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# Chaotropic anion–phosphatidylcholine membrane interactions: an ultra high field NMR study

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NMR studies on the interaction of the linear chaotropic anions, $SCN^-$ and $SeCN^-$, with sonicated egg phosphatidylcholine (EPC) vesicles have been carried out at field strengths up to 14.1 Tesla. At 600 MHz, both anions cause splitting or increased splitting of the choline $N^+(CH_3)_3$, $CH_2N^+$ and $O_3POCH_2$ $^1H$ resonances with $SeCN^-$ being somewhat more effective in this action than is $SCN^-$. No changes were observed in the glycerol $CH_2OP$ and $CH_2OCO$ $^1H$ resonances and the phosphate $^{31}P$ resonance of the headgroup region. The $^{13}C$ spectrum was unchanged by the presence of the anions. After 18 h of exposure to the anion, the $^1H$ resonance splittings but not the chemical shift values returned to those prior to anion exposure. Increasing the temperature of the vesicles decreased the anion-induced splitting, but, upon return to the beginning temperature, the chemical shifts did not return to their original values. The results are considered in terms of the 'molecular electrometer' model recently developed by Seelig and co-workers [1].

## Introduction

The interaction of anions with the surface of phospholipid vesicles has been an active area of biomembrane structure and function research. The $^1H$-NMR signal from the choline headgroup of small EPC bilayer vesicles has been observed to be split [2–4]. Other workers have found that the $N^+(CH_3)_3$ resonance splitting is inversely related to vesicle size [5]. The downfield resonance has been assigned to choline groups in the outer monolayer of the bilayer wall of the vesicles whereas the upfield resonance is thought to arise from headgroups on the inner monolayer of the phospholipid bilayer. This chemical shift difference could arise from differences in the radius of curvature of the inner and outer monolayers although the exact molecular mechanism responsible for such splitting is still unknown. Moreover, the choline headgroup splitting is increased by the presence of chaotropic anions [2], in a manner consistent with the position of the anion in the lyotropic series [6]. Again, the mechanism for this increase in splitting has not yet been delineated, although it has been speculated [2] that breaking up of the structured water [7] in the headgroup region by the chaotropic anions may be involved.

In this paper we compare the effects of two different chaotropic anions, $SCN^-$ and $SeCN^-$, on not only the $N^+(CH_3)_3$ $^1H$ resonance but also on the $CH_2N^+$, $O_3POCH_2$, $CH_2OP$, and $CH_2OCO$ resonances for EPC. We have done this at both 600 and 500 MHz $^1H$-NMR frequencies. We have also studied the $^{31}P$ and $^{13}C$ resonances for EPC as they are affected by $SeCN^-$. Additionally, we have explored the independent effects of time and temperature on the various anion-induced splittings.

The results are considered in light of recent ideas of phospholipid headgroup–anion interactions put forth by Seelig and co-workers [8], who have used $^2H$-NMR techniques to examine the binding of $SCN^-$ to large phospholipid multibilayer vesicles. They postulate that $SCN^-$ is bound in the plane of the choline quaternary nitrogen. Seelig's vesicles, because of his method of preparation, are presumably much larger in size and multibilayer in structure compared to our smaller predominantly single bilayer walled vesicles. Since the radii of curvature for the two types of vesicles should be quite different, this may well be reflected in phospholipid packing differences between the inner and

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outer bilayers of the small vesicles vis à vis the larger vesicles. Further comments on our vesicle structure are given later. The anion chemical shift studies reported here extend our previous work [2,9] and complement the elegant studies of Seelig and co-workers.

## Materials and Methods

Egg phosphatidylcholine was obtained from Avanti Polar lipids (Birmingham, AL), and was used without further purification. Potassium thiocyanate (KSCN) was obtained from Fisher and potassium selenocyanate (KSeCN) was obtained from Aldrich. These salts were reagent grade (ACS certified) and were the highest purity available. Sodium 3-trimethylsilylpropionate-2,2,3,3-$d_4$ (TSP) and $D_2O$ (> 99% pure) were obtained from Aldrich.

EPC vesicles were prepared in the following manner. Egg PC was first prepared as a stock solution in chloroform. Chloroform was then removed from the lipid under a stream of dry nitrogen gas followed by freeze-drying overnight. The lipid was then dispersed in $D_2O$ and vesicles prepared by sonication in a sonicator bath, (Laboratory Supplies, Hicksville, NY). The use of this sonicator bath involves placing the lipid sample at a depth in the bath such that turbulence of the entire dispersion is obtained. No other instrument adjustments are necessary. The lipid sample was in a nitrogen atmosphere and was cooled in 20°C water. Typically, 5 min of sonication followed by 5 min of nonsonication was continued for approximately 1 h. Under these conditions, an optical density of 0.5 or less at 546 nm was routinely obtained. Nicomp Particle Size measurements indicated that the vesicles were about 300 Å in diameter. The ratio of the $N^+(CH_3)_3$ downfield signal to the upfield signal, after anion addition, was approx. 1.9. We have studied our anion effects for larger vesicles, 1000 Å or somewhat greater, OD ≅ 1, and find that while the resonance resolution was somewhat less than for the smaller vesicles, as were the original resonance splittings, nevertheless, the chaotropic anion effects, reported here, were unchanged. It should be mentioned here that under our preparation conditions, the $^1H$ spectra attained were as in the figures shown in this paper; very reproducible spectra and precise anion shifts were obtained.

All NMR experiments were carried out at the Duke University NMR spectroscopy Center. NMR data were obtained on 0.5 ml vesicle preparations in $D_2O$ in 5-mm NMR tubes. $^1H$-NMR spectra were recorded on Varian Unity 600 and 500 NMR spectrometers. 600 MHz $^1H$-NMR parameters were: spectral window (SW) 4804 Hz; digital resolution (DR) 0.292 Hz/pt or 0.00048 ppm/pt; pulse width (PW) 8 μs (62° flip angle); acquisition time (AT) 3.4 s; and delay (Dl) of 1 s. 500 MHz $^1H$-NMR parameters were: SW 3958 Hz; DR 0.242 Hz/pt or 0.00048 ppm/pt; PW 10 μs (67° flip angle); AT 4.1 s and Dl 1 s.

$^{31}P$-NMR spectra were obtained on the Unity 500 Spectrometer at 202.33 MHz. $^{31}P$-NMR shifts were calibrated with a 85% $H_3PO_4$ sample (0.00 ppm). Phospholipid $^{31}P$-NMR parameters were: SW 4464 Hz; DR 0.272 Hz/pt; PW 15 μs (67° Flip angle); AT 3.68 s and Dl 1 s. No $^1H$ decoupling was used.

Proton decoupled $^{13}C$-NMR spectra were obtained on a General Electric GN300WB spectrometer at 75.47 MHz. $^{13}C$-NMR parameters were: SW 15,624 Hz (from −6 to +201 ppm); DR 1.22 Hz/pt; PW 10.10 μs (90° flip angle); AT 1.05 s and Dl 3 s.

## Results

Fig. 1 shows the complete 600 MHz $^1H$-NMR spectrum with assignments for egg PC vesicles in $D_2O$. The major $(CH_2)_n$, $CH_3$ and choline $N^+(CH_3)_3$ resonances of phosphatidylcholines are well recognized, [10–15]. We carried out 2-dimensional (2D) COSY, RELAY and TOCSY NMR experiments to firm the assignments of the glycerol backbone and fatty acyl resonances of EPC. The cross peaks from direct connections and relayed magnetization confirm:

(1) The *sn*-2-glycerol methine (CHOCO) resonance overlaps with the vinyl (CH=CH) resonance of double bonded acyl chains at ≅ 5.3 ppm.

(2) The two *sn*-1 glycerol methylene protons ($CH_2OCO$) are not equivalent. One $^1H$ resonance is resolved at 4.43 ppm. A second $^1H$ resonance overlaps on the high-field side of the choline $OPO_3CH_2$ resonance at 4.25 ppm.

(3) The two *sn*-3 glycerol methylene protons ($CH_2OP$) resonate in the broad signal at 4.02 ppm.

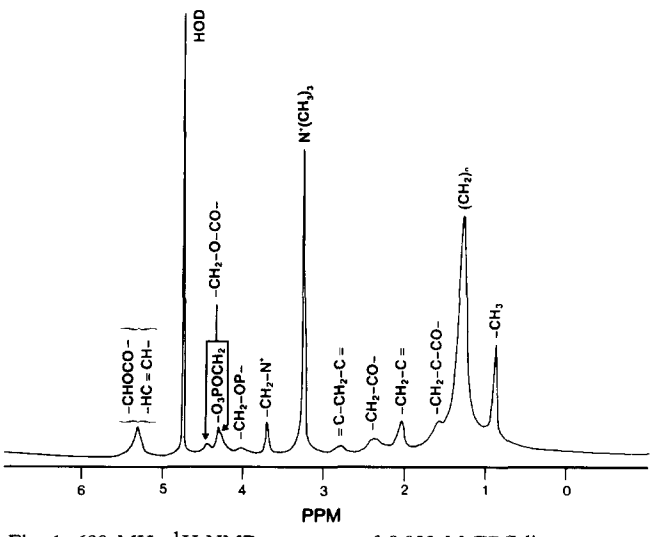


Fig. 1. 600 MHz $^1H$-NMR spectrum of 0.029 M EPC liposomes at 26°C. Resonance assignments were obtained from 2D NMR experiments (see text). The reference is set to TSP at 0 ppm.

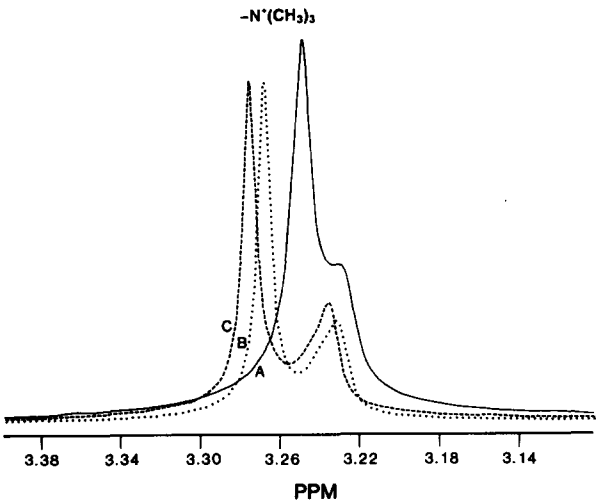


Fig. 2. Choline $N^+(CH_3)_3$ proton NMR resonance at 600 MHz from EPC liposomes (A) in the presence of 0.2 M KSCN (B) or 0.2 M KSeCN (C). EPC concentration and temperature as in Fig. 1. In (A), the two resonances are at 3.230 and 3.248 ppm (about 11 Hz apart). In (B), they are at 3.231 and 3.267 ppm (about 22 Hz apart) while in (C) they are at 3.235 and 3.275 ppm (about 24 Hz separation).

(4) The choline $CH_2N^+$ and $O_3POCH_2$ methylene protons, respectively, resonate at 3.71 and 4.32 ppm.

The 2D NMR assignments clarify those in the literature [10–15]. The 2D spectra are not primary to the thrust of this paper and are not shown. The nonequivalence of the *sn*-1 $CH_2OCO$ protons and the location of the *sn*-2 CHOCO proton has often been neglected. Only the headgroup resonances, i.e., 3.2–4.6 ppm, are shown in what follows since the remainder of the EPC spectrum (mainly from the hydrophobic acyl chains) is not altered by anions at the concentrations used here.

In Fig. 2 it is apparent that the initial splitting of the $N^+(CH_3)_3$ resonance, approx. 11 Hz, is further increased by addition of either anion to the liposome solution. The total splitting in the presence of the SeCN is $\geq 22$ Hz. With the use of the 600 MHz instrument, $SeCN^-$ is seen to be slightly more effective in increasing the splitting than is $SCN^-$. The increased splitting clearly results from the downfield $N^+(CH_3)_3$ resonance moving further downfield upon anion addition. The chemical shift of the upfield $N^+(CH_3)_3$ resonance is changed very little by the presence of either anion.

In Fig. 3 the $CH_2N^+$ resonance, initially a single peak, is now split by the addition of either anion. The original 'single' resonance is transformed into resolved downfield and upfield components; the upfield resonance is essentially at the same chemical shift value as that of the single $CH_2N^+$ resonance before anion addition. The resulting splitting is 20 Hz for $SCN^-$ and 23 Hz for $SeCN^-$ at 0.2 M anion concentration in the NMR tube.

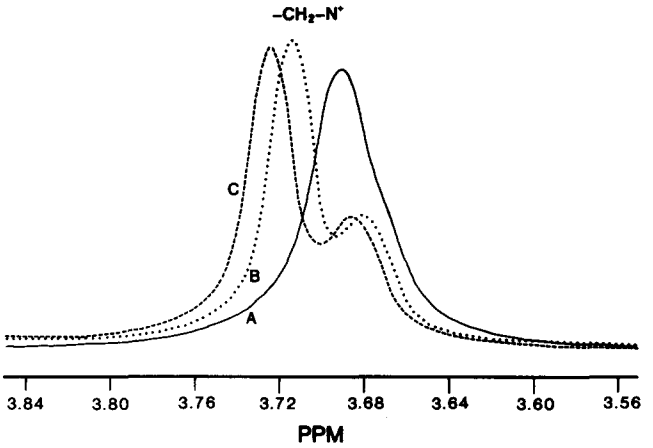


Fig. 3. 600 MHz choline $CH_2N^+$ proton NMR resonance from EPC liposomes (A), in the presence of 0.2 M KSCN (B), or 0.2 M KSeCN (C). In (A) the resonance is at 3.691 ppm, in (B) it is split into signals at 3.681 and 3.714 ppm (about 20 Hz apart) while in (C) the resonances are at 3.685 and 3.723 ppm (about 23 Hz separation).

In Fig. 4 the choline $O_3POCH_2$ resonance exhibits a shoulder resonance about 15 Hz upfield from a taller, downfield resonance. Addition of $SCN^-$ or $SeCN^-$ shifts the low-field, taller resonance 22.9 Hz downfield for $SCN^-$ and 24.9 Hz for $SeCN^-$, whereas the upfield shoulder remains at its original position. The split resonance is centered at about the position of the taller resonance before anion addition. The glycerol $CH_2OP$ and $CH_2OCO$ resonances are not de-

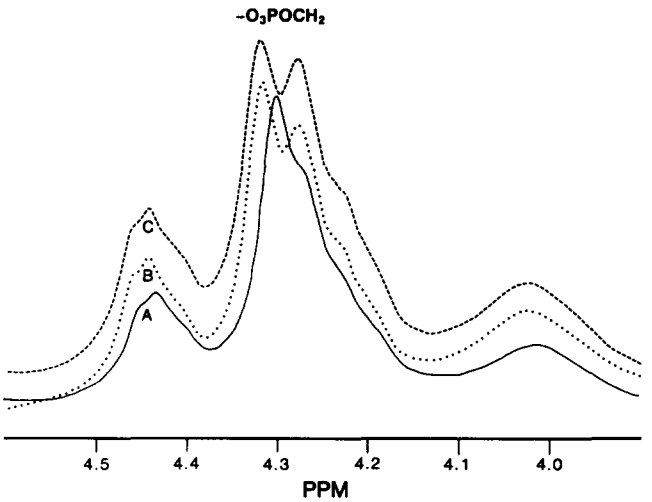


Fig. 4. 600 MHz choline $O_3POCH_2$ and glycerol $CH_2OCO$ and $CH_2OP$ proton NMR resonances from EPC liposomes (A) in the presence of 0.2 M KSCN (B) or 0.2 M KSeCN (C). The glycerol $CH_2OCO$ methylene protons are not equivalent: one $^1H$ resonance is resolved at approx. 4.43 ppm and the second $^1H$ resonance is the overlapped shoulder resonance at approx. 4.23 ppm. The $O_3POCH_2$ methylene initially shows $^1H$ resonances at 4.277 and 4.302 ppm (about 15 Hz separation). Upon addition of the anion, the resonances are at 4.276 and 4.314 ppm (about 23 Hz separation) for $SCN^-$ and 4.277 and 4.318 ppm (about 25 Hz separation) for $SeCN^-$.

tectably affected by exposure of the lipid to either of the two anions.

The use of 0.4 M $SeCN^-$ rather than 0.2 M $SeCN^-$ increases the headgroup splittings by only a small amount, suggesting that saturation of the vesicle $SeCN^-$ binding sites is being approached. This saturation is about 14 $SeCN^-$ molecules per EPC molecule. Interestingly, we found [16] previously that the maximum number of water molecules bound per EPC molecule is approximately 14. The anion induced splittings are independent of the pH since essentially the same magnitude of splittings is obtained at acidic, basic and pH values near neutrality. Deconvolution analysis reveals that after anion addition, the ratio of the downfield $N^+(CH_3)_3$ peak to the upfield $N^+(CH_3)_3$ peak is about 1.9. This value is characteristic of small vesicles of about 200 Å diameter [5]. The difference in the two peak areas is thought to arise from the fact that the surface area of the outer monolayer is greater than that of the inner monolayer thereby allowing for a greater number of $N^+(CH_3)_3$ groups in the outer monolayer. Additionally, we find that the downfield resonance is consistently narrower than the upfield resonance and this we ascribe to more constricted packing of the headgroups in the inner monolayer, resulting from the smaller radius of curvature.

$^{31}P$-NMR measurements show a single phosphorus resonance for EPC which is also unaffected by the presence of $SeCN^-$. This single resonance indicates NMR isotropy. The $^1H$ decoupled $^{13}C$-NMR spectrum is also unchanged by the presence of $SeCN^-$ except for the appearance of a resonance at about 122 ppm due to the CN group carbon. The $^{31}P$- and $^{13}C$-NMR spectra are not shown here.

Fig. 5 shows the 500 MHz spectra of EPC plus anion as a function of time. Careful analyses of the spectra shows that after 18 h the anion-induced splitting of the choline $N^+(CH_3)_3$ peak returns to about the value it had before anion addition; this decrease in splitting over the 18-h time period is attained by the upfield resonance moving downfield and merging with the downfield peak. A similar behavior occurs for the $CH_2N^+$ resonance; after 18 h it is once again a single resonance but now at the chemical shift value of the downfield resonance of the anion split pair, i.e., downfield shifted from its original position. The $O_3POCH_2$ resonance also returns to an unsplit condition, however, the resonance is significantly broadened compared to its width before anion addition, and its chemical shift appears downfield shifted from its original position. The $N^+(CH_3)_3$ resonance occurs at 3.228 and 3.245 ppm before anion addition; 3.232 and 3.273 ppm immediately after anion addition and 3.252 and 3.272 ppm 18 h. later. For $CH_2N^+$, the corresponding chemical shift values are 3.686 ppm, 3.682 ppm and 3.722 ppm and 3.719 ppm at 18 h. For the $OPO_3CH_2$ reso-

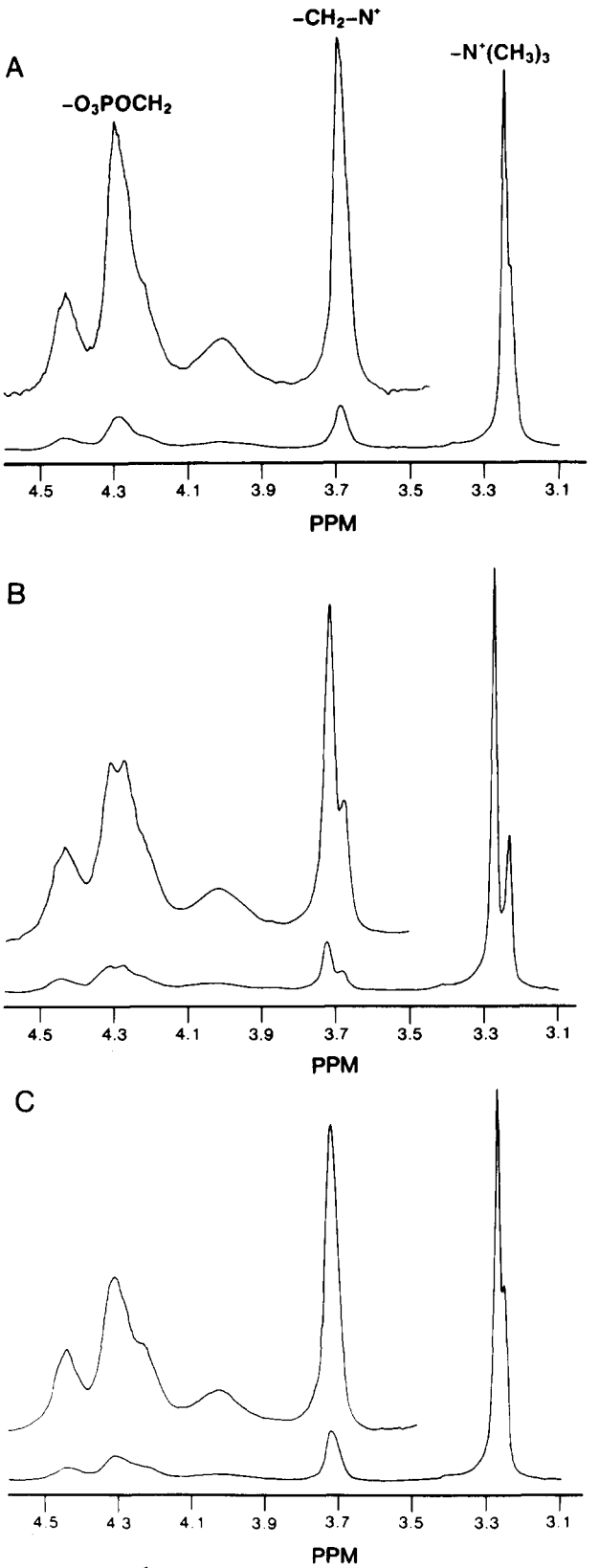


Fig. 5. 500 MHz $^1H$-NMR headgroup spectrum of EPC liposomes (A), immediately after exposure to 0.2 M KSeCN (B) and 18 h. later (C). EPC concentrations and temperature as in Fig. 1. The $N^+(CH_3)_3$ peak is initially split by 8.5 Hz; by 26.5 Hz after $SCN^-$ addition and 9.4 Hz 18 h. later. For the $CH_2N^+$ peak the corresponding values are 0, 20 Hz and 0; for the $OPO_3CH_2$ resonance, the corresponding splittings are 0, 20 Hz and 0.

TABLE I

*Temperature dependence of EPC headgroup chemical shifts and splittings at 500 MHz*

| | $N(CH_3)_3^+$ | | $CH_2N^+$ | | $OPO_3CH_2$ | |
|---|---|---|---|---|---|---|
| | $\delta$ (ppm) | $\Delta\delta$ (Hz) | $\delta$ (ppm) | $\Delta\delta$ (Hz) | $\delta$ (ppm) | $\Delta\delta$ (Hz) |
| No $SeCN^-$ at 26°C | 3.246, 3.265 | 9.5 | 3.707 | – | 4.317 | – |
| With $SeCN^-$ at 26°C | 3.249, 3.294 | 22.5 | 3.699, 3.742 | 21.5 | 4.292, 4.334 | 21.0 |
| With $SeCN^-$ at 60°C | 3.256, 3.274 | 9.0 | 3.703 | – | 4.317 | – |
| With $SeCN^-$ after cooling to 26°C | 3.266, 3.292 | 13.0 | – 3.738 | – | 4.319 | – |

nance, the values are 4.292 ppm, 4.273 ppm and 4.313 ppm and 4.310 ppm at 18 h.

We have carried out $^1$H-NMR studies of the vesicle-anion interaction at 200, 300, 400, 500 and 600 MHz and find that the headgroup splittings and anion effects increase in Hz with the Zeeman magnetic field but are essentially the same on the ppm scale.

Table I lists the chemical shift values for the EPC headgroup region signals at 26°C, after anion exposure at 26°C, heating to 60°C and recooling to 26°C. The splitting of the choline $N^+(CH_3)_3$ and $CH_2N^+$ resonances due to the $SeCN^-$, is greatly reduced at 60°C, due to an upfield movement of the downfield $N^+(CH_3)_3$ and $CH_2N^+$ resonances. Upon recooling,

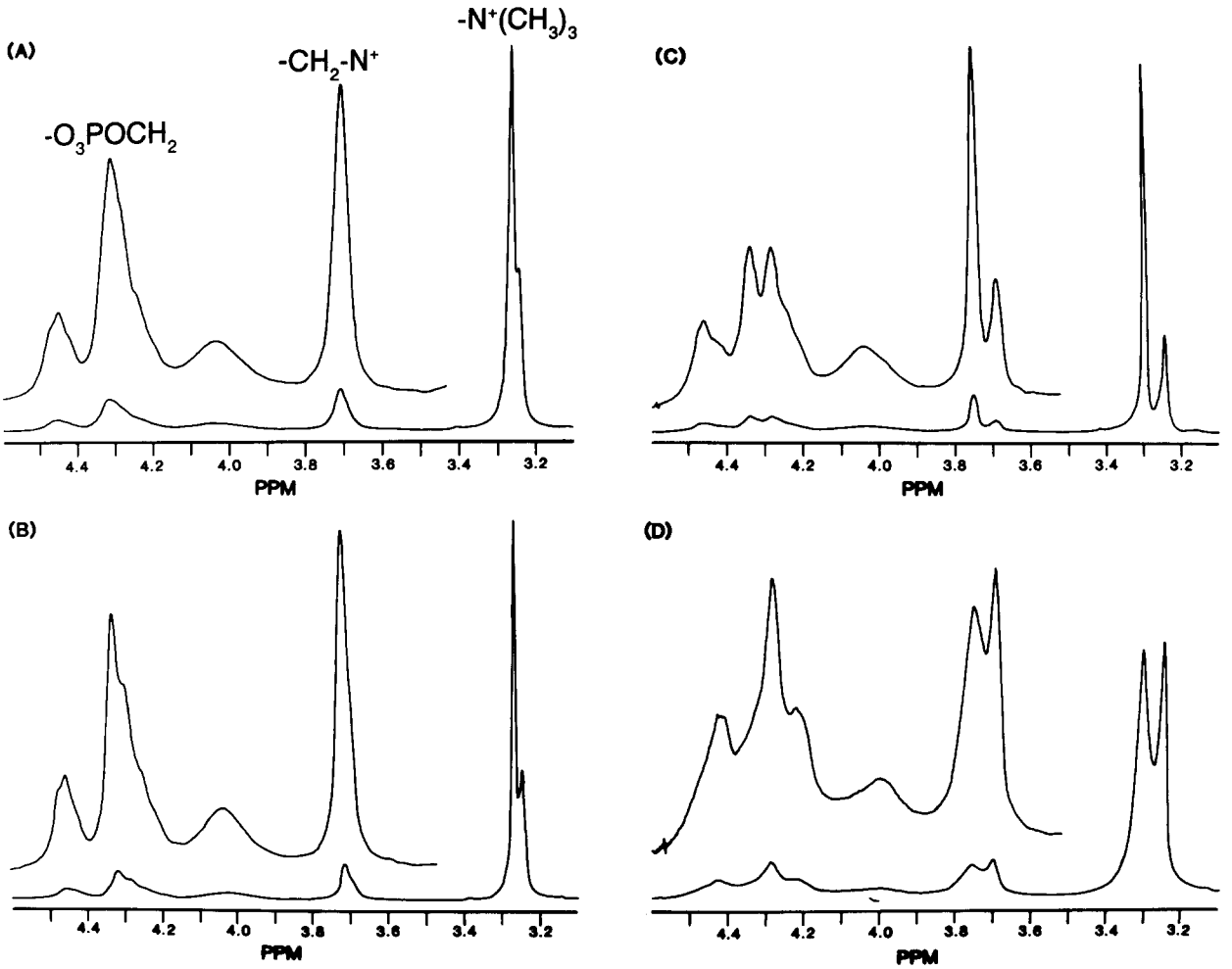


Fig. 6. 600 MHz $^1$H-NMR spectrum of headgroup resonances of EPC liposomes (A), EPC liposomes exposed to 0.2 M NaCl (B) EPC liposomes exposed to 0.2 M NaCl and 0.4 M KSeCN (C) and EPC liposomes exposed to 0.2 M NaCl +0.4 M KSeCN+$10^{-5}$ M $MnCl_2$ (D). Note relatively small effects of NaCl on the choline $N^+(CH_3)_3$, $CH_2N^+$ and $O_3POCH_2$ resonances and the lack of interference with the SeCN induced splitting by the NaCl. Identical numbers of scans were taken for spectra C and D, with D plotted at about 3-times higher gain. Note the broadening of the low-field components of the choline $N^+(CH_3)_3$, $CH_2N^+$ and $O_3POCH_2$ resonances by the paramagnetic $Mn^{2+}$ ion, and the lack of broadening of the upfield components. EPC concentration and temperature are as in Fig. 1.

both peaks are downfield shifted with respect to their chemical shift values prior to anion addition; the greater part of the original anion-induced splitting has not returned, however, because of a downfield movement of the upfield resonance, upon heating and recooling. The choline $O_3POCH_2$ resonance displays a somewhat different behavior with temperature: Upon heating to 60°C, the downfield resonance shifts upfield and the upfield component shifts downfield, merging into a single narrow resonance at about the center of the original split peaks. The temperature behavior of this peak is thus more complex than for the $N^+(CH_3)_3$ and $CH_2N^+$ resonances. After cooling back to 26°C, the $O_3POCH_2$ resonance shows a shift change in at least one, if not both, component signals. It clearly does not resolve into two split components. The resonance chemical shifts obtained with $SeCN^-$ after the heat/cool cycle approach those obtained with $SeCN^-$ at 26°C but waiting 18 h.

As a control experiment (data not shown here), the same temperature cycling was done for EPC vesicles in the absence of anion. Upon heating from 26°C to 60°C, the small initial choline $N^+(CH_3)_3$ proton resonance splitting is decreased; this decrease is attained primarily by the downfield resonance moving upfield. Upon cooling back to 26°C, the $N^+(CH_3)_3$ resonance splitting is about that before heating. Heating causes the choline $CH_2N^+$ peak to move upfield. Upon cooling, the peak is back to its original appearance and chemical shift. For the choline $O_3POCH_2$ resonance, the shoulder moves upfield and exhibits a rather complex lineshape. Upon recooling, the resonance returns to its original condition.

Figs. 6 A and B show that the presence of NaCl induces only small chemical shift changes of the headgroup resonances and does not prevent the $SeCN^-$ induced splittings (Figs. 6 B and C).

In Figs. 6 C and D are shown the headgroup region resonances for EPC + 0.2 M $Cl^-$ + 0.4 M $SeCN^-$ with and without the addition of $10^{-5}$ M $MnCl_2$. The downfield components of the split choline $N^+(CH_3)_3$, the $CH_2N^+$ and the $O_3POCH_2$ resonance pairs are greatly broadened by the presence of the $Mn^{2+}$ whereas the upfield components of *all three* resonance pairs are unaffected. This argues strongly for the downfield resonances arising from the lipid in the outer monolayer and the upfield resonances coming from the inner monolayer lipid [2]. The other $^1H$ resonances from the glycerol backbone region exhibit little, if any, changes due to the $Mn^{2+}$. This is less clear, however, since these resonances are broad even in the absence of $Mn^{2+}$.

## Discussion

The electric charge effects on the headgroup, as found by Seelig et al. and the anion effects obtained here should be related: We found [2] that the $N^+(CH_3)_3$ resonance splitting increases upon exposure of the liposomes to chaotropic anions and that the splitting increase is related to the position of the anion in the lyotropic series [6]. Clearly, (Fig. 6) the nonchaotropic anion $Cl^-$ has only a small effect on the $^1H$ headgroup resonance splittings whereas both $SeCN^-$ and $SCN^-$ produce a greater splitting increase consistent with the position of these two anions in the lyotropic series; $SeCN^-$ is more effective in increasing this splitting, in agreement with the binding studies of Wolff [17]. This would argue against a simple electrostatic interaction *. Macdonald and Seelig [8], find in their $^2H$-NMR studies that only anions which are chaotropic influence the headgroup arrangement to any significant extent; our results clearly agree. Scherer and Seelig [1] find that regardless of the chemical nature of the compounds involved, whether small inorganic ions or negatively charged lipids, they qualitatively exert the same effect on the phosphocholine headgroup as long as their electric charge has the same sign. In contrast, we find [9] that the presence of phosphatidic acid (PA) in EPC liposomes produces somewhat different effects on the choline resonance splitting and chemical shift values than does exposure to chaotropic anions.

The $CH_2N^+$ proton resonance splitting by chaotropic anions shows that this group, also, is exposed to the anions. Again, exposure results in a downfield resonance as well as a resonance at the chemical shift value of the original single peak. In contrast, the $^1H$ resonance from the adjacent glycerol $CH_2OP$ group and the $^{31}P$ resonance of the phosphate group show no observable changes with the presence of the chaotropic anions, perhaps because of the 'protective' effect of the negatively charged phosphate group.

Our studies also reveal little change in the glycerol $CH_2OCO$ resonance upon anion exposure; its rather 'interior' position in the headgroup should limit its accessibility to the anions. The $O_3POCH_2$ proton resonance, however, is split upon anion exposure, even though the $CH_2$ is also near a negatively charged moiety and one might expect that its exposure to anions would be minimized as for the $CH_2OP$.

Macdonald and Seelig [8] find $^2H$ quadrupole splitting changes for the $CD_2N^+$ and $OPO_3CD_2$ groups in the presence of SCN which are *equal* in magnitude but *opposite* in sign for vesicles where 'inside' and 'outside' were equilibrated using 'freeze-thaw' cycles. Our chemical shift changes for these groups are clearly *equal* in magnitude but in the same direction for vesicles with distinct 'inside' and 'outside' monolayers.

Our work is consistent with studies on the binding of the organic anion tetraphenylboride (TPB) to EPC

* We thank a reviewer for bringing this to our attention.

bilayers [18,19]: it was suggested that the TPB lies at the membrane/water interface in a manner which permits electrostatic interaction between the anion and choline $N^+(CH_3)_3$ group while minimizing repulsion from the negatively charged phosphate group. The largest $^1H$ chemical shift changes occurred in the choline $N^+(CH_3)_3$ and $CH_2N^+$ resonances with a smaller change for the $O_3POCH_2$ resonance. The glycerol $CH_2OP$ and $CH_2OCO$ resonances as well as the $^{31}P$ resonance showed a much smaller change upon anion addition.

Both the time and temperature measurements (Fig. 5 and Table I) indicate that chaotropic anions enter the interior of the liposomes and cross the lipid bilayer affecting the headgroups both interior and exterior to the liposomes. In either case, after the anion-induced splitting is decreased, the upfield peaks for the $N^+(CH_3)_3$, $CH_2N^+$ and $O_3POCH_2$ resonances have merged with the downfield peaks. The $SCN^-$-induced downfield shift of both upfield and downfield resonances is similar to that obtained for the headgroup where no inside/outside differentiation exists. Macdonald and Seelig [8] suggest, that $SCN^-$ is excluded from the bilayer interior; our results indicate $SCN^-$ and $SeCN^-$ move across the bilayer interior under a concentration gradient. Their results are obtained under equilibrium conditions with respect to the presence of $SCN^-$ inside and outside the lipid vesicle.

Macdonald and Seelig [8] find that the $SCN^-$ association constants were not much altered by the presence of charged lipids and, therefore, the level of binding represents primarily the effects of electrostatics. We have found [9] that the $N^+(CH_3)_3$ resonance splitting increase, caused by $SCN^-$, is less for PC-PA liposomes containing a greater mole fraction of negatively charged PA. As shown in Fig. 6, however, the presence of $Cl^-$ at a concentration equal to that of $SeCN^-$ has little, if any, effect on the $SeCN^-$-induced splitting of the $N^+(CH_3)_3$ resonance.

According to Macdonald and Seelig the effect of the anions would be to move the headgroup esterified oxygen axis closer to the bilayer surface due to the electrostatic attraction of the choline $N^+(CH_3)_3$ group to the plane of the anion [8]. The chemical shift changes found by us using chaotropic anions might well be an indication of this axis movement. We have not here examined the effects of positive surface charge on the headgroup splittings, although, in our earlier work [2], we used a variety of cations, both monovalent and divalent, with the anions and found no differences in the $N^+(CH_3)_3$ resonance splitting. Recently, we have found [20] that the presence of the positively charged amphiphilic drug, amiodarone, in the EPC vesicles has a significant, but qualitatively different effect on the $N^+(CH_3)_3$ resonance than do the chaotropic anions; $SCN^-$ addition, however, still causes a downfield chemical shift change of the choline $N^+(CH_3)_3$ resonance.

That the $^{31}P$ and $^{13}C$ resonances are unaffected by the presence of the anion is not surprising: the negative charge around the phosphate group would tend to shield the phosphorus from direct interaction with the anion. Protons on the carbon skeleton would also present a certain hindrance to the presence of the anions near the carbons. Macdonald and Seelig find an anion effect on the $^{31}P$ resonance [8] which they ascribe to a decrease in the absolute value of the chemical shift anisotropy. Since he presumably is using larger liposomes than we are and is, therefore, looking at anisotropy, the fact that we see no $^{31}P$ effect is not in disagreement with his results. In small EPC vesicles, the $^{31}P$ signal has been found to be isotropic.

The results shown in Fig. 6 are interesting in that they show that the $Mn^{2+}$ cation has access to the same headgroup 'sites' as do the chaotropic anions. From electrostatic considerations alone, one might expect a repulsive effect at the choline $N^+(CH_3)_3$ and $CH_2N^+$ sites and an attractive interaction near the phosphate group. This, however, is not the case. As for anions, three of the five headgroup resonances are affected by $Mn^{2+}$ whereas the other two are not obviously affected. This would argue against a simple electrostatic interaction occurring between the headgroup and the anions.

In summary, the chemical shift changes and resonance splittings seen by us upon exposure of liposomes to chaotropic anions are consistent with the 'molecular electrometer' model [21] put forth by Seelig et al. The $^2H$ results are ascribed to changes in the conformation of the headgroup relative to the bilayer normal whereas we interpret our chemical shift changes as 'interactions' between the anions and the respective headgroup moieties. We believe, as we have previously stated [2,9], that this interaction involves the structured water in the headgroup region [16] being altered by the anions [6,22] and this alteration itself rather than a simple electrostatic interaction between the anion and headgroup moieties may result in the observed changes in chemical shift values. Indeed the initial choline $^1H$ resonance splitting observed before anion exposure may arise from a dissimilarity in structured water near the inner and outer headgroups [23], resulting from headgroup packing differences between the two monolayers.

A paper by Rydall and Macdonald [24] came to our attention after our work was submitted. These workers find that the $^2H$ quadrupole splittings of the headgroup moieties, upon anion exposure, increase in the order $NO_3^- \ll I^- < SCN^- < ClO_4^-$, an order similar to what we found for the $N^+(CH_3)_3$, resonance splitting increase [2]. It should be noted, however, that where the quadrupole splitting follows a Hofmeister series for

anions, our resonance splitting studies suggest a somewhat more complicated picture: the splittings follow a Hofmeister series for point and linear anions whereas for tetrahedral anions ($ReO_4^-$ and $ClO_4^-$) the situation is more complex [9]. We also found that at high anion concentrations, ($\geq 0.5$ M), the hydrocarbon chain $^1H$ resonances were affected suggesting that the vesicle structure might be changing. We attempted to relate headgroup chemical shift changes (unpublished results) to the thyroid anion binding studies of Wolff [17], however, we found that the anion effects for EPC and for Wolff's 'thyroidal' phospholipid did not differ. In any event, both the quadrupole studies [24] and our chemical shift measurements provide information on anions at the vesicle surface although the parameters measured in the two studies need not behave in an exactly parallel manner. Further work on water structure in the phospholipid headgroup region as affected by chaotropic ions is necessary and such studies are in progress.

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