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FACTORS AFFECTING THE MOTION OF THE POLAR HEADGROUP IN PHOSPHOLIPID BILAYERS

A ^{31}P NMR STUDY OF UNSONICATED PHOSPHATIDYLCHOLINE LIPOSOMES

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SUMMARY

(1) The 129 MHz and 36.4 MHz ^{31}P NMR spectra of unsonicated liposomes consisting of phosphatidylcholines of varying chain length and unsaturation have been investigated.

(2) In the liquid crystalline state the ^{31}P NMR liposome spectra are similar for both saturated and unsaturated phosphatidylcholines, demonstrating that the motion of the polar headgroup is not sensitive to the fatty acid composition in the disordered liquid crystalline state.

(3) Below the hydrocarbon phase transition temperature there is a marked increase in the linewidth of the ^{31}P NMR liposome spectra, indicating a reduction in the motion of the polar headgroup.

(4) The addition of equimolar concentrations of cholesterol to phosphatidylcholine eliminates phase transition effects experienced by the polar headgroup. The motion of the polar headgroup is then very similar to that obtained in the liquid crystalline state for pure phosphatidylcholine bilayers.

(5) In the liquid crystalline state the motion of the polar headgroup in the phosphate region is insensitive to changes in the available area per phosphatidylcholine molecule.

INTRODUCTION

^{31}P NMR is an increasingly important technique in the study of model and biological membranes. In the case of sonicated phospholipid vesicles, high resolution ^{31}P NMR spectra are observed [1–3]. These narrow spectra arise because the small vesicle entities experience fast isotropic tumbling [4]. The larger liposomes (produced by mechanical agitation of aqueous dispersions of phospholipid) do not experience such rapid tumbling, and the much broader ^{31}P NMR spectra obtained reflect only the local anisotropic motions available to the phospholipid in the polar headgroup region [4]. In this regard, the membrane fragments obtained by osmotic lysis of cell

membranes are of similar size to liposomes. The fact that similar ^{31}P NMR spectra are observed for cell membrane preparations and derived liposomes [5, 6] indicates that the bulk of the phospholipid polar headgroups in biological membranes have similar motion to that of liposomes.

^{31}P NMR studies on vesicles have shown that the motion experienced by the polar headgroup is sensitive to the gel-liquid crystalline phase transition of the hydrocarbon chains [2, 4, 7]. Monolayer studies [8, 9] indicate that the area per phospholipid molecule in the condensed gel phase is appreciably less than in the more expanded liquid crystalline phase. The increased motion of the polar headgroup in the liquid crystalline phase would therefore appear to be associated with the larger area available to it. Furthermore, monolayer studies show that the area per phospholipid molecule may be affected by the degree of unsaturation of the hydrocarbon chains and by the incorporation of cholesterol [8, 10, 11]. It is of interest to determine whether the motion in the polar headgroup also relects these changes in the available area per molecule.

In this work we have therefore investigated the effects of fatty acid chain length and degree of unsaturation and the effects of equimolar concentrations of cholesterol on the allowed motion of the polar headgroup in phosphatidylcholine liposomes, using ^{31}P NMR. Such studies are difficult using sonicated vesicle systems, as the vesicle ^{31}P NMR linewidths are sensitive to the vesicle size [4, 7], which depends on the length and unsaturation of the hydrocarbon chains, and aggregation phenomena, as well as the cholesterol content [7].

MATERIALS AND METHODS

Lipids

1,2-Dilauroyl-*sn*-glycero-3-phosphorylcholine (12 : 0/12 : 0-phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0-phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorycholine (16 : 0/16 : 0-phosphatidylcholine), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (18 : 0/18 : 0-phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (18 : 1_c/18 : 1_c-phosphatidylcholine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18 : 1_t/18 : 1_t-phosphatidylcholine) and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphorylcholine (16 : 1_c/16 : 1_c-phosphatidylcholine) were synthesised as described previously [20]. All synthetic phosphatidylcholines were synthesised and generously supplied by the biomembranes group of the Department of Biochemistry, State University of Utrecht (The Netherlands). Egg phosphatidylcholine was isolated from hen eggs according to established procedures. Soya phosphatidylcholine was generously supplied by Dr. H. Eikermann of Natterman and Cie, Köln, Germany. Cholesterol was obtained from Fluka (Buchs, Switzerland) and was recrystallised twice from absolute ethanol. Soya phosphatidylcholine contained approximately 3 % of lysophosphatidylcholine, but all other lipids were at least 99 % pure as indicated by thin-layer chromatography.

Chemicals

^2H_2O was obtained from Ryvan, Southampton, U.K. All other chemicals were Analar grade.

Preparation of liposomes

Approx. 50 μmol of phosphatidylcholine dissolved in chloroform was placed in a glass vial and the chloroform evaporated under a stream of nitrogen. Residual chloroform was then removed by placing the sample in a high vacuum overnight. The phospholipid was deposited as a thin film on the bottom of the vial. Liposomes were produced after the addition of 1 ml of ^2H_2O containing 25 mM Tris/acetic acid ($p^2H = 7.0$) and 0.2 mM EDTA by vortexing the resulting mixture above the phase transition temperature of the lipid. This procedure appeared to produce more homogeneous aqueous phospholipid dispersions than may be obtained by vortexing a mixture of dry phospholipid powder and the aqueous solution. Liposomes produced by the latter method showed a large narrow ^{31}P NMR resonance superimposed on the usual broad liposome resonance. This narrow resonance presumably arises from small phospholipid systems, such as vesicles. This narrow signal was either eliminated or appreciably reduced for liposomes prepared by the first method (see Fig. 1).

Nuclear magnetic resonance

Two ^{31}P NMR spectrometers were employed in this study, both of which were interfaced with Nicolet B-NC 12 computers and operated in the Fourier transform mode. The lower frequency (36.4 MHz) instrument was a Bruker WH-90, and was equipped with temperature control, a deuterium "lock" and proton decoupling facilities. The high frequency (129 MHz) instrument was constructed in this laboratory [12] and was similarly equipped except for proton decoupling. The 129 MHz spectrometer was of significantly greater sensitivity than the 36.4 MHz spectrometer. All spectra were therefore obtained at 129 MHz except in cases where proton decoupling was needed, in which case the 36.4 MHz instrument was employed. Accumulated free induction decays were obtained from up to 1000 transients employing a 0.2–0.5 s interpulse time.

RESULTS

The ^{31}P NMR spectra of unsonicated phosphatidylcholine liposomes at high magnetic field strengths exhibit a "solid state" type of lineshape which may be attributed to the chemical shift anisotropy of the phosphate phosphorus [4]. The distinguishing feature of these spectra is a lineshape with a low field shoulder (see Fig. 1). It is convenient to characterize the linewidths of these spectra as the width at half height. This parameter may be directly related to the allowed motion in the phosphate group region of the polar headgroup (see Appendix).

The 129 MHz ^{31}P NMR spectra of liposomes consisting of 12 : 0/12 : 0, 14 : 0/14 : 0, 16 : 0/16 : 0 and 18 : 0/18 : 0 phosphatidylcholine in the liquid crystalline state are shown in Fig. 1. It may be observed that within signal to noise limitations these spectra are identical to each other. (The narrow line that is superimposed on the spectra of 16 : 0/16 : 0 and 18 : 0/18 : 0 liposomes presumably arises from phospholipids in small vesicles. See Materials and Methods.) Below the hydrocarbon phase transition temperature there is a significant change in lineshape and a dramatic increase in linewidth of the liposome spectra. This effect is illustrated in Fig. 2 for 18 : 0/18 : 0-phosphatidylcholine liposomes at 20 °C.

The 129 MHz ^{31}P NMR linewidths of spectra arising from liposomes consist-

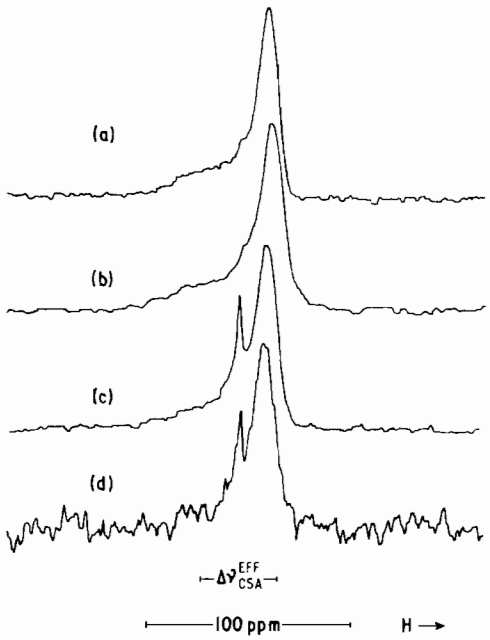

Fig. 1. 129 MHz ^{31}P NMR spectra of liquid crystalline saturated phosphatidylcholine liposomes. (a) 12:0/12:0-phosphatidylcholine (50 °C), (b) 14:0/14:0-phosphatidylcholine (50 °C), (c) 16:0/16:0-phosphatidylcholine (50 °C), (d) 18:0/18:0-phosphatidylcholine (40 °C).

ing of saturated phosphatidylcholine as a function of temperature are shown in Fig. 3. As in the case of the corresponding vesicle spectra [7], a sudden increase in linewidth occurs below the hydrocarbon phase transition temperature. The transition temperature may be estimated to be 25 °C for 14 : 0/14 : 0, 40 °C for 16 : 0/16 : 0 and 55 °C for 18 : 0/18 : 0 phosphatidylcholine, in good agreement with vesicle results [7]. Above the phase transitions the observed liposome linewidths are very similar for all the saturated phosphatidylcholines, and are remarkably insensitive to further increase of temperature.

The spectra of liposomes consisting of unsaturated phosphatidylcholine are

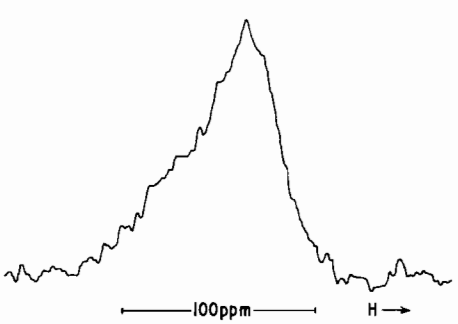

Fig. 2. 129 MHz ^{31}P NMR spectrum of 18:0/18:0-phosphatidylcholine liposomes at 20 °C (below the phase transition temperature).

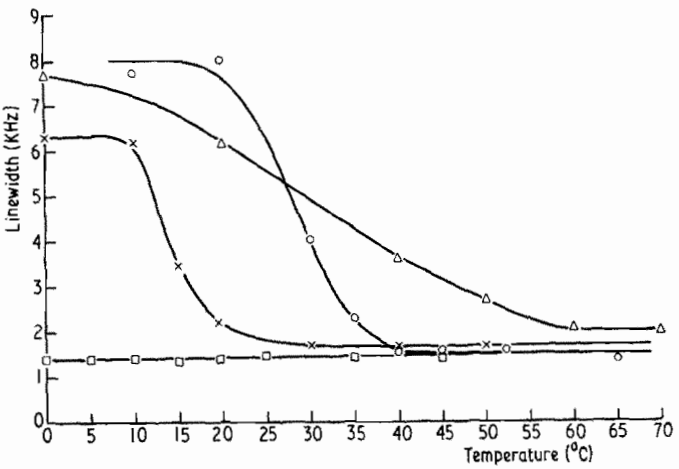

Fig. 3. Temperature dependence of the full width at half height of the 129 MHz ^{31}P NMR spectra arising from liposomes of saturated phosphatidylcholine: □, 12:0/12:0-phosphatidylcholine; ×, 14:0/14:0-phosphatidylcholine; ○, 16:0/16:0-phosphatidylcholine; △, 18:0/18:0-phosphatidylcholine.

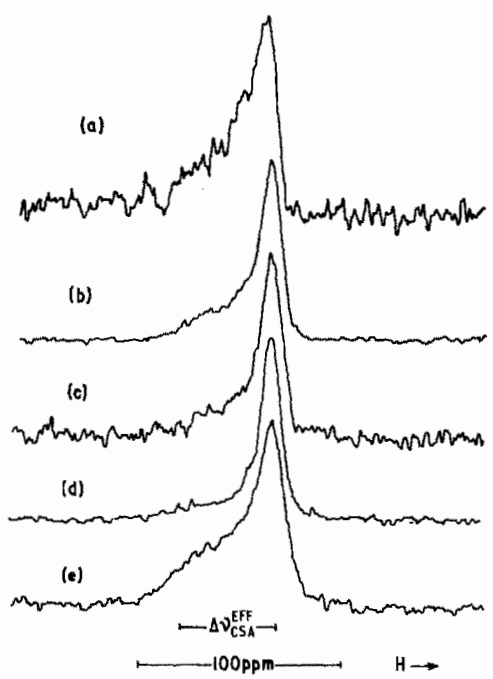

Fig. 4. 129 MHz ^{31}P NMR spectra of liquid crystalline unsaturated phosphatidylcholine liposomes at 50 °C: (a) soya phosphatidylcholine, (b) egg phosphatidylcholine, (c) 18:1_c/18:1_c-phosphatidylcholine, (d) 18:1_t/18:1_t-phosphatidylcholine, (e) 16:1_c/16:1_c phosphatidylcholine.

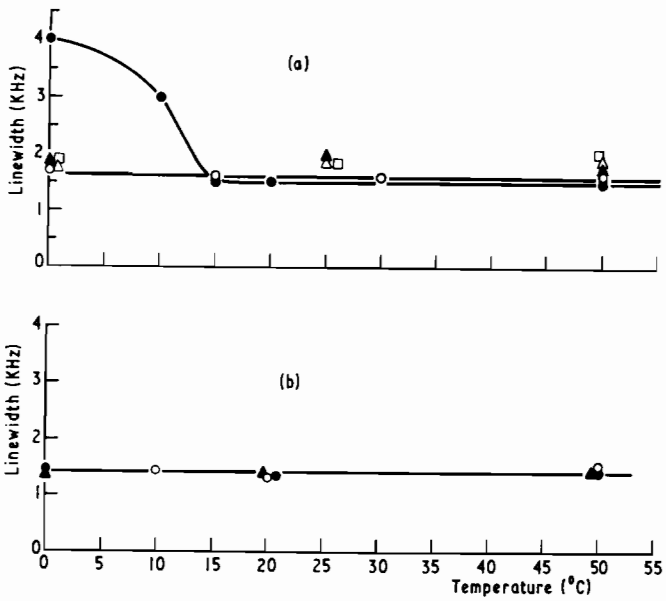

Fig. 5. Temperature dependence of the full widths at half height of the 129 MHz ^{31}P NMR spectra arising from phosphatidylcholine liposomes. (a) Unsaturated phosphatidylcholines: □, soya phosphatidylcholine; ▲, egg phosphatidylcholine; ○, 18:1_c/18:1_c-phosphatidylcholine; △, 16:1_c/16:1_c-phosphatidylcholine; ●, 18:1_t/18:1_t-phosphatidylcholine. (b) Phosphatidylcholine/cholesterol (1:1) mixtures: ○, 18:1_c/18:1_c-phosphatidylcholine+cholesterol (1:1); ●, 18:1_t/18:1_t-phosphatidylcholine+cholesterol; ▲, 14:0/14:0-phosphatidylcholine+cholesterol (1:1).

shown in Fig. 4. Qualitative comparison of Figs. 1 and 4 reveals that the low field shoulders may be somewhat more pronounced for the unsaturated liposome spectra than for the (liquid crystalline) saturated liposome spectra. The observed widths at half height corresponding to both saturated and unsaturated liquid crystalline phosphatidylcholines are, however, very similar, as may be noted from Figs. 3 and 5a. Furthermore, the liposome spectra of unsaturated phosphatidylcholines are again very insensitive to temperature, except for 18 : 1_t/18 : 1_t-phosphatidylcholine liposomes, which exhibit a phase transition at 5 °C [13].

The spectra obtained at 20 °C for liposomes consisting of equimolar mixtures of 14 : 0/14 : 0, 18 : 1_t/18 : 1_t and 18 : 1_c/18 : 1_c phosphatidylcholine with cholesterol are shown in Fig. 6. These spectra have very similar lineshapes, and no phase transition effects may be observed as the linewidths are insensitive to temperature (see Fig. 5b). This result is in agreement with previous calorimetric studies in which it has been shown that the presence of equimolar cholesterol eliminates the hydrocarbon phase transition [14–16]. It may be observed that the low field shoulder of these spectra is fairly pronounced, as in the case of the unsaturated phosphatidylcholine liposomes. The linewidth of 1.4 kHz is very similar to that observed for liposomes of saturated and unsaturated phosphatidylcholine in the liquid crystalline state.

As has been previously noted [4, 6], and is further discussed in the Appendix, the lineshape and linewidth of a ^{31}P NMR liposome spectrum are both influenced by two interactions. These are the dipolar interactions the phosphorus atom experiences

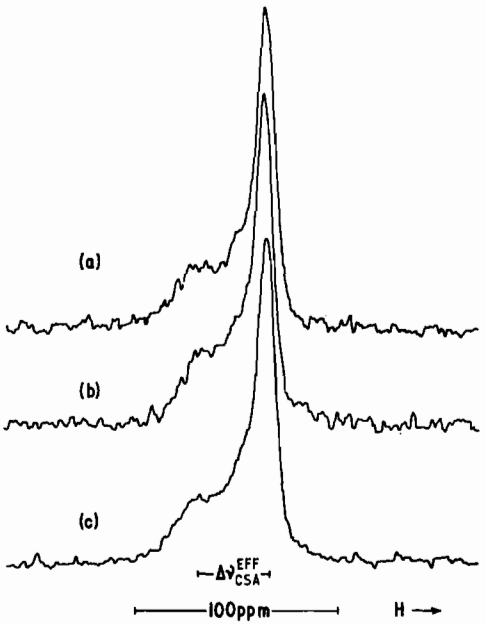

Fig. 6. 129 MHz ^{31}P NMR spectra of liposomes consisting of phosphatidylcholine/cholesterol (1:1) at 20 °C: (a) 14:0/14:0-phosphatidylcholine/cholesterol (1:1); (b) 18:1_t/18:1_t-phosphatidylcholine/cholesterol (1:1); (c) 18:1_c/18:1_c-phosphatidylcholine/cholesterol (1:1).

with the two nearest neighbouring methylene groups, and the effective chemical shift anisotropy $\Delta\nu_{CSA}^{EFF}$ (the frequency separation between the low field shoulder and the main peak). It may be noted from Fig. 1 and Fig. 4 that $\Delta\nu_{CSA}^{EFF}$ is difficult to measure in situations where proton decoupling is not employed, as the dipolar broadening obscures accurate resolution of the low field shoulder. However proton-decoupled liposome spectra (obtained at 36.4 MHz in our case) reveal the low field shoulder far more clearly, as is shown in Fig. 7a for 16 : 0/16 : 0-phosphatidylcholine at 50 °C, and $\Delta\nu_{CSA}^{EFF}$ may be more accurately estimated. This is in agreement with previous observations [17]. The values of $\Delta\nu_{CSA}^{EFF}$ obtained for various saturated and unsaturated species of phosphatidylcholine are summarized in Figs. 7b and 7c. Also included in Fig. 7 are the observed values of $\Delta\nu_{CSA}^{EFF}$ for certain saturated and unsaturated species of phosphatidylcholine in the presence of equimolar concentrations of cholesterol. It may be observed for 14 : 0/14 : 0 and 16 : 0/16 : 0 phosphatidylcholine that $\Delta\nu_{CSA}^{EFF}$ decreases only slightly ($\lesssim 10\,\%$) on going from the gel state to the liquid crystalline state, in strong contrast to the width at half height of the non-decoupled spectra (see Fig. 3). Also the values of $\Delta\nu_{CSA}^{EFF}$ for both saturated and unsaturated phosphatidylcholines in the liquid crystalline state are very similar (in the range 43–47 ppm) and do not vary appreciably ($\lesssim 5\,\%$) over the temperature range investigated. The largest change in $\Delta\nu_{CSA}^{EFF}$ arises on the addition of equimolar concentrations of cholesterol to 16 : 0/16 : 0-phosphatidylcholine, for which $\Delta\nu_{CSA}^{EFF}$ is decreased from 45 ppm or more to approx. 36 ppm. It is interesting to note that, in the case of unsaturated phosphatidylcholines, equimolar concentrations of cholesterol do not produce such a strong reduction of $\Delta\nu_{CSA}^{EFF}$ (see Fig. 7c). Furthermore, $\Delta\nu_{CSA}^{EFF}$ is

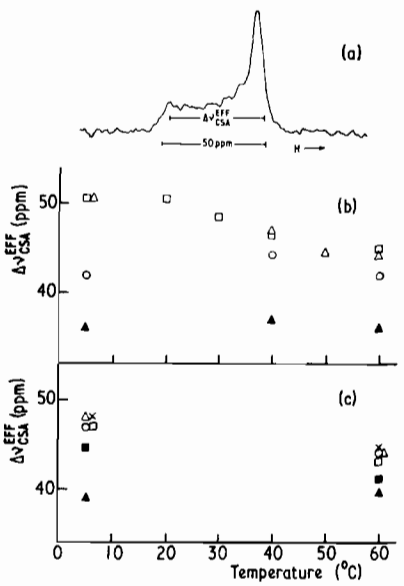

Fig. 7. (a) 36.4 MHz ^{31}P NMR spectra of 16:0/16:0-phosphatidylcholine liposomes at 50 °C in the presence of broad band proton decoupling. (b) Effective chemical shift anisotropy as a function of temperature for various saturated phosphatidylcholines: △, 16:0/16:0-phosphatidylcholine; □, 14:0/14:0-phosphatidylcholine; ○, 12:0/12:0-phosphatidylcholine; ▲, 16:0/16:0-phosphatidylcholine/cholesterol (1:1). (c) Effective chemical shift anisotropy as a function of temperature for various unsaturated phosphatidylcholines: △, 18:1_t/18:1_t-phosphatidylcholine; □, 18:1_c/18:1_c-phosphatidylcholine; ○, 16:1_c/16:1_c-phosphatidylcholine; ×, egg yolk phosphatidylcholine; ▲, 18:1_t/18:1_t-phosphatidylcholine/cholesterol (1:1); ■, 18:1_c/18:1_c-phosphatidylcholine/cholesterol (1:1).

smaller for 18 : 1_t/18 : 1_t-phosphatidylcholine/cholesterol (1 : 1) than for 18 : 1_c/18 : 1_c phosphatidylcholine/cholesterol (1 : 1).

DISCUSSION

The observed non-decoupled ^{31}P NMR spectra arising from all species of liquid crystalline phosphatidylcholine investigated have approximately similar lineshapes and linewidths. As the observed values of the effective chemical shift anisotropy $\Delta\nu_{CSA}^{EFF}$ are very similar for all saturated and unsaturated liquid crystalline phosphatidylcholines (see Fig. 7), any small differences in lineshape which are observed (compare, for example, Figs. 4c and 4e) arise because of changes in the dipolar interactions experienced by the phosphate phosphorus. In this regard computer simulations (see Appendix) show that larger dipolar interactions, for constant values of $\Delta\nu_{CSA}^{EFF}$, result in more prominent low field shoulders relative to the main high field peak. It is interesting to note that the largest low field shoulder exibited by the liquid crystalline liposomes is observed for 16 : 1_c/16 : 1_c-phosphatidylcholine (see Fig. 4), thus suggesting that the dipolar broadening experienced by the phosphate phosphorus is largest for this species. This is somewhat unexpected, as logical extrapolation of existing differential scanning calorimetry data [9, 13, 14] implies that

16 : 1_c/16 : 1_c-phosphatidylcholine at 60 °C is the most fluid (i.e. the furthest above the hydrocarbon phase transition temperature) of any of the pure phosphatidylcholines investigated in this study. The observation of larger dipolar interactions would, however, imply that the motion of the methylene-phosphate-methylene region is more restricted for this most fluid species. Further experiments to clarify this point are in progress.

In general, the fact that the observed ^{31}P NMR spectra arising from the liquid crystalline phosphatidylcholine liposomes all have similar linewidths and values of $\Delta\nu_{CSA}^{EFF}$ shows that the motion in the phosphate region of the polar headgroup is insensitive to the fatty acid composition of the phospholipid. Monolayer and X-ray studies show that the available area per molecule in the liquid crystalline state depends strongly on the degree of unsaturation of the phospholipid. For example, the limiting area per molecule for liquid crystalline saturated phosphatidylcholines is estimated as 56 Å^2 [19], whereas for 18 : 1_c/18 : 1_c-phosphatidylcholine the available area per molecule is 68 Å^2 [8, 18]. The fact that the ^{31}P NMR spectra do not exhibit corresponding effects (except for the relatively small differences in lineshape) shows that the motion of the methylene-phosphate-methylene region of the polar headgroup does not become larger as the available area per molecule is increased. This conclusion implies that the anisotropic motion is restricted by intramolecular rather than intermolecular effects.

Experiments on oriented partially hydrated 16 : 0/16 : 0-phosphatidylcholine bilayers show that the primary motional difference between phosphatidylcholine in the gel state and the liquid crystalline state is that in the liquid crystalline state the phospholipid molecule is free to rotate about its long axis (ref. 6 and Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., in preparation). Well below the phase transition this rotational motion is hindered, with a rotational correlation time $> 10^{-4}$ s. As the temperature is raised toward the phase transition temperature the spectra undergo motional narrowing owing to the onset of rapid axial rotation of the phospholipid. The narrowing observed for fully hydrated 14 : 0/14 : 0, 16 : 0/16 : 0 and 18 : 0/18 : 0 phosphatidylcholine liposomes (see Fig. 5) again shows the effects of this motional averaging. It should be noted, however, that this narrowing takes place over a temperature interval of approx. 15 °C, and is effectively complete approx. 5 °C below the actual phase transition temperature as detected by differential scanning calorimetry [7]. It would therefore appear that axial rotation of the phospholipid is allowed well below the hydrocarbon phase transition temperature, possibly owing to a small increase in the available area per molecule arising from thermal expansion in the plane of the bilayer.

As indicated in Fig. 3 the measured widths at half height ($\Delta\nu_{\frac{1}{2}}$) reach a limiting value approx. 20 °C below the phase transition temperature, except in the case of 18 : 0/18 : 0-phosphatidylcholine. The fact that the values of $\Delta\nu_{CSA}^{EFF}$ are similar for the four species of phosphatidylcholine investigated that exhibit phase transition effects implies that any differences in $\Delta\nu_{\frac{1}{2}}$ arise from differences in the dipolar interactions. In particular, the large limiting halfwidth of 8 kHz for 16 : 0/16 : 0-phosphatidylcholine may be compared to the progressively smaller limiting halfwidths of 6.3 and 4.0 kHz observed for 14 : 0/14 : 0 and 18 : 1_t/18 : 1_t phosphatidylcholine, respectively. These results suggest that shorter chain length and/or unsaturation introduces significant motion into the phosphate region of the polar headgroup well

below the phase transition. This may be interpreted as due to the decreased Van der Waals interactions that may occur between hydrocarbon chains of shorter length or containing *trans* double bonds. The greater degree of disorder in such systems would result in more motion of the phospholipid molecule. The anomalous behaviour of the widths of the 18 : 0/18 : 0-phosphatidylcholine liposome spectra is not yet understood. It would appear that the polar headgroup of distearoyl phosphatidylcholine experiences significant motion well below the phase transition temperature.

The width at half height ($\Delta\nu_{\frac{1}{2}}$) of the liquid crystalline phosphatidylcholine spectra of 1.5 kHz may be employed to obtain a measure of the dipolar order parameter (see Appendix) of 0.13. Thus the motion in the phosphate region of the polar headgroup is appreciably restricted in the liquid crystalline state. The fact that only negligible changes in lineshape and linewidth are observed on increasing the temperature well above the transition temperature shows that the extent of this motion is remarkably insensitive to temperature.

The introduction of equimolar concentrations of cholesterol into the phosphatidylcholine liposomes has several interesting effects. Firstly, all phase transition effects are eliminated as the ^{31}P NMR spectra of 16 : 0/16 : 0 and 18 : 1_t/18 : 1_t phosphatidylcholine/cholesterol (1 : 1) liposomes are insensitive to temperature changes over the interval 0–60 °C. Secondly, the values of $\Delta\nu_{CSA}^{EFF}$ obtained for the cholesterol-containing liposomes are sensitive to the degree of unsaturation of the hydrocarbon chains. It may be noted from Fig. 7 that on the addition of equimolar cholesterol $\Delta\nu_{CSA}^{EFF}$ is reduced by 8 ppm (at 60 °C) for 16 : 0/16 : 0-phosphatidylcholine, whereas $\Delta\nu_{CSA}^{EFF}$ is reduced by only 4 ppm and approx. 2 ppm (at 60 °C) for 18 : 1_t/18 : 1_t and 18 : 1_c/18 : 1_c phosphatidylcholine, respectively. In this regard monolayer studies indicate that the strongest phosphatidylcholine-cholesterol interactions (as evidenced by the "condensing" effect of cholesterol on the area per liquid crystalline phosphatidylcholine molecule) occur for more saturated molecular species [8, 10, 11]. This is attributed to stronger cholesterol-fatty acid Van der Waals interactions possible for the less disordered (more saturated) phospholipid species. Our results therefore indicate that $\Delta\nu_{CSA}^{EFF}$ is most reduced when the cholesterol-acyl chain interactions are strongest. This effect is surprising, as the most condensed phosphatidylcholine might be expected to have reduced motion in the phosphate region, and therefore larger values of $\Delta\nu_{CSA}^{EFF}$. There are, however, explanations other than increased motion which could account for the larger reduction of $\Delta\nu_{CSA}^{EFF}$ for 16 : 0/16 : 0-phosphatidylcholine/cholesterol (1 : 1) than for 18 : 1_t/18 : 1_t and 18 : 1_c/18 : 1_c phosphatidylcholine/cholesterol (1 : 1). These include a change in the angle between the director of the rigid lattice chemical shift anisotropy tensor and the axis of rotation, or, in other words, a change in the orientation of the polar headgroup in the phosphate region. Further investigation is necessary to clarify this point.

It may be calculated that $S_{DIP} = 0.11$ for all the 16 : 0/16 : 0, 18 : 1_t/18 : 1_t and 18 : 1_c/18 : 1_c phosphatidylcholine/cholesterol (1 : 1) spectra, indicating a slight increase in the motion in the phosphate group region, over that obtained in the normal liquid crystalline state, on adding cholesterol.

In general, as the observed dipolar order parameters and values of $\Delta\nu_{CSA}^{EFF}$ for the cholesterol/phosphatidylcholine (1 : 1) liposomes are close to those observed for pure liquid crystalline phosphatidylcholine liposomes, it would appear that the motion in the methylene-phosphate-methylene region of the polar headgroup is very

similar to that obtained in the normal liquid crystalline state. It may be concluded that the presence of cholesterol allows fast axial rotation of the phospholipid moleles below the normal phase transition temperature. It is interesting to note that ^{2}H NMR results [20] indicate that the motion in the $N(CH_3)_3^+$ region of liquid crystalline egg phosphatidylcholine is not affected by the presence of cholesterol, and that therefore the motion for the whole polar headgroup is not strongly affected by the presence of cholesterol. It may be noted from ^{13}C NMR studies [21] that cholesterol is situated in the hydrophobic core of the phospholipid bilayer, such that the hydroxyl group does not extend significantly past the glycerol backbone. These results suggest that the polar headgroup, in the presence of equimolar amounts of cholesterol, should be free to move over the whole area of the phospholipidcholesterol complex, which has been estimated as 84 Å^2 [19]. The fact that a significant increase in motion in the phosphate group region is not observed for the phosphatidylcholine/cholesterol (1 : 1) liposomes again indicates that the polar headgroup is not free to move over the larger area available to it.

APPENDIX

We briefly summarize the theoretical interpretation of the observed ^{31}P NMR liposome spectra. A more complete discussion is presented by McLaughlin et al. [4, 6] and Cullis et al. (Cullis, P.R., McLaughlin, A. C. and Hemminga, M. A., in preparation). If a phospholipid experiences rapid rotation about its long axis, as has been concluded for liquid crystalline phospholipids [6], and the magnetic field $\boldsymbol{H}_0$ is at an angle θ with respect to this axis, then the frequency of the observed resonance depends on the chemical shift anisotropy of the phosphate phosphorus according to

$$\nu = \nu_0 + \left(\frac{3\cos^2\theta - 1}{3}\right) \Delta\nu_{CSA}^{EFF} \tag{1}$$

where $\Delta\nu_{CSA}^{EFF}$ is the value of the rigid lattice chemical shift anisotropy after averaging over the allowed motion. Similarly, it may be shown that the dipolar interactions of the phosphorus atom with the nearest neighbouring methylene protons, in the presence of rapid axial rotation, results in a θ-dependent second moment given by

$$(\Delta W_G^{(\theta)})^2 = \left(\frac{3\cos^2\theta - 1}{2}\right)^2 (\Delta W_G^{EFF})^2 \tag{2}$$

where

$$(\Delta W_G^{EFF})^2 = \frac{\gamma_P^2 \gamma_H^2 \hbar^2 (I+1)}{3} \sum_i \frac{(3\cos^2\Delta_i - 1)^2}{r_i^6}$$

The angles Δ_i are the angles between the phosphorus-proton internuclear vectors r_i and the axis of rotation. Therefore the position of the resonance arising from a particular phospholipid depends on the chemical shift anisotropy as described by Eqn. 1, and the second moment of this line depends on the dipolar interactions through Eqn. 2. We may therefore simulate the spectra corresponding to non-oriented systems such as liposomes by convoluting over all angles θ. The type of

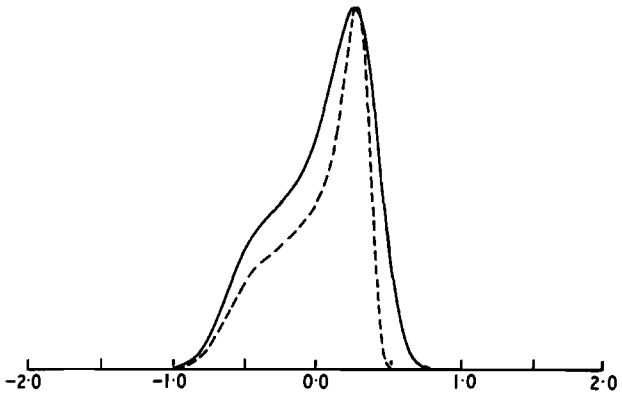

Fig. 8. Simulated "solid state" spectra. The solid line shows the lineshape expected for component linewidths, which are angularly independent and where the square root of the second moment of these component lines is given by $\Delta W_G^{EFF} = 0.14 \Delta \nu_{CSA}^{EFF}$. The dashed line shows the lineshape expected when the component linewidths are orientation dependent according to

$$\Delta W_G(\theta) = \Delta W_G^{EFF} \left(\frac{3\cos^2 \theta - 1}{2} \right)^{\frac{1}{2}} \text{ where } \Delta W_G^{EFF} = 0.14 \Delta \nu_{CSA}^{EFF}.$$

The X axis is in units of $\Delta \nu_{CSA}^{EFF}$.

lineshape obtained is illustrated by the dashed line in Fig. 8 for $\Delta W_G^{EFF}/\Delta \nu_{CSA}^{EFF} = 0.14$. Also included in this figure is the lineshape obtained when the second moments are independent of θ (*i.e.* there is no rapid rotation about the long axis of the molecule) but the frequency position of the lines arising from individual phospholipids still depend on θ as in Eqn. 1. This has been shown to correspond to the situation in the gel state, well below the phase transition temperature (ref. 6 and Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., in preparation). It may be noted that the (gel state) lineshape for the θ-independent dipolar widths exhibits a larger low field shoulder than does the corresponding (liquid crystalline) lineshape for θ-dependent widths.

In the liquid crystalline state, where there is rapid axial rotation, a molecular order parameter may be defined from the dipolar interactions according to

$$(S_{DIP})^2 = \frac{\overline{(\Delta W_G^{(\theta)})^2}}{(\overline{\Delta W_G^2})_{RL}} = \frac{(\Delta W_G^{EFF})^2}{5(\overline{\Delta W_G^2})_{RL}} \tag{3}$$

where the bars indicate averaging over all possible angles θ between the axis of rotation (or r_i, in the case of the rigid lattice second moment $(\overline{\Delta W_G^2})_{RL}$) and H_0. It may be calculated that $(\overline{\Delta W_G^2})_{RL}^{\frac{1}{2}} = 2.5$ kHz for phosphorus-proton internuclear distances of $r_i = 2.6$ Å [22]. If $\Delta \nu_{CSA}^{EFF}$ is known, ΔW_G^{EFF} may also be calculated from the observed halfwidths $\Delta \nu_{\frac{1}{2}}$ of the liquid crystalline ^{31}P NMR liposome spectra, using the computer-generated relationship for θ-dependent second moments seen in Fig. 9. Thus the order parameter S_{DIP}, which characterizes the allowed motion in the methylene-phosphate-methylene region of the polar headgroup, may be obtained.

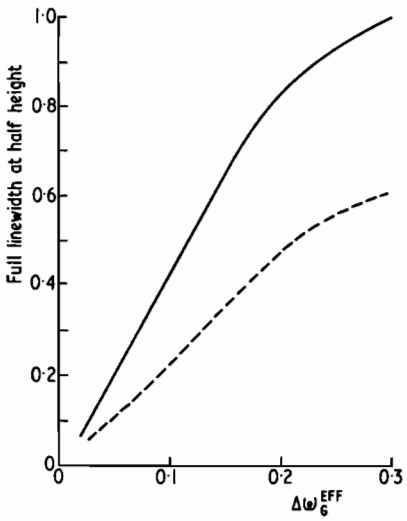

Fig. 9. Theoretical plot of the full width at half height of the solid state spectra as a function of the square root of the second moment ΔW_G^{EFF} of the component lines. Both axes are in units of $\Delta\gamma_{CSA}^{EFF}$. The solid line shows the theoretical dependence when ΔW_G^{EFF} is angularly independent, whereas the dashed line shows the dependence when

$$\Delta W_G(\theta) = \Delta W_G^{EFF} \left(\frac{3\cos^2\theta - 1}{2}\right)^{\frac{1}{2}}.$$

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