3812-32-6; Ac^-, 64-19-7; Cl^-, 16887-00-6; Br^-, 24959-67-9; NO_3^-, 14797-55-8; I^-, 20461-54-5; SCN^-, 302-04-5.

References

Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373.

Altenbach, C., & Seelig, J. (1984) *Biochemistry 23*, 3913–3920.

Altenbach, C., & Seelig, J. (1985) *Biochim. Biophys. Acta 818*, 410–415.

Aveyard, R., & Haydon, D. A. (1973) *An Introduction to the Principles of Surface Chemistry*, Cambridge University, Cambridge, England.

Barsukov, L. I., Volkova, V. I., Shaprio, Yu. E., Viktorov, A. V., Bystrov, V. F., & Bergelson, L. D. (1977) *Bioorg. Khim. 3*, 1344–1361.

Chung, F.-L., Juchatz, A., Vitarius, J., & Hecht, S. S. (1984) *Cancer Res. 44*, 2924–2930.

Chung, F.-L., Wang, M. Y., & Hecht, S. S. (1985a) *Carcinogenesis (London) 6*, 539–547.

Chung, L., Kaloyanides, G., McDaniel, R., McLaughlin, A., & McLaughlin, S. (1985b) *Biochemistry 24*, 442–452.

Collins, K. D., & Washabough, M. W. (1985) *Q. Rev. Biophys. 18*, 323–422.

Conway, B. E. (1978) *J. Solution Chem. 7*, 721–770.

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

Deveaux, P. F., & Seigneuret, M. (1985) *Biochim. Biophys. Acta 822*, 63–125.

Fendler, J. (1980) *Acc. Chem. Res. 13*, 7–13.

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

Honig, B. H., Hubbell, W. L., & Flewelling, R. F. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 163–193.

Jarvis, N. L., & Scheiman, M. A. (1968) *J. Phys. Chem. 72*, 74–78.

Kunitake, T. (1979) *J. Macromol. Sci., Chem. A13*, 587–602.

Kunitake, T., Ando, R., & Ishikawa, J. (1986) *Mem. Fac. Eng., Kyushu Univ. 46*, 221–243.

Lau, A., McLaughlin, A., & McLaughlin, S. (1981) *Biochim. Biophys. Acta 645*, 279–292.

Macdonald, P. M., & Seelig, J. (1987a) *Biochemistry 26*, 1231–1240.

Macdonald, P. M., & Seelig, J. (1987b) *Biochemistry 26*, 6292–6298.

McLaughlin, S. A. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.

McLaughlin, S., Bruder, A., Chen, S., & Moser, C. (1975) *Biochim. Biophys. Acta 394*, 304–313.

Müller, G.-O. (1959) *Praktikum der quantitativen chemischen Analyse*, pp 332–334, Hirzel-Verlag, Leipzig.

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

Scherer, P. G., & Seelig, J. (1987) *EMBO J. 6*, 2915–2922.

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

Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.

Seelig, J., Tamm, L. K., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3933.

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

Stankowski, S. (1983a) *Biochim. Biophys. Acta 735*, 341–351.

Stankowski, S. (1983b) *Biochim. Biophys. Acta 735*, 352–361.

Stankowski, S. (1984) *Biochim. Biophys. Acta 777*, 167–182.

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

Tatulian, S. A. (1983) *Biochim. Biophys. Acta 736*, 189–195.

Winiski, A. P., McLaughlin, A. C., McDaniel, R. V., Eisenberg, M., & McLaughlin, S. (1986) *Biochemistry 25*, 8206–8214.