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Investigation of Anion Binding to Neutral Lipid Membranes Using ^2H NMR†

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ABSTRACT: The binding of aqueous anions (ClO_4^-, SCN^-, I^-, and NO_3^-) to lipid bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated using deuterium (^2H) and phosphorus-31 (^{31}P) nuclear magnetic resonance (NMR) spectroscopy. The ability of these anions to influence the ^2H NMR quadrupole splittings of POPC, specifically labeled at the α or β position of the choline head group, increased in the order $NO_3^- \ll I^- < SCN^- < ClO_4^-$. In the presence of these chaotropic anions, the quadrupole splitting increased for α-deuterated POPC and decreased for β-deuterated POPC, indicating a progressive accumulation of negative charge at the membrane surface. Calibration of the ^2H NMR quadrupole splittings with the amount of membrane-bound anion permitted binding isotherms to be generated for perchlorate, thiocyanate, and iodide, up to concentrations of 100 mM. The binding isotherms were analyzed by considering electrostatic contributions, according to the Gouy-Chapman theory, as well as chemical equilibrium contributions. For neutral POPC membranes, we obtained ion association constants of 32, 80, and 115 M^{-1} for iodide, thiocyanate, and perchlorate, respectively. These values increase in the order expected for a Hofmeister series of anions. We conclude that the factor determining whether a particular anion will bind to lipid bilayers is the ease with which that anion loses its hydration shell. A comparison of the calibrated sensitivity of the ^2H NMR quadrupole splitting to these and other ligands indicated that, in addition to charge, two factors dictate the level of the ^2H NMR response: first, whether the ligand is cationic or anionic; and second, whether the ligand is predominantly hydrophobic or hydrophilic in nature. Both of these factors can be seen to arise from the details of the "choline-tilt" model of the ^2H NMR response to surface charges.

Ion binding to membrane surfaces represents a probable regulatory mechanism in biology (Hille, 1984). The polar groups of membrane lipids are involved in that they themselves contribute to the surface charge and in that they constitute ion-binding sites. It has been shown that phosphatidylcholines respond to the presence of bound ions and that this response can be detected using deuterium nuclear magnetic resonance (^2H NMR)[1] spectroscopy (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b). Recent evidence indicates that this sensitivity of phosphatidylcholine involves a concerted conformational change of the choline head group, undergone in response to changes in the membrane surface charge. In this sense, phosphatidylcholine behaves like a "molecular voltmeter". Models of this conformational change suggest that the entire choline group tilts with respect to the plane of the membrane as its quaternary nitrogen is either attracted to or repelled by opposite or like surface charges (Scherer & Seelig, 1989; Roux et al., 1989; Macdonald et al., 1991).

In this report, we describe a detailed, comparative investigation of the binding of aqueous anions (ClO_4^-, SCN^-, I^-, NO_3^-) to neutral phosphatidylcholine membranes using ^2H NMR. We have determined ion-binding isotherms and extracted association constants by taking into account both electrostatic and equilibrium binding considerations. In addition, this comparison has delineated certain limitations of ^2H NMR for the investigation of such binding equilibria, while clarifying other aspects of the ^2H NMR response to surface charges.

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[1] Abbreviations: ^1H NMR, proton nuclear magnetic resonance; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; ^2H NMR, deuterium nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TLC, thin-layer chromatography; CSA, chemical shift anisotropy; DSC, differential scanning calorimetry.

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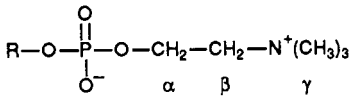
Materials and Methods

The following nomenclature is employed to indicate deuteron positions in the phosphocholine head group:



1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) was purchased from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), selectively deuterated at the α or β-methylene segment, was synthesized starting with POPA as described by Harbison and Griffin (1984). The deuterated POPC was purified via CM-52 column chromatography as described by Comfurius and Zwaal (1977). The purity of the synthesized lipids was assessed by thin-layer chromatography (TLC) and ^{1}H NMR. All final products resulted in a single spot on an overloaded TLC plate, migrating with an R_f identical to authentic POPC. The ^{1}H NMR spectra were identical to that of nondeuterated POPC except for the changes expected in the presence of α- or β-deuterons. All salts, present in their sodium form, were of the highest grade available.

^{2}H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz by employing the quadrupole echo technique (Davis et al., 1976), and by using quadrature detection with complete phase cycling of the pulse pairs (Griffin, 1981). Specifics regarding the 90° pulse length (2 μs), the interpulse delays (40 μs), the recycle delay (250 ms), the spectral width (50 kHz), the data size (2K), and the number of acquisitions (5000) are those noted in parentheses.

^{31}P NMR spectra were recorded at 121.25 MHz by using a Hahn echo pulse sequence with phase cycling of the pulses and proton decoupling as described by Rance and Byrd (1983). Specifics regarding the 90° pulse length (4.25 μs), the interpulse delay (40 μs), the recycling delay (1 s), the spectral width (100 kHz), the data size (1K), and the number of acquisitions (6400) are, again, those noted in parentheses.

Samples were prepared for NMR spectroscopy as follows. A volume of chloroform containing 26 μmol of deuterated POPC was dried under a stream of nitrogen, and any remaining solvent was removed under high vacuum. The lipids were dispersed in 400 μL of aqueous buffer (10 mM HEPES, pH 7.4) containing the desired concentration of a particular salt. In order to completely equilibrate the anion concentration between the various lamelli of the resulting multilamellar vesicles, the suspension was subjected to repeated cycles of vortexing and freeze–thawing as described by Macdonald and Seelig (1988). The equilibrated suspension was centrifuged at 13000*g* for 30 min. While the pellet was taken for NMR measurement, the clear supernatant was assayed for ion content.

The anion concentration in the sample supernatants was assayed using ion chromatography on a Dionex QIC IonChrom analyzer equipped with a conductivity meter for quantitation. Specifics regarding the column matrix (Dionex AG-3 Separator), flow rate (3 mL/min), eluent (8 mM Na_2CO_3), and regenerant (25 mM H_2SO_4) are noted in parentheses. Supernatants were diluted into the linear response region of standard curves (0–2 mM), and their anion concentrations were determined by back-calculation. We measured X_b, the moles of anion bound per mole of lipid, by calculating the difference between the anion concentration in the supernatant before and after the addition of lipids. This independent assay served as a means of calibrating the relationship between the size of the quadrupole splitting and the amount of bound anion in the concentration range 0–10 mM. The inherent limitations in this method of calculating X_b constrain the range of concentrations over which the assay may be employed. Specifically, at higher concentrations, the measured difference between the nominal and free anion concentrations becomes small relative to the experimental error of the measurement.

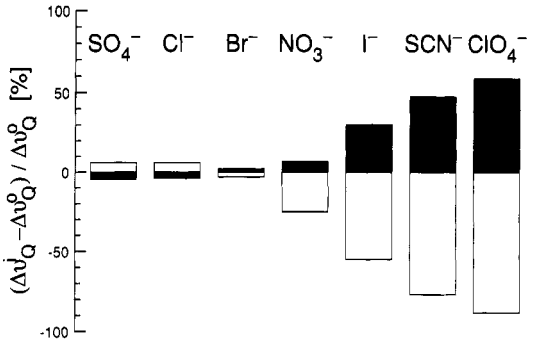


FIGURE 1: Survey of the effects of various aqueous anions on the quadrupole splittings from α- and β-choline-deuterated POPC. The change in the value of the quadrupole splitting measured with either POPC-α-d_2 (dark bars) or POPC-β-d_2 (light bars) containing membranes in the presence of 0.5 M of the indicated anion is expressed as the percent change of the quadrupole splitting relative to its value in the absence of anions.

Results

Specific binding of aqueous anions to lipid bilayers has been demonstrated previously [cf. Chapman et al. (1977), Tatulian (1983, 1987), and Macdonald and Seelig (1988)] by using a variety of experimental techniques. Macdonald and Seelig (1988) used ^{2}H NMR to investigate in detail the binding of thiocyanate to neutral and to positively charged membranes. To expand upon this previous work, we surveyed a group of aqueous anions, representing a Hofmeister series, for their ability to influence the conformation of the choline head group of POPC. Figure 1 compares the effects of those various anions on the size of the quadrupole splittings, $\Delta\nu_Q$, from α-deuterated and from β-deuterated POPC membranes. Only those anions with known water structure breaking (chaotropic) properties (Collins & Washabough, 1985) significantly influenced the quadrupole splitting. Their effects on the quadrupole splittings from α- versus β-deuterated POPC were opposite in direction and roughly equal in magnitude. This is in keeping with the "choline tilt" model of the "molecular voltmeter" effect (Seelig et al., 1987). The direction in which the quadrupole splittings changed upon the addition of aqueous anions is that which is expected for the accumulation of negative surface charge. SO_4^{2-} and Cl^-, both regarded as being water structure makers (kosmotropes), only marginally influenced the quadrupole splitting. It is possible that the small effects observed for these latter salts arise as a consequence of weak Na^+ binding to the membranes (Lau et al., 1981; Macdonald & Seelig, 1987a). Since these changes in the quadrupole splittings are ion-specific, they cannot be ascribed merely to changes in ionic strength. Moreover, the activity coefficients of the various ions are each very similar (lying between 0.76 and 0.78 at 0.1 *m*; CRC Handbook, 1974) so that differences in ionic activities cannot explain the quadrupole splitting effects. Instead, we attribute these changes to the binding of specific anions to the membrane surface.

A more detailed, comparative binding study was undertaken for those anions (ClO_4^-, SCN^-, I^-, NO_3^-) which showed the largest influence on the quadrupole splitting. Figure 2 shows examples of the effects of these aqueous anions on the ^{2}H NMR and ^{31}P NMR spectra from POPC membranes. The

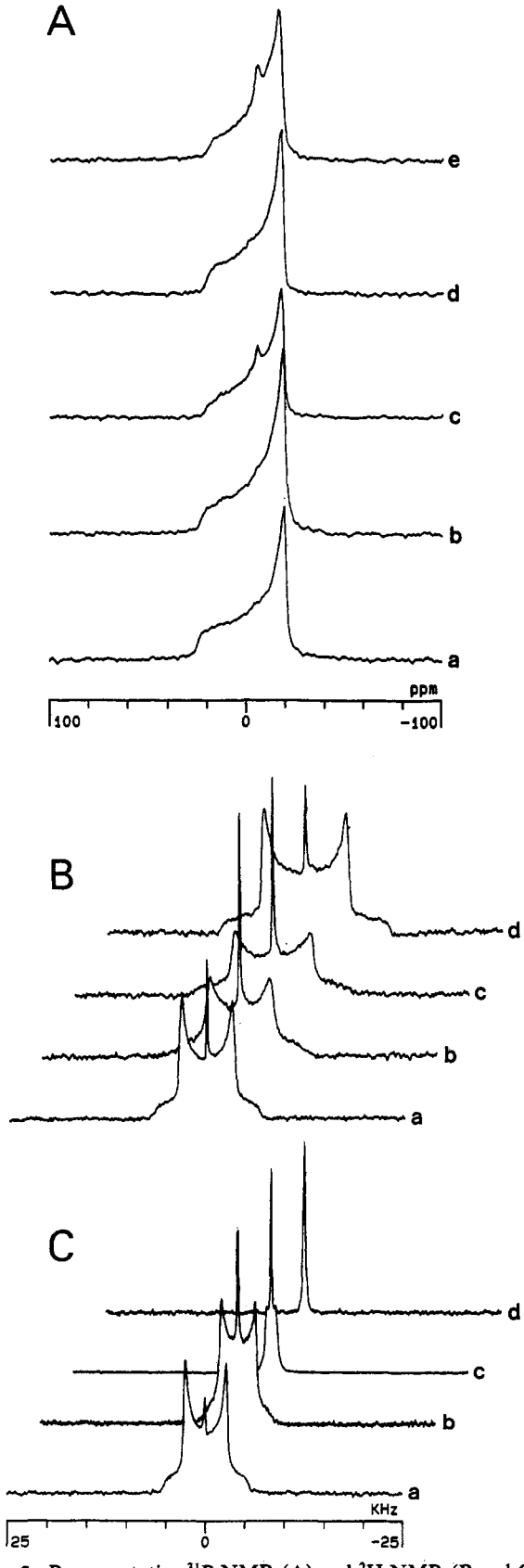


FIGURE 2: Representative ^{31}P NMR (A) and ^{2}H NMR (B and C) spectra in the presence of various aqueous anions. In (A), ^{31}P NMR spectra are shown which were obtained for POPC membranes in the presence of (a) buffer alone, (b) $NaNO_3$, (c) NaI, (d) NaSCN, (e) $NaClO_4$, and all (b–e) at a concentration of 0.5 M. In (B), ^{2}H NMR spectra from POPC-α-d_2 are shown for increasing concentrations of $NaClO_4$: (a) no salt, (b) 8 mM, (c) 250 mM, (d) 1000 mM. In (C), ^{2}H NMR spectra for POPC-β-d_2 are shown for increasing concentrations of $NaClO_4$: (a) no salt, (b) 10 mM, (c) 250 mM, (d) 1000 mM.

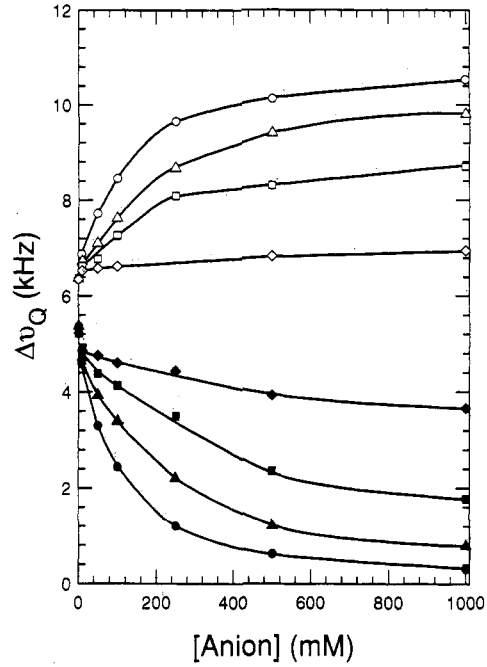


FIGURE 3: Variation of the ^{2}H NMR quadrupole splittings from POPC-β-d_2 (closed symbols) and POPC-α-d_2 (open symbols) as a function of the concentration of various anions: perchlorate (circles), thiocyanate (triangles), iodide (squares), and nitrate (diamonds).

^{31}P NMR spectral line shape is particularly sensitive to the macroscopic phase state of the membrane lipids (Seelig, 1977; Culllis & De Kruiff, 1979). The ^{31}P NMR spectra in Figure 2A demonstrate that, even at extremely high concentrations of these anions (i.e., 0.5 M), the membrane lipids retain an overall bilayer configuration. Nevertheless, the ^{31}P chemical shift anisotropies (CSA) did respond to the presence of these anions, showing a reduced magnitude relative to neutral POPC membranes (e.g., from −43 to −37 ppm in the presence of 0.5 M $NaClO_4$). The influence of the various anions on the ^{31}P CSA increased in the order $NO_3^- < I^- < SCN^- < ClO_4^-$. Figure 2B,C shows ^{2}H NMR spectra for α- and β-deuterated POPC, respectively, in the presence of increasing concentrations of $NaClO_4$. It is clear that the effects of ClO_4^- on the size of the quadrupole splittings are progressive with increasing anion concentration, and opposite in direction for α- versus β-deuterated POPC. The direction of the changes observed in the size of the quadrupole splittings upon exposure to ClO_4^-, SCN^-, I^-, or NO_3^- was that expected in the presence of a negative membrane surface charge density (Seelig et al., 1987). The ^{2}H NMR spectra themselves were each typical of fluid lipids in a bilayer configuration, regardless of the concentration of anion or the deuterium labeling position. For all anions, neither the ^{31}P nor the ^{2}H NMR spectra showed any evidence of separate "ion bound" and "free" components, signifying that the equilibrium exchange rates for these anions are fast on the ^{31}P and ^{2}H NMR time scales (i.e., less than 10^{-5}–10^{-6} s).

The effects on the quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ of increasing anion concentrations over the range 0–1.0 M are shown in Figure 3 for the anions NO_3^-, I^-, SCN^-, and ClO_4^-. The influence of the aqueous anions on the quadrupole splitting increased in the order $NO_3^- \ll I^- < SCN^- < ClO_4^-$. We observed that the magnitude of the change in $\Delta\nu_Q$ at a given concentration, arising from ligand binding, was greater for the β-deuterated position compared to the α-position. These variations in the quadrupole splitting occurred progressively with increasing anion concentrations.

In order to convert these NMR data to binding isotherms for the anions of interest, it is necessary to calibrate the change

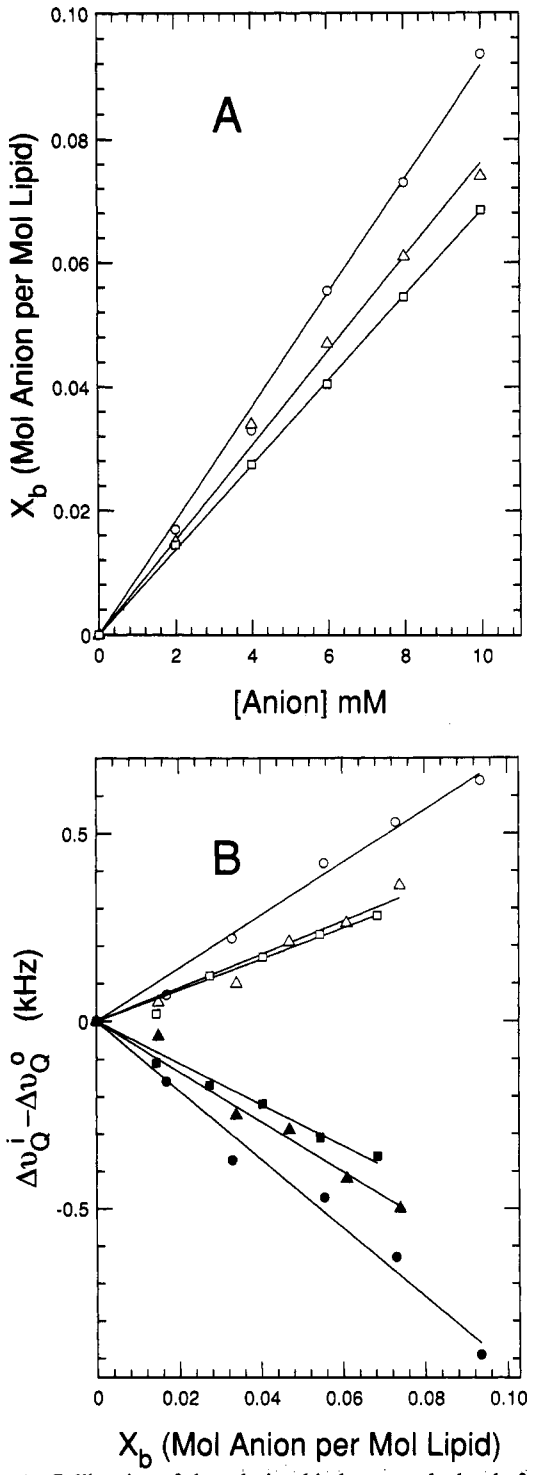


FIGURE 4: Calibration of the relationship between the level of anion binding (X_b) and the quadrupole splitting ($\Delta\nu_Q$). Part A shows the dependence of X_b on the anion concentration for the cases of perchlorate (circles), thiocyanate (triangles), and iodide (squares), where X_b was determined as described in the text. Part B shows the interdependence of X_b and the corresponding quadrupole splittings from POPC-α-d_2 (open symbols) and POPC-β-d_2 (closed symbols).

in quadrupole splitting with the number of moles of anion bound per mole of lipid, X_b. This we accomplished by using an independent assay of the extent of anion binding, based on ion chromatography (cf. Materials and Methods). The results in Figure 4A, obtained from the independent assay, clearly show that anion binding indeed occurs. Over the limited concentration range investigated by using the independent binding assay, the level of anion binding increased in a linear fashion with increasing anion concentration. Note that this

Table I: Calibration of the Relationship between the ^{2}H NMR Quadrupole Splitting and the Level of Anion Binding from Equation 1

	slope (kHz/mol)		coefficient of determination (r^2)	
anion	m_α, POPC-α-d_2	m_β, POPC-β-d_2	POPC-α-d_2	POPC-β-d_2
ClO_4^-	7.03	−9.19	0.98	0.99
SCN^-	4.83	−6.68	0.97	0.98
I^-	4.11	−5.53	0.97	0.98

Table II: Correlation of the Quadrupole Splittings from POPC-α-d_2 and POPC-β-d_2 for Equivalent Anion Concentrations[a]

anion	slope ($m_{\alpha\beta}$)	intercept ($b_{\alpha\beta}$)	r^2
ClO_4^-	−1.36 (−1.20)	13.90 (12.79)	0.93 (0.99)
SCN^-	−1.67 (−1.28)	15.91 (13.35)	0.89 (0.99)
I^-	−1.15 (−1.34)	12.47 (13.70)	0.95 (0.98)

[a] The slopes ($m_{\alpha\beta}$) and intercepts ($b_{\alpha\beta}$) from eq 3 were determined by using linear regression analysis over the limited data range from 0 to 10 mM (first values) or the entire data range from 0 to 1000 mM (values in parentheses).

linearity is fortuitous and will not continue at higher anion concentrations (e.g., see Table IV). The overall level of anion binding at any one concentration of anion increased in the order $I^- < SCN^- < ClO_4^-$. One cannot directly extract anion association constants from such data because, first, only a minute portion of the total binding isotherm is present and, second, the effects of surface electrostatics must be considered (see Discussion). Figure 4B illustrates the interdependence of the level of anion binding and the quadrupole splittings from both α- and β-deuterated POPC. It is evident that the variation of $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ with X_b is linear for each of perchlorate, thiocyanate, and iodide. Calibration curves for nitrate could not be confidently produced, as the changes in the quadrupole splitting and the values of X_b were small relative to the experimental errors in their determination. In all three cases shown in Figure 4B, the relationship between $\Delta\nu_Q$ and X_b took the form

$$\Delta\nu_i = m_i X_b + b_i \quad (1)$$

where the subscript i refers to either the α- or the β-deuteron labeling position and b_i equals the quadrupole splitting measured in the absence of anions. The slopes and regression analysis results for all situations are summarized in Table I.

Normally one estimates values of X_b from ^{2}H NMR data at higher ligand concentrations by extrapolation of eq 1. An estimate of the range of concentrations over which such an extrapolation is valid was obtained in the following fashion. Plots of the quadrupole splittings obtained under otherwise identical conditions of ligand binding for α- versus β-deuterated phosphatidylcholines are generally linear (Seelig et al., 1987). Such α–β correlation plots are shown in Figure 5 for three of the anions (ClO_4^-, SCN^-, and I^-) investigated here. To a first approximation, the α–β correlations are linear for all three cases over the entire range of anion concentrations. Fitting the correlation plots to equations of the form

$$\Delta\nu_\beta = m_{\alpha\beta}\Delta\nu_\alpha + b_{\alpha\beta} \quad (2)$$

provides the slopes, $m_{\alpha\beta}$, and intercepts, $b_{\alpha\beta}$, listed in Table II for the individual anions, for different ranges of anion concentration. In fact, Table II shows that one obtains different values of $m_{\alpha\beta}$ and $b_{\alpha\beta}$ depending on the range of concentrations evaluated, which suggests that the linearity of such α–β correlations is only approximate. Noting that, for any one anion at any one concentration, the value of X_b should be

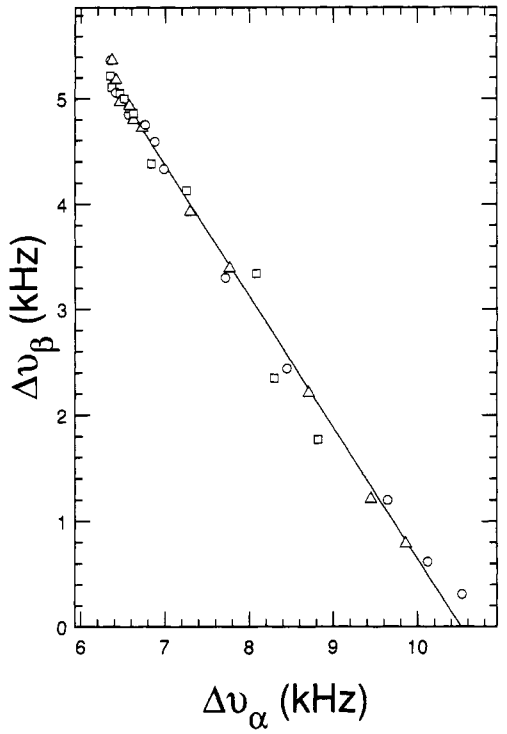


FIGURE 5: Correlation plot of the quadrupole splittings from POPC-α-d_2 and POPC-β-d_2. The quadrupole splittings from the two deuteron labeling positions, obtained under identical conditions of anion concentration, are plotted with respect to one another for perchlorate (circles), thiocyanate (triangles), and iodide (squares). The best linear fit to the global data set is shown as the solid line. The fits to the individual data sets are listed in Table II.

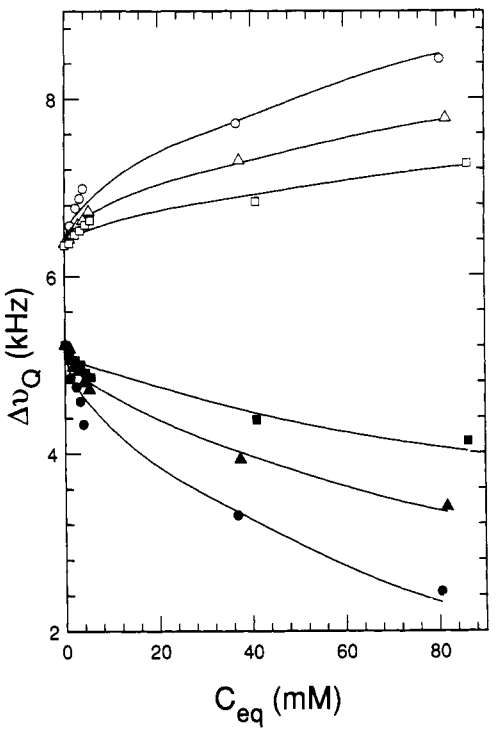


FIGURE 6: Binding isotherms as measured from the variation of the quadrupole splittings of POPC-α-d_2 (open symbols) and POPC-β-d_2 (closed symbols) with the eqilibrium $NaClO_4$ (circles), NaSCN (triangles), and NaI (squares) concentration, C_i^{eq} (plus 10 mM HEPES, pH 7.4, 25 °C). The solid lines represent the nonlinear least-squares best fit to the data, calculated assuming a 1:1 anion/lipid binding model, and association constants of 32, 80, and 115 M^{-1} for I^-, SCN^-, and ClO_4^-, respectively.

Table III: Slope and Intercept Identities[a] Evaluated from Equation 5

	$m_{\alpha\beta}(m_\alpha/m_\beta)$ for range (mM)		$[b_\beta - (m_\beta/m_\alpha)b_\alpha]/b_{\alpha\beta}$ for range (mM)	
anion	0–100	500–1000	0–100	500–1000
ClO_4^-	1.05	0.59	0.97	1.62
SCN^-	0.97	0.74	1.02	1.29
I^-	0.92	0.83	1.06	1.18

[a] Values of the slope and intercept identities in the ideal case equal unity.

the same for either α- or β-deuterated POPC, allows us to rearrange eq 1 to give

$$(\Delta\nu_\alpha - b_\alpha)m_\beta = (\Delta\nu_\beta - b_\beta)m_\alpha \quad (3)$$

which upon further rearrangement yields the expression:

$$\Delta\nu_\beta = \left[\frac{m_\beta}{m_\alpha}\right]\Delta\nu_\alpha + \left[b_\beta - \frac{m_\beta}{m_\alpha}b_\alpha\right] \quad (4)$$

A comparison of eq 2 and 4 yields the identities

$$m_{\alpha\beta} = m_\beta/m_\alpha;\ b_{\alpha\beta} = b_\beta - \left(\frac{m_\beta}{m_\alpha}\right)b_\alpha \quad (5)$$

Table III summarizes the results that we obtained when these identities were evaluated over different ranges of the α–β correlations. For concentrations up to 100 mM, both the slope and intercept identities showed excellent agreement, within experimental error (i.e., ± 5%). However, for higher concentration ranges (e.g., 250–1000 mM), a progressive decline in the quality of the agreement between the identities became apparent, such that at extreme concentrations the slope of the α–β correlations deviated from the expected values by as much as 40%. This deviation from ideality was substantial for all anions, but increased in severity in the order $I^- < SCN^- < ClO_4^-$. We conclude that an extrapolation of eq 1 is not justified beyond concentrations of approximately 100 mM, because of the extreme values reached by the quadrupole splittings.

Figure 6 illustrates the correspondence between simulated and real ^2H NMR data over the concentration range up to 100 mM. As will be explained under Discussion, the quadrupole splittings were simulated by taking into account the combined effects of the contribution of the binding equilibrium, the influence of surface electrostatics, and the calibrated relationship between the quadrupole splittings and X_b. Within this limited range, we have been able to extract binding association constants and to generate the dependence of the membrane surface potential on the bulk anion concentration.

Discussion

Anion Binding to POPC Membranes. ^2H NMR has proven enormously useful in the study of ligand binding to membrane surfaces [e.g., see Seelig and Macdonald (1987)]. In order to extract anion association constants from ^2H NMR data, we have to consider the combined effects of chemical equilibria, surface electrostatics, and the calibrated relationship between the quadrupole splittings and the amount of bound anion. The simplest model that describes lipid bilayer surface electrostatics is the Gouy–Chapman–Stern theory (Aveyard & Haydon, 1973). In this approach, the surface charge density, σ, originates from the individual contributions of all charged species (lipids and/or ions) at the membrane surface (Mclaughlin, 1977) and gives rise to a surface potential, Ψ_0, which in turn influences the concentration of ions at the membrane surface relative to their corresponding concentration in the bulk solution.

Table IV: Anion Binding to POPC Membranes (10 mM HEPES, pH 7.4, 25 °C)

anion	C^{eq} [a] (mM)	X_b [b] (mol/mol)	σ [c] (mC/m²)	Ψ_0 [d] (mV)	C^m [e] (mM)
ClO_4^-	0	0	0	0	0
	0.9	0.017	−7.4	−29.5	0.29
	1.2	0.033	−8.5	−33.0	0.32
	2.4	0.056	−11.9	−42.0	0.46
	3.2	0.073	−13.8	−45.9	0.54
	3.9	0.094	−15.0	−48.3	0.60
	36.8	0.205	−48.3	−71.9	2.24
	80.5	0.307	−74.8	−76.8	4.04
SCN^-	0	0	0	0	0
	1.0	0.015	−6.6	−26.5	0.37
	1.8	0.034	−8.8	−33.2	0.49
	2.9	0.047	−11.3	−39.4	0.63
	4.0	0.061	−13.2	−43.4	0.74
	5.2	0.074	−14.9	−46.4	0.84
	37.5	0.193	−43.2	−66.5	2.81
	81.1	0.282	−67.4	−71.7	5.02
I^-	0	0	0	0	0
	1.1	0.015	−4.0	−16.5	0.55
	2.2	0.028	−6.3	−23.9	0.86
	3.3	0.041	−7.9	−28.6	1.10
	4.4	0.055	−9.3	−31.6	1.30
	5.5	0.069	−10.4	−34.1	1.46
	41.0	0.136	−32.8	−53.5	5.11
	86.3	0.209	−51.2	−58.7	8.79

[a] Measured by using ion chromatography, average of separate determinations on POPC-α-d_2 and POPC-β-d_2. [b] Calculated using eq 1, with parameters from Table I, and averaged for separate determinations of membranes containing POPC-α-d_2 or POPC-β-d_2. [c] Calculated using eq 2. [d] Calculated using eq 3. [e] Calculated using eq 4.

We calculate the surface charge density (σ) from the mole fraction (X_b) of bound anion (X_b = moles of ion bound per mole of lipid) of valence z by using

$$\sigma = (ez/S)X_b \quad (6)$$

where S is the cross-sectional area occupied by a lipid molecule at the surface (assumed to equal 68 Å for POPC) and e is the unit charge (Macdonald & Seelig, 1988). In eq 6, it is assumed that only one species of ion is binding to the surface [surface binding of sodium ions is usually considered to be quite low (e.g., see Lau et al., 1989) and should not dramatically influence the surface charge density] and that the cross-sectional area of the lipids is independent of the amount of bound ion. Table IV lists values for X_b and σ for ClO_4^-, SCN^-, and I^- binding to neutral POPC membranes. Note that X_b is obtained from quadrupole splitting data by using eq 1.

Having obtained values for the surface charge density, we calculate the surface potential, Ψ_0, according to the Graham equation (Aveyard & Haydon, 1973):

$$\sigma^2 = 2000\epsilon_0\epsilon_D RT\sum_i C_i^{eq}[e^{-z_iF_0\Psi_0/RT} - 1] \quad (7)$$

where ϵ_0 is the permittivity of free space, ϵ_D is the dielectric constant of water, T is the absolute temperature, R is the gas constant, C_i^{eq} is the concentration at equilibrium of ion i, having valence z_i, in the bulk aqueous phase, and F_0 is the Faraday constant. The summation is over all ions i (both anions and cations) in solution. Values of Ψ_0 for each anion are listed in Table IV.

Incorporating the surface potential into the Boltzmann equation below permits one to determine the concentration of free anions, C_i^M, at the plane of ion binding:

$$C_i^M = C_i^{eq}\exp(F_0\Psi_0/RT) \quad (8)$$

We are now in a position to consider the equilibrium binding of anions located near the surface–aqueous interface to binding sites located on the surface, using the Langmuir adsorption isotherm:

$$K_aC_i^M = X_b/(1 - nX_b) \quad (9)$$

where K_a is the association constant for the particular anion and $1 - nX_b$ is the mole fraction of free binding sites (each binding site consisting of n lipids).

Table V: A Comparison of Anion Association Constants and Anion Surface Charge Densities

anion	radius (Å)	charge density, σ [a] (mC/m²)	K (M^{-1})			
SO_4^{2-}	2.30[e]	−482				
Cl^-	1.82[f]	−385	1.67[b]			
Br^-	1.98[f]	−325	4.03[b]	3.6[c]	2.0[d]	
NO_3^-	1.96[f]	−332	23.65[b]	2[c]	2.8[d]	
I^-	2.24[f]	−254		40[c]		32[e]
SCN^-	2.20[g]	−263		10[c]		80[e]
ClO_4^-	2.36[h]	−229		222[c]	70[d]	115[e]

[a] Charge density $\sigma = ze/4\pi r^2$ where r is the radius of the anion. [b] Barsukov et al. (1977). [c] Tatulian (1983). [d] Tatulian (1987). [e] This work. [f] Waddington (1966). [g] Iwadate et al. (1982). [h] Kapustinskii (1956).

Figure 6 shows the correspondence between the experimental and the simulated quadrupole splitting data used to extract anion binding constants and binding stoichiometries. In practice, we employed a nonlinear least-squares fitting program based on a Marquardt routine which, by considering the combined effects of chemical equilibrium, surface electrostatics, and the calibrated relationship between the quadrupole splittings and the level of binding, minimized the error between actual and simulated quadrupole splittings as evaluated using a χ^2 test, through an iterative adjustment of the binding constant K_a (Altenbach & Seelig, 1984; Altenbach, 1985).

The best fit to the quadrupole splittings from both POPC-α-d_2 and POPC-β-d_2 simultaneously was obtained by assuming a 1:1 anion/lipid binding stoichiometry and association constant of 32, 80, and 115 M^{-1} for I^-, SCN^-, and ClO_4^-, respectively. The average deviation between the simulated and experimental data points was ±114 Hz (63 Hz, 66 Hz) for ClO_4^- (SCN^-, I^-), which is close to the experimental accuracy of the quadrupole splitting measurements. When we assumed a binding stoichiometry greater than 1:1, the quality of the fit of the simulated to the experimental data deteriorated substantially. For example, in the case of $n = 2$, the average deviation between experiment and simulation rose to ±140 Hz (107 Hz, 75 Hz) for ClO_4^- (SCN^-, I^-). For $n = 3$, the deviations were even larger, i.e., ±316 Hz (217 Hz, 80 Hz) for ClO_4^- (SCN^-, I^-). Simulations have shown that the ability of ^2H NMR data to differentiate between different binding stoichiometries improves as the degree of saturation of binding increases (Macdonald & Seelig, 1987a). This is clearly the case here where for ClO_4^- different stoichiometries lead to large differences in the quality of the fit of simulations, while for I^- different stoichiometries yield simulated data which differ little in the quality of fit to experiment. We conclude that a 1:1 binding stoichiometry is most likely for all these anions, but with reservations as described above for I^-.

Anion association with phospholipid vesicles has been investigated in numerous previous studies (McLaughlin et al., 1975; Barsukov et al., 1977; Hauser, et al., 1977; Tatulian, 1983, 1987; Macdonald & Seelig, 1988). Table V lists the association constants reported by various laboratories for anion binding to phosphatidylcholine model membranes. We also list the corresponding ionic radius and the effective surface charge density for each of the anions.

The anion association constants in Table V are listed in order

of their increasing propensity to act as chaotropes, i.e., water structure breakers, with ClO_4^- being the most and Cl^- and SO_4^{2-} being the least chaotropic (Collins & Washabough, 1985). They represent a so-called Hofmeister series. In general, regardless of the technique employed to determine binding isotherms, the measured association constants increase with increasing chaotropic tendency. The inconsistencies in the magnitude of the observed association constants must be attributed to the details of the particular technique employed. For instance, in a previous ^2H NMR study, Macdonald and Seelig (1988) determined a value of 12.6 M^{-1} for the association constant of SCN^- binding to POPC membranes. It is important to note that the values of the quadrupole splittings at particular SCN^- concentrations for both POPC-α-d_2 and POPC-β-d_2 reported by Macdonald and Seelig (1988) are virtually identical to those reported here. The factor of 6 difference between the values of K_a for SCN^- estimated in the two studies can be traced back to the factor of 6 difference between the slopes calibrating the relationship between X_b and $\Delta\nu_Q$ used in the two studies. Macdonald and Seelig (1988) employed a colorimetric assay of SCN^- concentrations before and after binding to lipids, in the concentration range up to 100 mM. The present study employed an ion chromatography based assay, in the concentration range up to only 10 mM. The latter approach should be considered technically superior because, in the lower range of concentrations tested, both X_b and $\Delta\nu_Q$ vary in an approximately linear fashion with the anion concentration and because errors in the determination of X_b by either type of independent assay increase with increasing overall concentration. A comparison between the two studies is further complicated by the fact that Macdonald and Seelig (1988) used the entire range of quadrupole splittings up to 1.0 M SCN^- to obtain K_a while we have constrained our data range to 100 mM. As shown in this study, at the extremes reached by the quadrupole splittings at high levels of anion binding, the relationship between X_b and $\Delta\nu_Q$ is not the same as at low levels of binding. The net effect is to decrease the apparent level of binding at high concentrations and to thereby reduce the apparent association constant. We conclude that, rather than focusing on the absolute values of the association constants, one should concentrate on the trends across the Hofmeister series of anions instead.

Within the Hofmeister series of anions, it is not uncommon for the relative order of a pair of anions to change depending on the details of the physical conditions or techniques employed. Tatulian (1983), for example, has observed an inversion between I^- and SCN^- affinities, while other studies have determined anion orders in which thiocyanate has greater effects than iodide. In particular, anion effects upon the ^1H NMR chemical shift of the N-terminal methyl groups of egg PC increase in the order $Cl^- < Br^- < I^- < SCN^-$ (Jendrasiak, 1972), while differential scanning calorimetry (DSC) experiments reveal that the ability of anions to influence the melting point of DMPC liposomes increases in the order $Cl^- < NO_3^- \leq Br^- < I^- < SCN^- < ClO_4^-$ (Jain & Wu, 1977).

The order in which the association constants for anion binding to POPC increase resembles a Hofmeister series in which $NO_3^- < I^- < SCN^- < ClO_4^-$. The factor which appears to correlate with the binding affinity of these anions for POPC is the charge density at the surface of the anion, as listed in Table V. A lower charge density correlates with a higher association constant. The ionic charge density influences the ease with which ions lose their waters of solvation. Binding to the membrane surface implies at least some loss of the anion's solvation shell. The largest anions with the lowest charge densities lose waters of solvation most readily and bind with the highest affinity.

From a biological point of view, it is interesting to note the work by Wolff (1982) and Schneider and Wolff (1965), who have shown that the affinity of thyroid tissue for anions increases in the order $Cl^- < Br^- < I^- < SCN^- < ClO_4^-$. They discuss the possible role of a "thyroid trap", in which phosphatidylcholine-like compounds might be involved, as a means of concentrating I^- in thyroid tissue. In this work, we have demonstrated that the binding behavior of anions to phosphatidylcholine follows the same order as the anion affinity for thyroid tissue.

Sensitivity of the "Molecular Voltmeter". The results of these investigations also provide a new perspective on the details of the working of the "molecular voltmeter" itself. The sensitivity of the ^2H NMR quadrupole splittings from head group deuterated phosphatidylcholines to ligand binding is considered to arise as a consequence of the resulting changes in the membrane surface charge density. Within the context of the "molecular voltmeter" concept, the choline group is believed to alter its angle of tilt with respect to the plane of the membrane surface as its positively charged quaternary nitrogen is either attracted to or repelled by opposite or like charges, respectively. The resulting "choline-tilt" is monitored by the ^2H NMR quadrupole splitting. However, a number of complications arise when converting quadrupole splittings to surface charge densities. First, it is known that quadrupole splittings do not vary linearly with the amounts of added charged species even when their amounts and location within the membrane are certain (Sixl & Watts, 1983; Scherer & Seelig, 1987; Macdonald et al., 1991). Secondly, it is known that not all charged species have equal effects at equal charge densities.

Beschiaschvili and Seelig (1991) recently catalogued the slopes, calibrating the relationship between the amount of bound charged species and the quadrupole splittings for a range of chemically diverse cationic "ligands". They concluded that, despite the huge variations in the actual values of the calibrated slopes, all cationic species follow a pattern in which the ratio m_β/m_α remains close to −0.5. In Figure 7, we have plotted selected data from Table I of Beschiaschvili and Seelig (1991), along with data for various anionic "ligands", but where we have normalized the slope values to remove variations due merely to the differences in the valence charge borne by a particular ligand. We see that, in addition to the unique motif characterizing the cations, all anionic species display a pattern in which the ratio m_β/m_α remains close to −1.0. Scherer and Seelig (1989) have pointed out that all cations (anions) yield α–β correlation plots having slopes $m_{\alpha\beta}$ in the region of −0.5 (−1.0). This is the result predicted by the identities of eq 5. In addition to the differences noted between cations and anions, the data in Figure 7 reveal that hydrophobic ligands, which intercalate between the membrane lipids, are sensed more readily by the "molecular voltmeter" than hydrophilic ligands, which bind only to the membrane surface. Each of these effects, cationic versus anionic and hydrophobic versus hydrophilic, are in fact features predicted by the "choline-tilt" model (Roux et al., 1989; Macdonald et al., 1991). The low values of m_α and m_β found in the present study for the chaotropic anions would indicate that they are better able to penetrate the lipid bilayer than their kosmotropic counterparts but occupy a plane of binding which is not so deep as that of anionic hydrophobic molecules.

Summary

We have shown that ^2H NMR can be used to quantitate

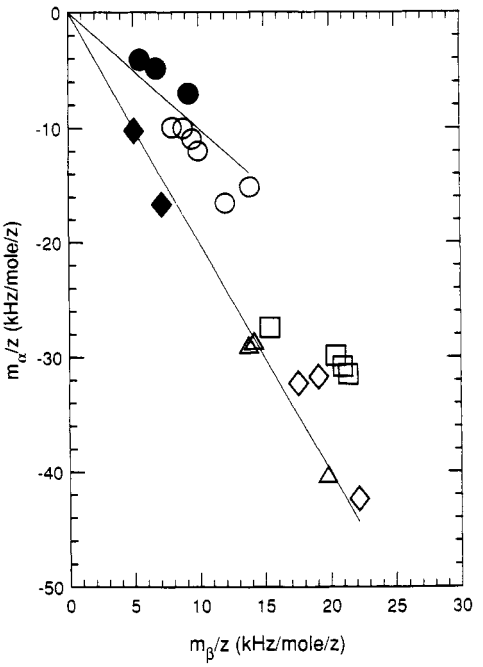


FIGURE 7: Correlation of the sensitivities of the quadrupole splittings from α-deuterated POPC (m_α) with those from β-deuterated POPC (m_β). All values of m_α and m_β have been normalized with respect to the charge borne by a particular ligand. Anionic ligands: aqueous anions (closed circles; present study), anionic phospholipids (open circles; Scherer & Seelig, 1987). Cationic ligands: all data adapted from Beschiaschvili and Seelig (1991) with aqueous cations (closed diamonds), cationic peptides (open diamonds), cationic amphiphiles (open squares), and cationic anaesthetics (open triangles). The solid lines indicate the case for $m_\beta/m_\alpha = -1.0$ and $m_\beta/m_\alpha = -0.5$.

anion binding to lipid membranes. Anion association constants for I^-, SCN^-, and ClO_4^- have been determined for binding to POPC membranes and were found to increase in the order expected for a Hofmeister series, suggesting a role for the water of solvation in dictating binding affinities. In addition, we have been able to suggest a correlation between the sensitivity of the ^2H NMR response to surface charges and the depth of penetration into the bilayer of a particular ligand. This has implications for our understanding of the "molecular voltmeter" effect, both in terms of the nature of the response and in terms of its limitations for monitoring ligand binding to membrane surfaces.

Registry No. POPC, 26853-31-6; I^-, 20461-54-5; SCN^-, 302-04-5; ClO_4^-, 14797-73-0; NO_3^-, 14797-55-8; SO_4^{2-}, 14808-79-8; Cl^-, 16887-00-6; Br^-, 24959-67-9.

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