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A COMPARISON OF THE HEADGROUP CONFORMATION AND DYNAMICS IN SYNTHETIC ANALOGS OF DIPALMITOYLPHOSPHATIDYLCHOLINE

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^{14}N-NMR spectra and relaxation times for dipalmitoylphosphatidylcholine and three analogs were obtained in both the liquid crystal and gel phases. The analogs either changed the PO_4^- to $N^+(CH_3)_3$ distance (P-N) within the headgroup by increasing the number of CH_2 groups from two in the phosphocholine headgroup (PN-2) to six in the phospho-(*N*′,*N*′,*N*′-trimethyl)hexanolamine headgroup (PN-6), or replaced the ester linkages to the hydrocarbon chains with ether linkages. ^{31}P-NMR spectra were obtained for the four samples in the liquid-crystal phase. (1) The ^{14}N- and ^{31}P-NMR spectra and ^{14}N relaxation times all indicate that increasing the P-N distance within the headgroup causes changes in both the average orientation of the C-N bond and its dynamics. (2) The ^{14}N-NMR spectra provide evidence for a change in orientational order of the headgroup as a result of changing the linkage to the acyl chains. On the other hand, the relaxation time measurements indicate that the molecular motion for the headgroup is independent of the type of linkage. (3) The thermal behaviour of the four samples is clearly reflected in the ^{14}N-NMR spectra. The second moments of the spectra show distinct changes at each of the phase tansitions. (4) The ^{14}N-NMR spectra show that the average conformation of the headgroups is not significantly altered by the main phase transition. For the PN-2 samples, T_{2e}, the decay of the quadrupolar echo, decreases discontinuously in the P_β, phase, which is evidence for a possible exchange process between two molecular states within this phase.

Introduction

Lipid molecules are important structural constituents of biological membranes and their conformational and dynamic properties have been the subject of intense study [1–3] using a wide variety of physical techniques [4,5]. These amphiphilic molecules aggregate spontaneously in an aqueous environment to form ordered, usually lamellar, bilayer structures. It is the lipid bilayer which is the basic structural matrix in which membrane-bound protein molecules function [6]. One outstanding feature of the membrane lipids is the great variety of individual molecules which occur in differing amounts in the composition of natural membranes [7]. Why does this chemical heterogeneity occur in biological membranes? A comprehensive picture of the lipid bilayer will answer this question as well as outline the features common to all lipid molecules that make them important structural components of the membrane.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; PC, phosphatidylcholine; diester PN-2, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; diether PN-2, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; diester PN-6, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(*N*′,*N*′,*N*′-trimethyl)hexanolamine; diether PN-6, 1,2-dihexadecyl-*sn*-glycero-3-phospho-(*N*′,*N*′,*N*′-trimethyl)hexanolamine.

Considerable progress has been made in elucidating the conformation and dynamics of the hydrocarbon chains of membrane lipids [1]. In model systems consisting of a single lipid dispersed in water there occurs a first-order phase transition of the chains from a gel to a liquid-crystalline state which results in a sudden change in the number of conformational states available to each lipid molecule [8,9]. The temperature at which this so-called chain-melting transition occurs depends in a systematic way on the chain length and type of bonding within the chain. It is also known that modifications of the polar headgroup cause changes in the cooperative properties of lipid bilayers [10]. For example, the gel to liquid-crystal phase transition occurs at a significantly higher temperature for DPPE than DPPC [11]. The role of the polar headgroups is of particular importance because ion-binding to the headgroup or pH-induced charge alterations can cause changes both in the long-range and short-range order in the lipid matrix [12–14]. In order to understand the influence the headgroup has on the properties of the lipid bilayer, a knowledge of the orientation and flexibility of the polar groups is essential. There are several recent articles [15–18] reviewing the present state of knowledge of the headgroup conformation and dynamics. Another segment of the lipid molecule which can influence the physical properties of the bilayer is the linkage between the glycerol backbone and the hydrocarbon chains. Recent results for sphingomyelin suggest that the amide linkage modifies the average conformation of these molecules when compared to lipids such as DPPC which have ester linkages to both chains [19]. The phase behavior of lipid molecules with a variety of different linkages is reviewed by Boggs [20] with particular reference to hydrogen bonding.

NMR has made a very significant contribution to our knowledge of the lipid conformation and dynamics. All segments of the lipid molecules can be probed in a nonperturbing way using the NMR technique. Deuterons selectively incorporated into phospholipids have been used to investigate not only the acyl chains, but also the glycerol backbone and headgroup regions [21,22]. Further studies of the headgroup have used natural-abundance ^{31}P, ^{14}N and ^{1}H resonances [18,23–26]. Recently, ^{13}C has been incorporated in the region of the ester linkage in DPPC and DPPE [27,28].

In this study, we report modifications in the dynamic structure of the headgroup region in several synthetic analogs of DPPC. The analogs alter the separation between the phosphate and trimethylammonium group and the interface to the hydrocarbon region by substituting alkyl ether linkages for corresponding diester linkages. For convenience, the samples are labelled either diester or diether to denote the linkage and either PN-2 or PN-6 to distinguish the two headgroups. Nitrogen NMR spectroscopy is the primary investigative probe used to monitor changes in the headgroup region. Some ^{31}P-NMR results, however, will also be presented.

Materials and Methods

Both forms of the PN-2 samples were purchased from commercial sources (Sigma Chemical Co., St. Louis, MO in the case of diester PN-2 and Calbiochem, La Jolla, CA in the case of diether PN-2) and were used without further purification. Both diester and diether PN-6 were synthesized as previously described [29]. Phospholipid purity was checked by TLC on silica-gel microslide plates developed in a chloroform/methanol/water (65 : 25 : 4, v/v) solvent system, and all lipids tested yielded a single spot at the expected position.

Multilayer dispersions of lipid in excess water (at least 40% of the total weight, lipid plus water) were prepared under argon at room temperature by the addition of distilled deionized water to approx. 300 mg dry lipid in a 10 mm outer diameter glass ampule. The ampule was then sealed under 0.5 atm. N_2. In some cases, the resulting dispersion was heated to approx. 15°C above the phase transition for approx. 1 h, and then vortexed to ensure adequate mixing.

The ^{14}N-NMR spectra were obtained at a frequency of 19.438 MHz using a home-built spectrometer [30] and a Brüker superconducting solenoid (6.3 tesla), as previously described [19,24]. The ^{31}P-NMR spectra were acquired on a Brüker WH-400 spectrometer at 162.1 MHz.

The modified quadrupole echo sequence [19,31] was used to obtain the ^{14}N-NMR spectra. Typically, radio-frequency pulse-widths of 6 μs with a

separation between the two pulses of 120 μs were used. Because of the relatively poor signal-to-noise ratio, 64–132K scans were averaged for each spectrum. T_1 was measured by observing the recovery of the magnetization following the application of a single 180° pulse. The quadrupole echo sequence was applied at various intervals following the initial pulse. The spectra obtained by Fourier transforming the echo signal were integrated under the peaks in the quadrupolar spectra. All T_1 relaxation time measurements were carried out in the liquid-crystal phase for each sample where there are distinct powder pattern spectra, with well-defined peaks. The recovery of the equilibrium magnetization was found to be well described by an exponential relationship. The T_1 value was obtained by a least-squares fit to the amplitude data. The decay of the quadrupolar echo, T_{2e}, was measured by acquiring spectra as a function of the pulse separation in the quadrupolar echo sequence. The integrated area of the entire spectrum was used as a measure of the amplitude of the signal. The amplitudes were found to decay exponentially with pulse separation. The T_{2e} values were obtained by a least-squares fit to the amplitude results.

The temperature of the sample was electronically controlled to within 0.1°C over the course of measurement. The sample was allowed at least 1 h to reach equilibrium before data acquisition. The actual temperature was measured with a copper-constantan thermocouple referenced to a triple point cell. The absolute temperature of the sample was known to at least 1°C.

Results

^{14}N-NMR spectra

The spectra shown in Fig. 1 for DPPC and the three analogs have the same qualitative temperature behavior. Above the gel to liquid-crystal phase transition temperature, T_c, the line shapes are sharp, well-defined powder patterns [18] characteristic of the liquid-crystal phase. For comparison, Table I shows the phase transition temperatures for all four compounds as determined by previous workers using calorimetric techniques. The observed lineshapes arise in unoriented samples from the nuclear Zeeman interaction perturbed in first order by an axially symmetric nuclear quadrupole

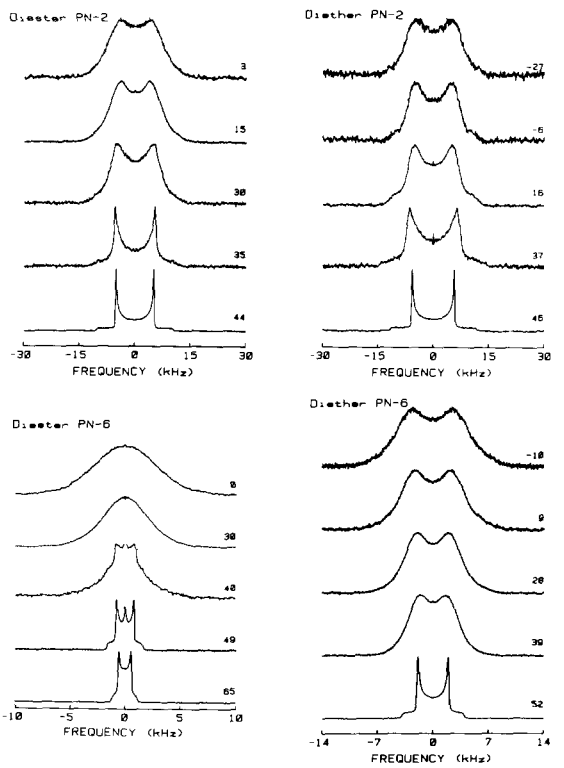

Fig. 1. Temperature dependence of the ^{14}N-NMR spectra from unsonicated dispersions of DPPC and each of the three analogs. The spectra were obtained using a modified quadrupole echo sequence with a repetition rate of 0.17 s. Typically, either 64K or 132K scans were averaged to obtain these spectra. For the lipids with a PN-2 headgroup, typical pulse spacings τ were 180 μs, while for the PN-6 lipids, τ values varied between 250 and 350 μs. In the case of the PN-6 lipids, T_{2e} is of the order of several milliseconds, and the spectral lineshapes are completely insensitive to τ values of the order of several hundred microseconds. For the PN-2 lipids the spectra have a 200 Hz line-broadening due to exponential multiplication of the echo signal prior to Fourier transformation. For the diether PN-6 spectra, the spectra have a 100 Hz line-broadening due to exponential multiplication and for the diester PN-6 spectra in the liquid-crystal phase the spectra have a 40 Hz line-broadening. In the gel phase, line-broadenings are the same as for diether PN-6.

interaction. The nuclear quadrupole moment of the ^{14}N nucleus ($I = 1$) interacts with the time-averaged electric field gradient at the nuclear site arising from the surrounding distribution of electronic and nuclear charges. The time average is over times which are long compared to the reciprocal of the static quadrupole frequencies (approx. 10^{-5} s). Below T_c there is a definite broadening of

TABLE I

PHASE TRANSITION TEMPERATURES

References: (a) Ref. 57; (b) Ref. 70; (c) Refs. 32, 33; (d) this paper.

	Main transition T_c (°C)	Pretransition T'_c (°C)
Diester PN-2	41	35 (a)
Diether PN-2	43	33 (b)
Diester PN-6	43 (c) 43 (d)	none
Diether PN-6	45 (d)	none

the sharp peaks in the liquid-crystal spectra. The broadening increases with decreasing temperature until the spectra are no longer observable. It should be noted that the quadrupolar splitting, the separation between the peaks in the spectra, shows little change on crossing the phase boundary. Careful inspection of the spectra for both the diester and diether PN-2 samples show that in the region between the main transition and the so-called pretransition, T'_c, (the $P_{\beta'}$ phase) the spectra are distinctly different from those in either the liquid-crystal or low-temperature phase. The differences are seen in the broadening of the spectra, not in the changes in quadrupole splitting. Both of the PN-6 samples show an abrupt change in broadening at the phase transition. There is no pretransition for these samples [32,33]. The diether PN-6 sample is unique among the four samples. At T_c the well-defined spectrum characteristic of the liquid-crystal phase disappears and a broad featureless line appears. No quadrupole splitting is apparent in the low-temperature phase.

For all of the samples there is a temperature below which it is no longer possible to obtain a spectrum. In the PN-2 samples, this happens in the ether-linked lipid at a temperature which is at least 15–20°C below the corresponding temperature in the ester-linked lipid. The reason for the disappearance of the spectra is a combination of the severe broadening of the spectra and a very short decay of the quadrupolar echo.

The quadrupolar splittings in the liquid-crystal phase

The quadrupolar splittings, $\Delta\nu_Q$, for spectra in the liquid-crystal phase were determined by measuring the separation between the two points located on the outside of the two 90° peaks at about 85% of the maximum peak heights. A comparison of the results for the four analogs is shown in Fig. 2. Although the calorimetrically determined values of T_c for the diether lipids are slightly (2–4°C) higher than for the corresponding diester, the quadrupolar splittings at the same reduced temperature, $\theta = (T - T_c)/T_c$, are such that $\Delta\nu_Q(\text{ether}) > \Delta\nu_Q(\text{ester})$. Fig. 2 also shows that when the separation between the phosphate and trimethylammonium groups is increased, there is a significant decrease in the quadrupolar splitting, regardless of the type of linkage: $\Delta\nu_Q$ (PN-2) > $\Delta\nu_Q$ (PN-6).

^{31}P-NMR spectra in the liquid-crystal phase

Fig. 3 displays ^{31}P-NMR spectra for all four samples at the same temperature, 50°C. The powder pattern, which is a result of the anisotropic chemical shift interaction, is characteristic of phospholipids in a lamellar liquid-crystal phase. The chemical shift anisotropy is (within experimental error) the same for lipids with the same headgroup.

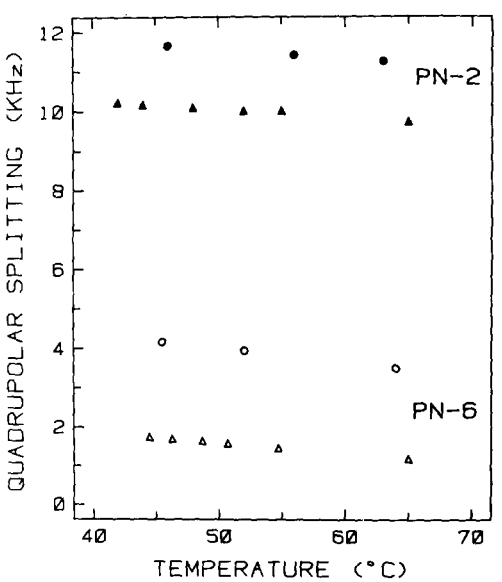

Fig. 2. A comparison of the quadrupolar splittings vs. temperature for each of the headgroups. Filled symbols are reserved for the PN-2 headgroup, while open symbols correspond to the PN-6 headgroup. Triangles denote the ester linkage, while circles denote the ether linkage. For lipids with a PN-2 headgroup, the splittings are known to ±0.1 kHz, while for the PN-6 lipids, they are known to ±0.05 kHz.

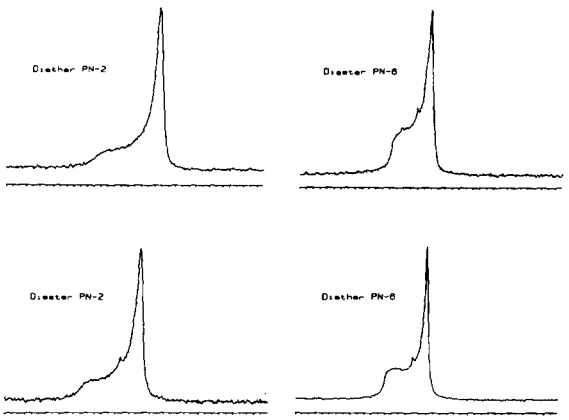

Fig. 3. A comparison of the ^{31}P-NMR spectra of DPPC and each of the three analogs at 50°C. The same samples were used for both the ^{14}N- and ^{31}P-NMR experiments. The ^{31}P spectra were recorded at 162 MHz using a Bruker WH-400 spectrometer. Between 100 and 5000 free-induction decays were averaged to obtain the spectra shown. Protons were decoupled using a broad-band decoupling power of approx. 5 W. All scales have a total width of 190 ppm.

It has an absolute magnitude of approx. 50 ppm for the PN-2 headgroup and a significantly different value, approx. 32 ppm, for the PN-6 headgroup. The fact that the values of the chemical shift anisotropy are the same for lipids with the same headgroup, irrespective of the linkage at the glycerol backbone, confirms earlier observations of Hauser [26] who noted that the ^{31}P-NMR spectra for diester PN-2 and diether PN-2 were superimposable. The measured absolute magnitude of the chemical shift anisotropy quoted by Hauser was 49 ± 2 ppm at 50°C.

The second moments of the spectra

The broadening below T_c is an important feature of the spectra shown in Fig. 1. A convenient parameter used to give a quantitative description of the width of the spectral lineshape, $F(\omega)$, is the second moment, M_2, [34] defined by the equation

$$M_2 = \int_0^\infty \omega^2 F(\omega)\, d\omega / \int_0^\infty F(\omega)\, d\omega.$$

Fig. 4 compares the calculated values of M_2 for all four samples. In Fig. 4a the diether PN-2 and diester PN-2 are compared, while Fig. 4b shows the results for diether PN-6 and diester PN-6. All

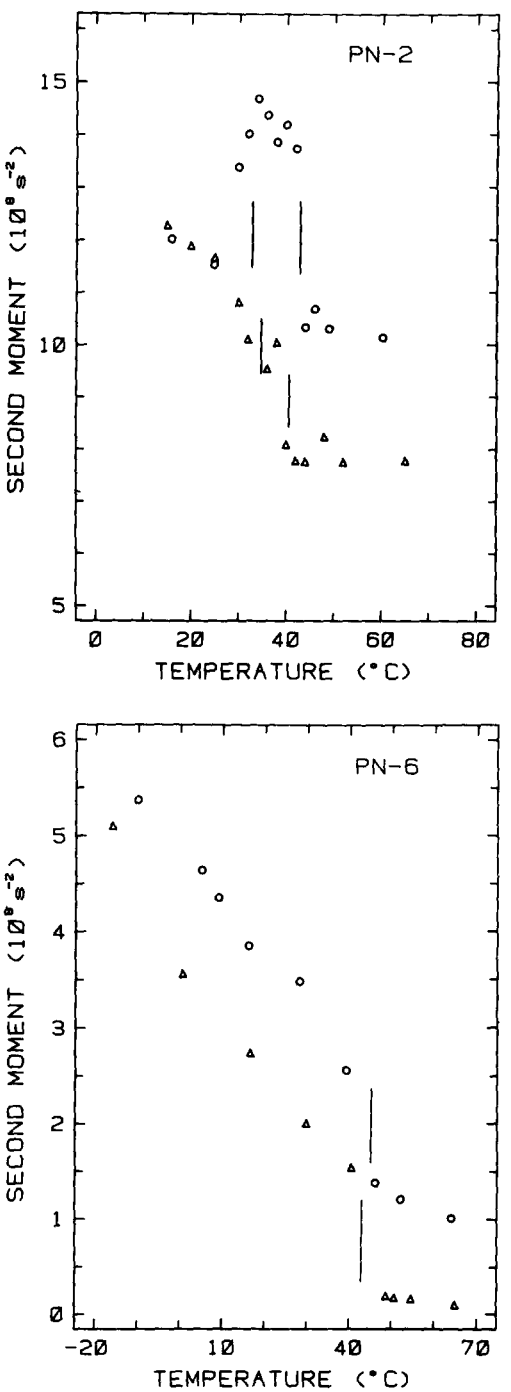

Fig. 4. A comparison of the second moment vs temperature for lipids containing either the PN-2 or the PN-6 headgroup. As in Fig. 2, circles denote the ether linkage, while triangles denote the ester linkage. The phase transition temperatures (and the pretransition temperatures in the case of lipids with a PN-2 headgroup) are indicated by the vertical lines.

four sets of results show a discontinuity at the corresponding gel to liquid-crystal phase transition. At all temperatures, the PN-2 samples have larger M_2 values because the $\Delta\nu_Q$ values are larger. For the PN-6 samples (Fig. 4b) there is a steady increase in M_2 as the temperature decreases below T_c. The diester PN-2 sample also shows a monotonic increase in M_2 below T_c, but the diether PN-2 sample has a maximum in the region between T_c and T_c'. The larger value of M_2 in the $P_{\beta'}$ phase for the diether PN-2 sample is not due to an increased broadening but rather to an increase in the quadrupolar splitting in this region.

Relaxation times

The spin-lattice relaxation times measured in the liquid-crystal phase for all four lipids are shown in Fig. 5. While the T_1 data are similar for headgroups of the same type, independent of the linkage to the glycerol backbone, the relaxation times are significantly shorter for the PN-6 headgroups. Within experimental error, the data for each lipid can be fit by a straight line on an Arrhenius plot. The activation energies derived from these fits are 31 kJ · mol^{-1} for either of the PN-2 headgroups, 16 kJ · mol^{-1} for diester PN-6 and 11 kJ · mol^{-1} for diether PN-6.

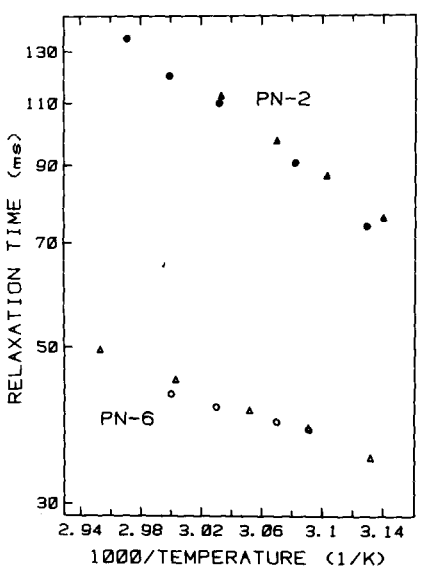

Fig. 5. A comparison of the spin-lattice relaxation times in DPPC and each of the three analogs. As in Fig. 2, filled symbols are reserved for lipids with a PN-2 headgroup, triangles denote ester linkages and circles denote ether linkages. The error in T_1 is estimated to be less than ±5%.

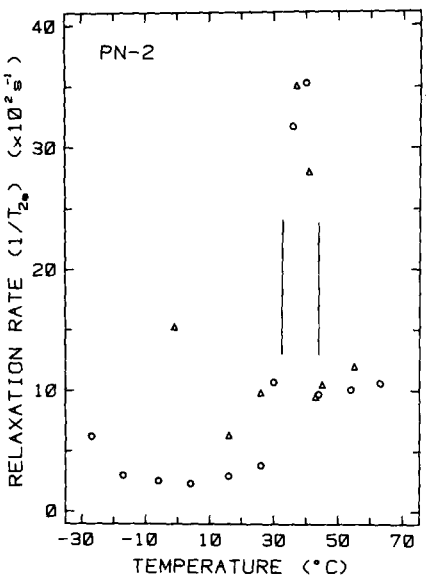

Fig. 6. The relaxation rate determined from the inverse of the time constant, T_{2e}, characterizing the decay of the quadrupolar echo. The spectral intensity $I(\tau)$ was measured as a function of pulse spacing τ, and a least-squares fit of the data using the relation $I(\tau) = A \exp(-2\tau/T_{2e})$ was used to determine T_{2e}. The standard error in T_{2e} determined by this method is less than ±3% in the L_α or $L_{\beta'}$ phase, and less than ±10% in the $P_{\beta'}$ phase.

The decay of the quadrupolar echo, T_{2e}, was measured for both of the PN-2 compounds because it was noticed that T_{2e} became very short in the $P_{\beta'}$ phase. This behavior is shown in Fig. 6. Below the pretransition, T_{2e} becomes even longer than in the liquid-crystal phase. At the lowest temperatures measured, T_{2e} again becomes short as the spectra broaden and ultimately become impossible to obtain. It should be pointed out that it was possible to obtain spectra for the ether-linked samples at 20°C lower than the corresponding ester-linked sample.

Discussion

In general, the headgroups of phospholipid molecules are exceedingly flexible. There are many different molecular conformations energetically possible in both the liquid-crystal and gel phases [35]. Ideally, each possible conformation of the

headgroup should be identified and the probability of finding each one determined. With the information presently available it is not possible to determine uniquely the average headgroup conformation [36]. It is therefore, essential to establish the limitations in the interpretation of the experimental measurements. In this next section a brief outline is given of the meaning of the measured NMR parameters.

Experimental data may often be interpreted in terms of the order parameters [21], for a rigid segment of the lipid molecule. If the axes X, Y, Z are fixed in that segment, the order parameters for that segment are

$$S_{ij} = \frac{1}{2}\overline{(3\cos\theta_i\cos\theta_j - \delta_{ij})} \quad (ij = X,Y,Z) \tag{1}$$

where the θ_i are angles between the X, Y, Z axes and the bilayer normal. The NMR frequency separation between the two peaks in the quadrupolar powder pattern observed for a spin 1 nucleus is given by

$$\Delta\nu_Q = (3e^2qQ/4h)\left\{S_{zz} + \frac{1}{2}\eta(S_{xx} - S_{yy})\right\} \tag{2}$$

where e^2qQ/h is quadrupole coupling constant, Q is the quadrupole moment of the nucleus and q is the field gradient parameter. In terms of the components of the electric field tensor, $V_{ij}(i, j = x, y, z)$,

$$eq = V_{zz}$$

and

$$\eta = (V_{xx} - V_{yy})/V_{zz}$$

For the particular case of ^{14}N in the choline headgroup, the major contribution to the electric field gradient comes from the electronic structure in the C_β-N-$(CH_3)_3$ moiety which has C_{3v} symmetry. The asymmetry parameter, η, is expected to be small and the axis of symmetry of the electric field gradient is along the C—N bond. As a result,

$$\Delta\nu_Q = (3e^2qQ/4h)S_{C-N}$$

It is reasonable to assume that e^2qQ/h is the same for each of the four lipid molecules studied. It is unfortunate, but at the present time the value of e^2qQ/h for the C_β-N-$(CH_3)_3$ group is not firmly established [24]. The best estimate of $e^2q\,Q/h$ at the moment is 135 kHz, determined from relaxation time studies.

The chemical shift anisotropy determined from the separation between the peak and shoulder of ^{31}P-NMR spectra can be expressed as

$$\Delta\sigma = S_{xx}(\sigma_{xx} - \sigma_{yy}) + S_{zz}(\sigma_{zz} - \sigma_{yy}) \tag{3}$$

The components of the chemical shift tensor, σ_{ij}, have been determined from low-temperature studies of fully hydrated DPPC and DPPE [37]. The X axis is roughly in the plane of the nonesterified oxygens and bisects the angle between them, while the Z axis is perpendicular to this plane and is nearly parallel to the direction of a vector joining the two esterified oxygens. The Y axis completes the right-handed orthogonal system.

It should be pointed out that the order parameters not only depend on the average orientation of the molecule fixed coordinate system but also on the amplitude of any fluctuations in the orientation. It is also important to recognize that Eqns. 2 and 3 may only be employed when the correlation time describing the molecular motion, τ_c, is short compared to the reciprocal of the quadrupolar splitting $(1/\Delta\nu_Q)$ or chemical shift anisotropy $(1/\Delta\sigma)$. When $\tau_c \approx 1/\Delta\nu_Q$ or $1/\Delta\sigma$ (the intermediate regime) spectra occur which do not exhibit distinct peaks and shoulders [38,39]. This intermediate regime is characterized by rather amorphous line shapes which depend dramatically on the spacing between the pulses in the quadrupole echo sequence. The best procedure for interpreting the data from this regime is to simulate the spectra starting from a particular model for the molecular motion and varying the rate of motion until a satisfactory fit is obtained [28,40].

In the absence of molecular motion the decay of the quadrupolar echo, T_{2e} is determined by the dipole-dipole interaction between neighboring nuclear spins. In the case of ^{14}N, the dominant interaction will be with surrounding protons. However, Woessner [41,42] pointed out many years ago that molecular motion in the intermediate regime results in a marked decrease in T_{2e} (called T_3 by Woessner). In fact, there is a minimum in

T_{2e} when $\tau_c \approx 1/\Delta\nu_Q$. It is interesting to note that standard techniques [43] where the fluctuating part of the quadrupole interaction is taken as perturbation on the time-averaged part are not valid in this region of correlation times. The entire quadrupolar Hamiltonian must be taken as being time-dependent and the usual solution involves setting up a number of equations describing the exchange of the molecule from one orientation to another [38].

The spin-lattice relaxation time describes the transfer of energy from the nuclear spin system to the surrounding environment which is a result of the fluctuating part of the nuclear quadrupole interaction made time-dependent by the molecular motion. In general, the relaxation time, T_1, due to the quadrupolar interaction is given by

$$(1/T_1) = (3/40)(e^2qQ/h)^2[J_1(\omega_0) + 4J_2(2\omega_0)] \quad (4)$$

where ω_0 is the Larmor precessional frequency and the spectral density functions

$$J_m(\omega) = [(-1)^m/4\pi]\int_0^\infty \overline{Y_{2m}(t)Y_{2-m}(t+\tau)}\exp(i\omega\tau)\,d\tau \quad (5)$$

The spherical harmonics $Y_{2m}(t)$ describe the time dependence of the C_β—N bond orientation in the case of ^{14}N relaxation in the choline headgroup. In general, relaxation can be dominated by one of many possible molecular reorientation mechanisms such as axial diffusion of the molecule about the long axis, wobble of this long axis about the bilayer normal, *trans-gauche* isomerization or translational diffusion if there is sufficient curvature of the membrane surface [44]. A very simplified approach is to assume that the motion of the C_β—N vector can be described by a single correlation time, τ_c, and in the region where $\tau_c\omega_0 \ll 1$ then [45,46]:

$$1/T_1 = (3/8)(e^2qQ/h)^2(1 - S_{C-N}^2)\tau_c \quad (6)$$

If the molecular motion is rate activated, then

$$\tau_c = \tau_0 \exp(E_a/kT) \quad (7)$$

where E_a is the acvtivation energy and k Boltzmann's constant. T_1 should increase with increasing temperature.

Comparison of the liquid-crystal results

I. PN-2 and PN-6 headgroups

For all four samples, the spectra in the liquid-crystal phase are well-defined powder patterns, indicating that the dominant components in the fluctuating part of the quadrupole interaction are described by correlation times $\tau_c \ll 1/2\pi\Delta\nu_Q$. Furthermore, T_1 increases with increasing temperature, indicating that the molecular motion(s) responsible for the spin-lattice relation must be such that $\tau_c \ll 1/\omega_0$ where ω_0 is the Larmor processional frequency. Eqn. 2 is valid, therefore, and the order parameter S_{C-N} is directly proportional to the observed $\Delta\nu_Q$. The question arises as to whether the large difference in $\Delta\nu_Q$ observed for the two PN-6 samples as compared to the PN-2 samples is a result of a different value of e^2qQ in the two cases. In general, e^2qQ is the result of the electronic structure in the immediate environment of the quadrupolar nucleus [47]. In both cases the nitrogen atom is surrounded by four carbon atoms in a nearly tetrahedral environment. In fact, the first change in the atomic structure does not occur until the third atom away from the nitrogen. There is little likelihood of significant differences in e^2qQ in the two different headgroups.

Consider first the differences between samples having the same linkage to the glycerol backbone but different headgroups. The order parameter, S_{C-N}, is very much smaller for the PN-6 group. An obvious reason for the difference is that the additional four methylene groups in the PN-6 moiety give rise to a more flexible headgroup resulting in a greater averaging of the $(3\cos\theta_{C\text{-}N} - 1)/2$ factor. This view is supported by the fact that the activation energy for the molecular motion derived from the T_1 results is significantly lower for the PN-6 groups as compared to that for the PN-2 groups. The values of T_1 at the same temperature, however, are smaller for the PN-6 samples, suggesting that the molecular motion in the PN-6 samples is described by a longer correlation time. The longer headgroup may be more flexible, but at the same time some of the molecular motion occurs on a slower time scale. While these results suggest that the longer headgroup maybe more flexible in the region of the nitrogen nucleus, the reduction in S_{C-N} for the PN-6 groups may also

be a direct result of a change in the average orientation of the headgroup. The ^{31}P results, which show that the chemical shift anisotropy is significantly smaller for the PN-6 groups, indicate that the whole headgroup is influenced by the increase in the number of CH_2 groups. The nitrogen S_{C-N} and T_1 results are, therefore, not just a result of the larger number of carbon-carbon bonds increasing the number of possible orientations of the C_β-N vector in the PN-6 samples as compared to the PN-2. The average orientation of the larger headgroup has probably also been changed.

To the present time, there has only been a small number of physical measurements on phospholipids with varying numbers of methylenes within the headgroup. In a recent study, Browning [48] has synthesized a number of phospholipids with simple alkyl headgroups having 0–3 methylenes. The NMR results on these compounds are strikingly similar to the results in this paper. The quadrupolar splittings for the C^2H_3 group and the chemical shift anisotropy for the ^{31}P resonance both decrease with increasing numbers of methylenes in the headgroup.

The phase transition temperatures of DPPC and the analogs [32] having 3–11 CH_2 groups show only a small variation (less than 4°C). Again, the phospholipids with simple alkyl headgroups [48] also show little variation in the phase transition temperature. Dielectric relaxation measurements [49] in the case of the dipalmitoylphosphatidylcholine analogs and ^2H-NMR results in the case of the simple alkyl groups [48] have been interpreted to infer a change in the average orientation of the headgroup with the addition of CH_2 groups. These analyses imply that, at least in the case where hydrogen bonding is not a factor, the size of the headgroup has little influence on the packing within the bilayer, little change in packing within the bilayer being reflected in similar gel to liquid-crystal transition temperatures. Oleate chains, for instance, disrupt the packing within the bilayer, causing the phase transition to decrease substantially. On the other hand, these results suggest that because the packing is determined by other factors, the various headgroups must conform to the available area at the bilayer surface. The smaller headgroup would be able to reorient within the plane of the membrane surface freely, while steric repulsion would become a factor for those containing many CH_2 groups.

II. Ester and ether linkages

The increase observed in the ^{14}N quadrupole splitting of the ether-linked lipids as compared to their ester-linked counterparts means that the S_{C-N} increases as a result of changing the bonding of the acyl chains to the glycerol moiety. The NMR results are unable to tell whether the observed differences are a result of changes in the average conformation or changes in the amplitude of the excursions of the C_β-N bond vector about its average orientation. The ^{31}P chemical shift anisotropy, however, is the same for lipids with identical headgroups. Hauser [26] earlier showed that the ^{31}P results for diester and diether PN-2 are the same, and from these and high resolution ^1H-NMR data on monomers and small micelles he concluded that the motionally averaged conformation of the headgroup was the same for both ether and ester linkages. The ^{14}N spin lattice relaxation time measurements shown in Fig. 5 are virtually identical for each pair of compounds with the same headgroup. The rate of the molecular motion for the headgroups would, therefore, seem to be independent of the type of linkage of the chains. The question which now must be addressed is, why are the ^{14}N quadrupolar splittings the only NMR parameters that show a significant dependence on the linkage to the glycerol moiety? The most probable answer is that the diether linkage causes only a small change in the packing of the hydrocarbon chains which in turn causes only a small alteration in the average orientation of the headgroup. A further result is that the molecular motion about the average orientation is similar for the two linkages. The ^{31}P results suggest that whatever conformational changes do take place, the major effect is near the quaternary nitrogen portion rather than near the phosphorus atom.

There is evidence from a wide variety of experimental techniques [50–55] to support the hypothesis that diether lipids do pack differently in the bilayer, and in most cases, this evidence would suggest that in both micelles and bilayers, diether-PC lipids are more tightly packed than their diester counterparts. It is now generally accepted that the headgroup is oriented in the plane of the

bilayer. In the liquid crystal phase there is free rotational diffusion of the lipid molecules about the bilayer normal. While there is room for each PN-2 headgroup to be in the plane of the bilayer [56], there is insufficient area per polar headgroup to allow free rotation in the plane without some steric hindrance. If the molecules are more closely packed within the bilayer, there will be more interaction between the headgroups. The steric repulsion may be manifested by an average headgroup orientation which is tilted out of the plane of the bilayer. The resulting time-average conformation for the more densely packed ether-linked molecules will be different from that of the ester-linked lipids.

Notice that although the presence of diether linkages increases the quadrupolar splitting by the same absolute amount in both the PN-2 and PN-6 headgroups (approx. 2 kHz) the relative increase of 100% in the PN-6 headgroup splitting is much larger than the 20% increase in the PN-2 headgroup. The presence of a larger headgroup appears to amplify the effects of a change in molecular packing on the dynamical structure of the headgroup. It is the PN-6 lipid which provides the conclusive proof that diether linkages do alter the dynamical structure of the headgroup.

The gel phase region

In comparison to DPPC, far less work has been carried out on the thermal behavior of the other three lipids used in this project. The known transitions are listed in Table I. Because there is a pretransition for the ether-linked PN-2 sample, it is reasonable to assume that the phase behavior of this sample is similar to that of ester-linked DPPC, the phase behavior of which has been well characterized by differential scanning calorimetry [57–60] and X-ray diffraction [61,62]. The PN-6 samples show no pretransition, but there is a well-defined gel to liquid-crystal transition.

The observed ^{14}N spectra change at each of the phase boundaries in aqueous dispersions of the four samples. There are not, however, large changes in the quadrupolar splittings but rather just a broadening of the spectra. At the main transition in each of the samples there is a marked change in the second moment of the spectra as shown in Fig. 4. Because the quadrupolar splittings do not change substantially, it is reasonable to suggest that the average conformation of the headgroup does not change. The various possible molecular motions must also continue through the phase transition, but the broadening is probably due to a slowing down of the rate of the molecular motion. Are these results consistent with other NMR results from the headgroup region? The chemical shift anisotropy in the ^{31}P spectra [63] of DPPC show no abrupt change at the main transition. There is only a gradual increase as the temperature decreases from 45 to 27°C. While the spectra [64] for the choline headgroup in DPPC selectively deuterated at the α, β and γ carbons show a discontinuity at the main transition, it is small in comparison to the change which occurs for deuterons on the acyl chains.

In a recent publication, Wittebort et al. [27] suggested on the basis of ^{13}C-NMR results that the $P_{\beta'}$ phase exhibits microscopic properties characteristic of the lower temperature $L_{\beta'}$ and higher temperature L_α phases. There coexist microscopic domains of molecules having gel and liquid-crystalline properties. The exchange between different domains results in an anomalously short T_2 for the ^{13}C nuclei. The ^{14}N spectra in the $P_{\beta'}$ phase do not appear to be a superposition of the spectra from the higher and lower phases. Because the quadrupolar splitting remains virtually unchanged throughout the whole temperature region, the ^{14}N spectra are not a good test to determine whether the $P_{\beta'}$ spectra are a superposition. However, T_{2e} is anomalously short (Fig. 6) in this region for both the ether- and ester-linked PN-2 samples. The PN-6 samples, which do not have a pretransition, do not show this effect. The anomalously short T_{2e} in the $P_{\beta'}$ phase could be a result of an exchange process in which molecules are moving from one molecular state to another. When the exchange rate $\tau \approx 1/\Delta\nu_Q$, short T_{2e} values result [41,42]. The coexistence of domains of liquid-crystalline and gel could be a possible explanation.

Gally et al. [64] were unable to detect the influence of the pretransition in DPPC from ^{31}P- or ^2H-NMR in the headgroup region. Füldner [65] has repeated the ^2H-NMR results on a $N(C^2H_3)_3$-DPPC sample and shown that there is only a slight broadening of the peaks at the pretransition. The change in the ^{14}N-NMR spectra

for the ether- and ester-linked PN-2 samples is also very subtle. Only a careful study such as that shown in Fig. 7 for the ether-linked PN-2 sample reveals that the shape of the peaks in the spectrum change in the region of 32°C. Because of the small effect on the headgroup spectra, Seelig [21] has suggested that the pretransition is associated with changes within the acyl chains and does not involve a conformational change of the choline moiety. It is now recognized that on the basis of

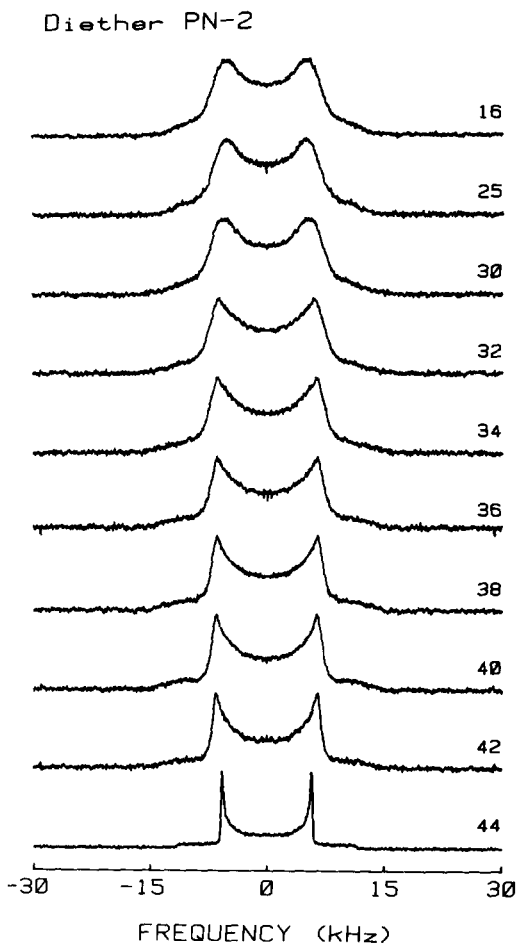

Fig. 7. Temperature dependence of the ^{14}N-NMR spectra for the diether analog of DPPC, diether PN-2. The repetition rate was 0.17 s, and 65K scans were signal-averaged for all spectra, except at 44°C, where 33K scans were averaged. The digitization rate was 10 μs, corresponding to a spectral width of 100 kHz, and the spectra have 200 Hz of line-broadening due to exponential multiplication. At 44°C, the pulse spacing, τ, was 180 μs, and for all of the gel phase spectra, τ was 140 μs.

X-ray diffraction [62], Raman [66,67] and infrared [68] spectroscopic studies that there is a change in the packing of the lipid molecules within the bilayer from hexagonal to orthorhombic. The rate of rotational diffusion about the long axis of the molecule also decreases at the pretransition [68,69]. Both of these factors could give rise to the observed broadening of the peaks in the ^{14}N and ^{2}H spectra. Below the pretransition, the rotational diffusion of the molecules may no longer have axial symmetry if the packing is orthorhombic. In this case there could be an axially asymmetric powder spectrum. While the observed spectra are not obviously asymmetric there could be a small asymmetry hidden within the broadened peaks.

In all the ^{14}N and ^{2}H spectra recorded from the headgroup to date there is severe broadening in the region below the pretransition and a disappearance of the NMR signal below 0°C. In DPPC, Davis [34] has shown that the axial rotation continues to slow down and stops on an NMR timescale at about -7°C. In this same temperature range, ^{31}P and ^{13}C [23,27] spectra are no longer axially symmetric, again showing that axial rotation has ceased. The disappearance of the ^{14}N spectra in all four samples must be a result of the slowing down of the axial rotation. As Speiss and Sillescu [38] have pointed out, the loss of intensity in the spectrum and the shortest T_{2e} occur when $\tau_c \approx 1/\Delta\nu_Q$. The slowing down of the molecular motion occurs at a lower temperature in the ether-linked lipids, since the disappearance of the NMR spectra occurs about 20°C lower for these as compared to the ester-linked lipids.

Conclusions

It has been shown that ^{14}N-NMR is a very sensitive probe of the choline headgroup conformation and dynamics. The ^{14}N- and ^{31}P-NMR results indicate that increasing the size of the headgroup with the addition of methylene groups results in changes in both the average orientation and dynamics of the headgroup. Altering the linkage to the glycerol moiety, however, only causes a change in the static orientation of the headgroup, possibly as a result of slightly tighter packing in the ether-linked lipids.

The thermal behavior of the four samples is

reflected in the ^{14}N spectra and relaxation times. The spectra show that the average conformation of the headgroup is not altered by the transition from the liquid-crystal to gel phase. Below T_c there is a progressive slowing down in the axial diffusion of the molecules which finally results in a disappearance of the spectra when $\tau_c \approx 1/\Delta\nu_Q$.

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