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A temperature study of diacetylenic phosphatidylcholine vesicles

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Abstract

Dispersions of the diacetylenic phosphatidylcholine, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine, $DC_{8,9}PC$, undergo a change from vesicles to hollow tubes on cooling. We report here a light scattering and multinuclear NMR study of the lipid vesicles over the temperature range 0–50°C. The 'splitting' of the $N^+(CH_3)_3$ resonance increases with decreasing temperature, consistent with the light scattering measurements which show a decrease in vesicle size with decreasing temperature. The NMR spectrum remains well-resolved over this temperature range, even at temperatures as low as 3°C. Phosphorus NMR also indicates that the 'bilayer structure' is maintained over this temperature range. The various proton resonances and the phosphorous signal from the lipid vesicles broaden as the temperature is lowered. These results will be helpful in developing a model for the tubule-forming ability of $DC_{8,9}PC$.

Key words: Diacetylenic phospholipid; Lipid vesicle; NMR

1. Introduction

1,2-Bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine($DC_{8,9}PC$) dispersed in excess water forms vesicles in its high temperature chain-disordered phase, above ca. 43°C, but as the temperature is dropped below ca. 37°C the liposomes reform into hollow cylinders, usually 0.5 to 0.75 μm in diameter, and tens of micrometers in length [1–4]. Bilayer thickness derived from X-ray electron density profiling indicates that the diacetylenic lipid must be tilted with respect to the bilayer normal in the tubule phase [5–7]. De Gennes has suggested that the tubule formation results from a tilted smectic C ferroelectric phase of the chiral lipid, whose electric polarization in the place of the lipid membrane could induce the membrane to roll up [8]. It has been shown that tubules will form in salt solution [9] which would shield against electric fields, lessening the likelihood of this explanation, but the possibility that the lipid forms smectic phases remains. Helfrich examined the mechanical forces on ribbons of lipid membrane and determined that there are conditions under which such structures acquire a helical twist [10]. Prost has suggested that the phase transition involves the creation of regions of hexatic tilt, and that this ordering may drive tubule formation [11]. Observation of faceting or 'roughening' of liposomal surfaces immediately before they transform into tubules lends experimental support to his suggestion [12].

It has been shown that dispersions of $DC_{8,9}PC$ sonicated at ~45°C form small unilamellar vesicles (SUVs) of the lipid which do not transform into tubules as the temperature is decreased through 38°C. If the temperature of the SUVs is further reduced to near 0°C the small vesicles fuse to form larger membrane structures, stacked bilayer sheets (SBS). Upon being warmed through the melting point, and then re-cooled below 37°C the lipid material is converted almost entirely to tubule microstructures. This fusion cycle insures efficient conversion of the lipid to the tubule microstructure [13].

In this work, we have studied the behavior of the small lipid vesicles as they are cooled from 50°C to near 0°C using light scattering and nuclear magnetic resonance (NMR) techniques. There have been few high resolution NMR studies of $DC_{8,9}PC$. Light scat-

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tering and NMR techniques provide complementary information about the vesicles; light scattering techniques quantitate size of the tubes, whereas NMR provides data regarding the molecular arrangement of the vesicles, primarily that of the polar headgroup region of the $DC_{8,9}PC$. Such information is necessary to understand the vesicle to tubule transition.

2. Materials and methods

$DC_{8,9}PC$ was obtained from Avanti Polar Lipids (> 99% pure by HPLC) and was further purified before use by three precipitations using acetone/chloroform. Residual solvent was removed under vacuum overnight, and samples were stored at −20°C in the dark.

Small unilamellar vesicles were prepared by hydration of 30 mg/ml samples with triple-distilled water, followed by sonication at 50°C in a sonicator bath (Laboratory Supplies, Hicksville, NY).

All anion measurements were obtained by adding KSCN to the NMR tube just prior to making the NMR measurements. The SCN^- concentration was 0.2 M in the NMR tube. Since the $DC_{8,9}PC$ concentration was ca. 0.03 M, the ratio of SCN^- to $DC_{8,9}PC$ was ca. 7:1.

Light scattering data was obtained at 90° in a thermostatted cell with a Langley-Ford CM-64 (64 channels) autocorrelator using He-Ne excitation. The cell temperature was controlled by a Neslab water bath. Cooling rates were generally 0.1–1 degree C per min. Autocorrelation functions were collected under computer control at constant time intervals. The sample temperature was monitored in situ with a type-K thermocouple/electronic ice-point with a Keithley 197 multimeter, and recorded at the beginning and end of each autocorrelation measurement. Data sets were analyzed by single-exponential curve fitting routines, and D, the apparent hydrodynamic diameter, evaluated from the fit by the standard relation,

$$D = 2k_BT\left[4\pi n \sin(\theta/2)\right]^2/(6\gamma\pi\eta\lambda^2)$$

where k_B is Boltzmann's constant, T is the absolute temperature, n is the refractive index of the fluid, θ is the scattering angle, η is the temperature-dependent viscosity of water, λ is the scattering wavelength (632.8 nm), and $\gamma = 1/2\tau$, where τ is the decay constant of the autocorrelation function.

^1H- and ^{31}P-NMR data were obtained using 0.5 ml liposome preparations in D_2O in 5 mm NMR tubes. The ^1H-NMR spectra were recorded at the Duke NMR Center on a General Electric GN-500 spectrometer, operating at 500.12 MHz. The ^1H 90° pulse for liposome preparations in the 5 mm ^1H probe was ≈ 10.5 μs. Survey ^1H-NMR spectra were recorded using a full spectral window of ±2645.5 Hz (240.5 ppm) with digital resolution of 0.322 Hz/pt. More detailed ^1H-NMR spectra were then recorded using a spectral window of ±1501 Hz with the transmitter frequency adjusted to focus on the 0–6 ppm region of the ^1H-NMR scale. These NMR spectra were digitized into 32768 computer points to yield a digital resolution of 0.183 Hz/point. The detailed one-pulse ^1H-NMR experiments used an 8 μs pulse (68° tip angle), an acquisition period of 5.46 s and a delay of 1 s.

The resonance from the CH_3 group was set at 0.9 ppm. Although we have used TSP as an internal reference, the use of the CH_3 resonance for this purpose presents no problem since in the work of this paper, we are primarily concerned with resonance line splittings and line broadening.

^{31}P-NMR spectra were obtained with a 10 mm broad-band NMR probe operating at 202.45 MHz. The ^{31}P-NMR shift scale was calibrated with a 85% H_3PO_4 sample set to 0.00 ppm. The phospholipid ^{31}P-NMR spectra were recorded using a spectral window of ±3356 Hz with the transmitter frequency adjusted to focus on the + 22 to − 11 ppm region of the ^{31}P-NMR scale. These NMR spectra were digitized into 32768 computer points to yield a digital resolution of 0.409 Hz/pt. Standard one-pulse ^{31}P experiments used a 15 μs pulse, an acquisition period of 2.44 s and a delay of 1 s.

The NMR probe temperature was monitored by a thermocouple and controlled by the system supplied with the NMR unit; the sample temperature was found to be within 1 degree C of that indicated by the thermocouple.

3. Results

Fig. 1a shows the size vs. temperature data for lipid vesicles formed from $DC_{8,9}PC$ at 50°C and cooled below 0°C. Fig. 1b shows the results for reheating the sample over the temperature range −5°C to 20°C. Note the gradual decrease in vesicle size as the temperature is lowered from 50°C to near 0°C and then the large increase in size of the lipid particles at lower temperatures. The detailed structure of these large particles is not known at this time. Upon reheating, the particle size continues to increase with temperature.

Figs. 2a and 2b show the complete proton spectra for $DC_{8,9}PC$ at temperatures of 50°C and 2.8°C, respectively. The spectra at intermediate temperatures are not shown since only small changes in spectral resolution are observed. Note especially the resolution decrease in the resonance at ca. 1.6 ppm as the temperature is decreased. This resonance arises from the CH_2-C-CO moiety of the phospholipid [14]. We ascribe

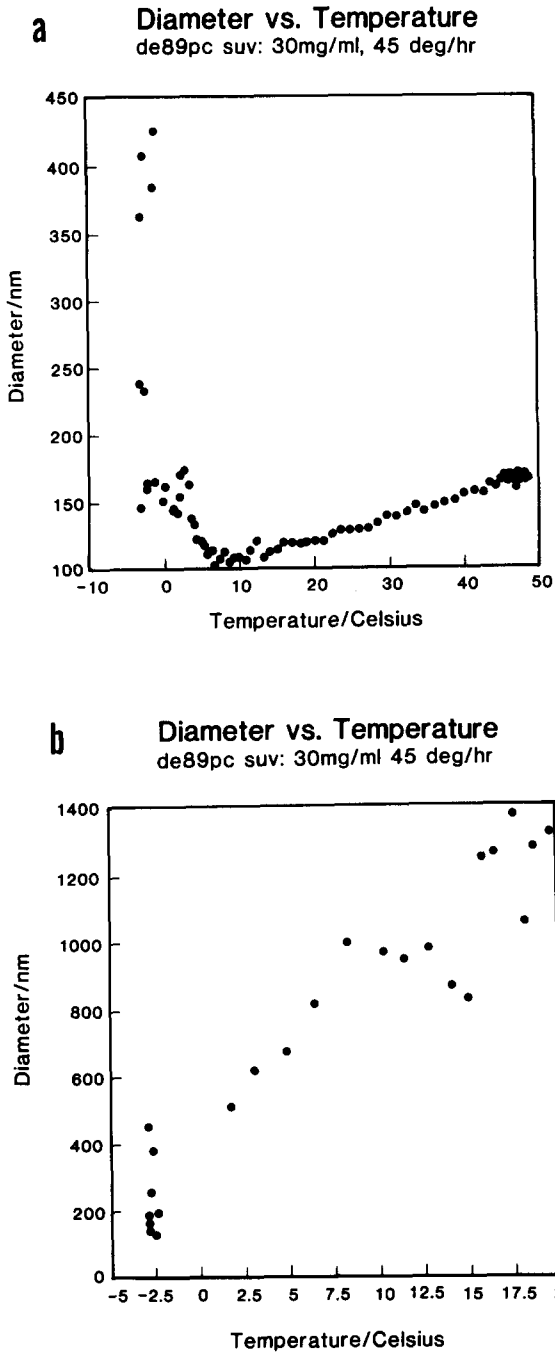

Fig. 1. Light scattering measurements of $DC_{8,9}PC$ vesicle size vs. temperature as the vesicles are cooled from 50°C (a) and then reheated to 20°C (b).

the splitting of this resonance, at 50°C, to the difference in chemical shifts for this group in the *sn*-1 and *sn*-2 hydrocarbon chains, respectively.

Fig. 3 shows the ^{31}P resonance for the vesicles at (a) 50°C, (b) 29.4°C and (c) 0°C. Note the broadening of the resonance at 29.4°C; at 0°C, the ^{31}P resonance is broadened beyond detection. This is consistent with the light scattering results which show the formation of large lipid particles at temperatures near 0°C.

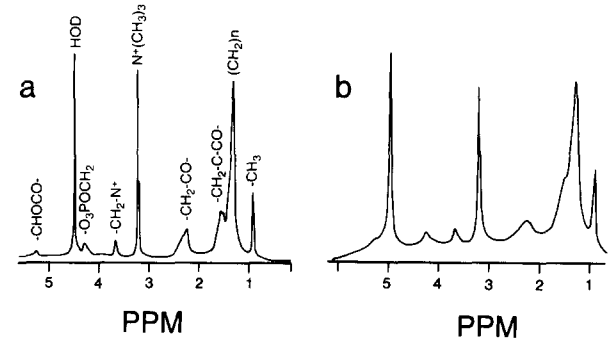

Fig. 2. Proton spectra for $DC_{8,9}PC$ vesicles at 50°C (a) and 2.8°C (b). Note the resolution of the spectra, even at temperatures below 3°C.

Figs. 4(a,c–e), respectively, show the $N^+(CH_3)_3$ resonance at 50°C, 29.4°C, 11.6°C and 2.8°C, while Fig. 4b shows the same resonance at 50°C after the addition of KSCN to the NMR tube. The splitting of the $N^+(CH_3)_3$ resonance at 50°C is increased by the addition of the chaotropic anion, SCN^-. The splitting is 12.1 Hz before and 18.9 Hz after anion addition. The resonance splitting with no anion present also increases as the temperature is lowered; we estimate the splitting to be 12.1 Hz at 50°C, 13.6 Hz at 29.4°C, and ca. 15 Hz at both 11.6°C and 2.8°C. Since there is a large increase in the line widths of the $N^+(CH_3)_3$ resonances as the temperature is lowered accurate measurements become problematic; deconvolution of such broad peaks is difficult and can be rather inaccurate.

Figs. 5(a,c–e), respectively, show the CH_2N^+ resonance at 50°C, 29.4°C, 11.6°C and 2.8°C while Fig. 5b shows the same resonance at 50°C after the addition of KSCN to the NMR tube. As for the $N^+(CH_3)_3$ resonance the presence of SCN^- induces a resonance splitting at 50°C; this splitting is 16.3 Hz. As the temperature is lowered, with no SCN^- present, the original CH_2N^+ resonance is broadened although no splitting is obvious.

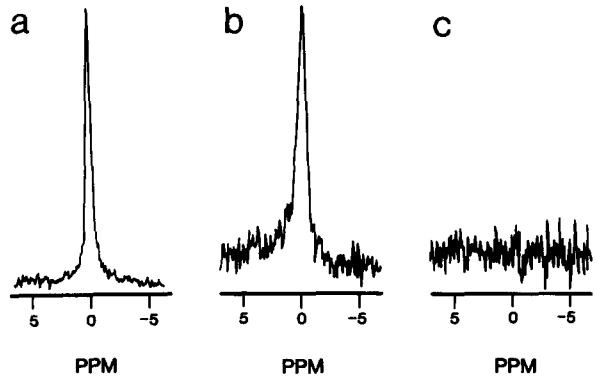

Fig. 3. The ^{31}P resonance at 50°C (a), 29.4°C (b) and the resonance broadening at 0°C (c).

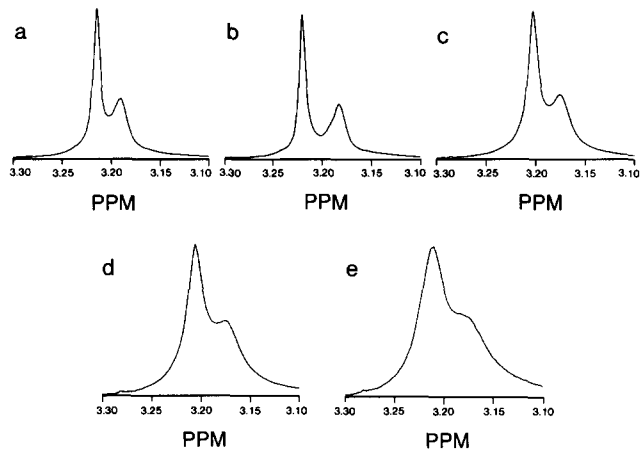

Fig. 4. The $N^+(CH_3)_3$ proton resonance at 50°C (a and b), 29.4°C (c), 11.6°C (d) and 2.8°C (e). In (b) is shown the split $N^+(CH_3)_3$ resonance from the $DC_{8,9}PC$ vesicles in the presence of SCN^- at a concentration of 0.2 M in the NMR tube. Note the broadening of the resonances with the decrease in temperature as well as the change in the resonance splitting.

The resonances from the CH_2-OP, ($\approx$ 3.95 ppm) and the O_3POCH_2 ($\approx$ 4.25 ppm) moieties are shown at 50°C and after the addition of KSCN in Figs. 6(a–e). In Figs. 6(c–e) these resonances are shown at 29.4°C, 11.6°C and 2.8°C; no anion is present at these latter three temperatures. Note the resonance splitting of the O_3POCH_2 resonance, at 50°C, after the addition of SCN^- to the NMR tube. The CH_2OP resonance is not observably affected by the presence of the anion. The O_3POCH_2 resonance, with no anion addition, may be split at 29.4°C; the resonance broadening at lower temperatures, however, precludes the measurement of this splitting as a function of temperature.

At temperatures of 0°C and lower, the sample becomes very milky, the internal light scattering increases greatly and the NMR resonances become so broad that a spectrum is no longer resolvable. This is consistent with the light scattering data (Fig. 1a) which shows a large increase in vesicle size and with the ^{31}P measurements (Fig. 3) which imply a large decrease in headgroup mobility. Upon reheating of the sample, the spectral resonances remained broad and unresolved, consistent with the light scattering data (Fig. 1b).

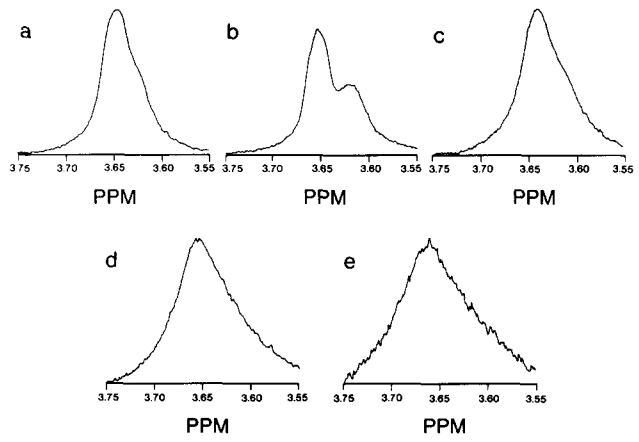

Fig. 5. The $(CH_2)N^+$ proton resonance at 50°C (a), 29.4°C (c), 11.6°C (d) and 2.8°C (e). There is a slight upfield shoulder of this resonance at 50°C but broadening of the resonance with decreasing temperature makes this discernment more difficult as the temperature is decreased. In (b) is shown the split CH_2N^+ resonance from vesicles exposed to 0.2 M SCN^- at 50°C.

4. Discussion

The $DC_{8,9}PC$ shows a well resolved proton spectrum, even at temperatures as low as 2.8°C. The NMR resonances become broader as the temperature decreases, consistent with decreased mobility of the various molecular groups present in the phospholipid. The spectrum is no longer observable at temperatures near 0°C, in accord with the large increase in lipid particle size (Fig. 1) and the ^{31}P resonance change (Fig. 3). This particle growth results from the formation of stacked $DC_{8,9}PC$ lamellae, i.e., bilayer sheets [15].

Another interesting feature of the spectrum shown in Fig. 2 is the resonance at about 5.3 ppm. In the typical spectrum of egg phosphatidylcholine (EPC), this resonance arises from both the overlapping *sn*-2-glycerol methine (CHOCO) and the vinyl (CH = CH) resonances [14]. Since $DC_{8,9}PC$ contains only acetylenic groups (C $\equiv$ C), the resonance shown in Fig. 2 is likely due only to the glycerol backbone CHOCO group. The resonances at $\approx$ 2.1 and $\approx$ 2.8 ppm, seen in EPC spectra and due to the $CH_2C=$ and $=CCH_2C=$ groups, respectively, are also absent in Fig. 2.

The $N^+(CH_3)_3$ resonance behavior, (Fig. 4), has been well-studied in other phosphatidylcholines [14,16,17]. As was found for EPC, this resonance is split with the downfield resonance arising from $N^+(CH_3)_3$ groups in the outer monolayer of the vesicle bilayer and the upfield resonance being due to groups in the inner monolayer of the vesicle bilayer. This splitting at 50°C is significantly increased by the presence of the chaotropic anion, SCN^-. This behavior was also found for other phosphatidylcholines including EPC and is due to the initial interaction of the SCN^- with the $N^+(CH_3)_3$ groups in the outer monolayer of the vesicle; this, in turn, results in a further downfield shift of the downfield $N^+(CH_3)_3$ resonance. We propose that the chemical shift change of the downfield resonance results from an alteration of the structured water [18] near the polar headgroup of the phosphatidylcholines. Water adsorption measurements (work in progress) for $DC_{8,9}PC$ films have been made; the adsorption isotherms indicate strong water binding [18] by the $DC_{8,9}PC$ as was found for other phos-

phatidylcholines (PC). In terms of number of water molecules adsorbed per PC molecule at each water vapor pressure, the isotherm for $DC_{8,9}PC$ lies between those for dioleylphosphatidylcholine and dipalmitoylphosphatidylcholine, in agreement with the neutron diffraction measurements of others [19]. Recent work [20] suggests a relationship between structured water, anion effects and tubule formation. We point out here, however, that the interaction of chaotropic anions with phospholipids is a complicated one: elegant studies, using deuterium-NMR, have been performed by Seelig and co-workers [21] as well as Rydall and McDonald [22]. Wolf [23] has nicely discussed this issue in thermodynamic terms while Tatulian [24] has addressed the issue of anion binding and vesicle surface charge.

Interestingly, Chappell and Yager [9], report a depression of the chain melting transition temperature of $DC_{8,9}PC$ due to the presence of the iodide anion. We have found [25] that I^- behaves in a manner very similar to SCN^- in terms of the EPC $N^+(CH_3)_3$ resonance splitting. I^- is less effective in this splitting action, however, than is SCN^-, consistent with the higher position of SCN^- in the lyotropic (Hofmeister) series [26]. The presence of I_3^-, formed when I^- interacts with the lipid headgroup [27], may, also influence both the NMR and transition temperature results.

The increase in splitting of the EPC $N^+(CH_3)_3$resonance, as a function of vesicle size has been studied by Brouillette and co-workers [28]. They found that for EPC, as the vesicle diameter becomes smaller at a given temperature, the $N^+(CH_3)_3$ NMR resonance splitting becomes larger. This work involved different size groupings of EPC vesicles using column chromatography. The NMR measurements were then made at a fixed temperature (24°C). Our light scattering results show that as the temperature is lowered from 50°C, the $DC_{8,9}PC$ vesicles become smaller and our NMR results indicate a concomitant increase in the $N^+(CH_3)_3$ resonance splitting. Our vesicle size determinations were done dynamically as the temperature was lowered; our NMR results, nevertheless, are in qualitative agreement with those of Ref. [28].

The CH_2N^+ resonance, observed at 3.65 ppm, is a single resonance at 50°C although the resonance does exhibit a small upfield shoulder (Fig. 5). In the presence of the chaotropic anion, SCN^-, this resonance for $DC_{8,9}PC$ is clearly split, behavior similar to that found for EPC [14]. Without the anion present, but as the temperature is decreased, the CH_2N^+ resonance from $DC_{8,9}PC$ broadens but does not split. The increased line broadening as the temperature is lowered, however, may obscure any splitting of the resonance.

In our previous work with EPC [14], we found that the 3.8–4.8 ppm chemical shift region contained the CH_2OP, the O_3POCH_2 and the CH_2CCO resonances. At 50°C, $DC_{8,9}PC$ shows the CH_2OP resonance at

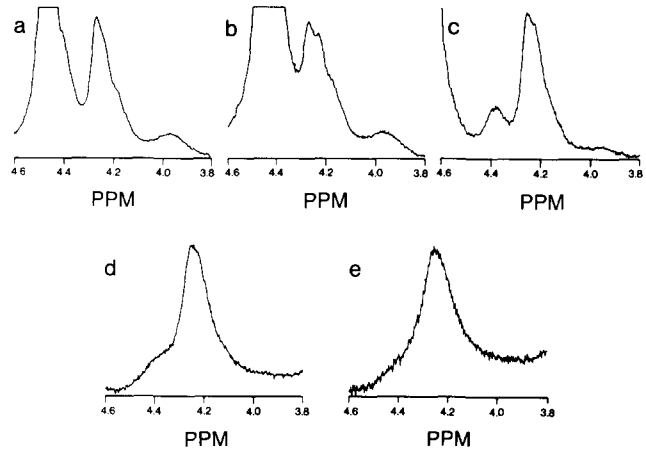

Fig. 6. The proton spectra of the O_3POCH_2 and the CH_2OP moieties at temperatures of 50°C (a), 29.4°C (c), 11.6°C (d) and 2.8°C (e). In (b) is shown the O_3POCH_2 split resonance from vesicles exposed to 0.2 M SCN^- at 50°C.

about 3.95 ppm as well as the O_3POCH_2 resonance near 4.3 ppm. The upfield shoulder ($\approx$ 4.4 ppm) for this resonance is also observable as it is in EPC and we assign this resonance to one of the glycerol methylene CH_2OCO protons since, as was found for EPC, these protons are not equivalent [14]. The other CH_2OCO proton resonates at about 4.5 ppm in EPC. Because of the chemical shift change of the HOD resonance at 50°C, the CH_2CCO resonance for $DC_{8,9}PC$ is masked although it is evident at lower temperatures (Fig. 6c). At 50°C, SCN^- does split the O_3POCH_2 resonance as it does for EPC [14]. With no anion present, as the temperature is lowered all of the resonances in the 3.8–4.8 ppm region broaden with only the O_3POCH_2 resonance still resolved at the lowest temperatures.

In summary, the one-dimensional NMR spectrum of $DC_{8,9}PC$ closely resembles that of EPC and other phosphatidylcholines although the resonances arising from moieties at or near the vinyl group are absent from the $DC_{8,9}PC$ spectrum. Expansion of the headgroup resonance spectra reveal that the headgroup moieties behave as they do in other phosphatidylcholines, with respect to chaotropic anion interactions. The headgroup resonance behavior as the temperature is lowered indicates that although the mobility of the group decreases, there is no severe molecular rearrangement even at temperatures just above that of the transition from vesicles to stacked bilayers. The increase in $N^+(CH_3)_3$ resonance splitting, and the appearance of the remainder of the NMR spectrum as the temperature is lowered toward 0°C indicates that the vesicles are decreasing in size but no dramatic rearrangement of the vesicle lipids is occurring. Solid-state NMR techniques will be necessary in order to study the $DC_{8,9}PC$ in the stacked bilayer and tubule

phases, which result from lowering the temperature below 0°C.

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