

BBA 77360

³¹P NMR STUDIES OF UNSONICATED AQUEOUS DISPERSIONS OF NEUTRAL AND ACIDIC PHOSPHOLIPIDS

EFFECTS OF PHASE TRANSITIONS, p²H AND DIVALENT CATIONS ON THE MOTION IN THE PHOSPHATE REGION OF THE POLAR HEADGROUP

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(Received December 19th, 1975)

SUMMARY

1. The 129 MHz (non-proton decoupled) and 36.4 MHz (proton decoupled) ³¹P NMR spectra arising from unsonicated aqueous dispersions of well defined species of phospholipid have been investigated. The phospholipids employed (and the parameters varied) include phosphatidylcholine (temperature), phosphatidylethanolamine (temperature), phosphatidic acid (temperature and p²H) and phosphatidylglycerol (temperature, p²H and Ca²⁺ (or Mg²⁺)) concentration.

2. At p²H = 7 the ³¹P NMR spectra arising from saturated species of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol become progressively broader as the temperature is reduced below the phase transition, demonstrating reduced motion in the phosphate region of the polar headgroup.

3. In the liquid crystalline state at p²H = 7 the molecular dipolar order parameters obtained for saturated species of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol are very similar, and are independent of the acyl chain length for species derived from lauric and myristic acid. Thus the motion in the methylene-phosphate-methylene region is similar for these different liquid crystalline phospholipid species.

4. The ³¹P NMR spectra of aqueous dispersions of 14 : 0/14 : 0 phosphatidic acid display anomalous temperature and p²H dependences. The effective chemical shift anisotropy ($\Delta\nu_{\text{CSA}}^{\text{EFF}}$) at 5 °C varies from 71 ppm at p²H = 8.5 to 38 ppm at p²H = 2.5. Further, the motion in the phosphate region is relatively insensitive to the gel or liquid crystalline nature of the hydrocarbon chains.

5. The addition of 40 mol % Ca²⁺ (or Mg²⁺) to saturated species of phosphatidylglycerol causes an increase of approx. 20 °C in the hydrocarbon phase transition temperature as indicated by ³¹P NMR. Equimolar concentrations of Ca²⁺ increase the transition temperature by approx. 70 °C, and no ³¹P NMR signal could be observed for the very condensed precipitate formed below this temperature. In the

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liquid crystalline state the motion in the phosphate region of the polar headgroup is not significantly affected by the presence of Ca^{2+} or Mg^{2+} .

6. The ^{31}P NMR spectra obtained from 18 : 1c/18 : 1c phosphatidylethanolamine are consistent with a phase transition from a lamellar to an hexagonal (H_{II}) phase in the region 10–15 °C.

7. The observed narrowing of the ^{31}P NMR spectra of aqueous dispersions of phospholipids as the temperature is raised toward the hydrocarbon transition temperature is discussed in terms of the "pretransition" observed in calorimetric studies.

INTRODUCTION

^{31}P NMR is a powerful technique in the study of model and biological membranes. Relatively narrow ^{31}P NMR spectra may be obtained from sonicated phospholipid vesicles [1–4], where the fast isotropic tumbling of the small vesicles is the dominant line narrowing mechanism [5]. The high resolution nature of these vesicle ^{31}P NMR spectra, in conjunction with appropriate shift reagents, allows the determination of vesicle sizes and the distribution of phospholipids across the vesicle bilayer membrane [1–3]. Further, effects due to hydrocarbon phase transitions may also be observed [4–5].

Alternatively, the much broader ^{31}P NMR spectra arising from unsonicated phospholipid "liposomes" may be studied. In these systems isotropic averaging mechanisms due to tumbling and lateral diffusion of the phospholipid in the plane of the membrane are not effective, and the spectra obtained reflect only the local anisotropic motions in the phosphate region of the polar headgroup [5]. It has been shown that equimolar concentrations of cholesterol eliminate phase transition effects in phosphatidylcholine and that the motion of the phosphate group is then similar to that observed in the normal liquid crystalline state [6]. Further, for phosphatidylcholine in the liquid crystalline state the motion in the phosphate group region is insensitive to the fatty acid composition [6]. Below the hydrocarbon phase transition the motion of the methylene-phosphate-methylene region is progressively restricted, with attendant increase in the ^{31}P NMR linewidth and changes in lineshape [5–7].

Membrane fragments obtained by osmotic lysis of biological membranes are of similar size as liposomes and would therefore be expected to show similar ^{31}P NMR spectra if the lipids are in a bilayer configuration. Such similarities are observed [7, 8]. In the case of membrane preparations obtained from *Acholeplasma laidlawii* B grown on elaidic acid phase transition effects analogous to those observed in liposomes are observed [8], and it may also be concluded that the motion in the phosphate regions of the bulk of the two phosphorus-containing lipids in this membrane are not influenced by the presence of the membrane proteins [8].

It is therefore established that ^{31}P NMR is capable of giving detailed motional information on the phosphate region of the polar headgroup, and that in the region of the phosphate group at least these motions are similar in liposomes and biological membranes. Previous liposome studies have been confined to phosphatidylcholine, however, whereas biological membranes may contain a variety of phospholipids. In this study we have therefore systematically investigated, using ^{31}P NMR, unsonicated aqueous dispersions of a variety of well defined phospholipids, differing both in the

fatty acyl constituents and in the nature of the polar headgroup. In particular, as it is well established that the packing properties of acidic phospholipids are strongly dependent upon the ionic environment [9–14] we have also investigated the effect of pH and divalent ions on the ^{31}P NMR spectra of various acidic phospholipids.

MATERIALS AND METHODS

Lipids

(i) *Phosphatidylcholines*. 1,2-Dilauroyl-*sn*-glycero-3-phosphorylcholine (12 : 0/12 : 0 phosphatidylcholine) and 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0 phosphatidylcholine) were synthesized as described previously [15].

(ii) *Phosphatidylethanolamines*. 1,2-Dilauroyl-*sn*-glycero-3-phosphorylethanolamine (12 : 0/12 : 0 phosphatidylethanolamine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylethanolamine (14 : 0/14 : 0 phosphatidylethanolamine), 1,2-dipalmitoleyl-*sn*-glycero-3-phosphorylethanolamine (16 : 1c/16 : 1c phosphatidylethanolamine) and 1,2-dioleoyl-*sn*-glycero-3-phosphorylethanolamine (18 : 1c/18 : 1c phosphatidylethanolamine) were synthesized from the corresponding phosphatidylcholines via the base exchange reaction with partially purified cabbage phospholipase D [16]. 2 g of phosphatidylcholine dissolved in 25 ml diethyl ether were vigorously shaken at room temperature with 50 ml 4 M ethanolamine/acetic acid (pH 5.5) and 50 ml phospholipase D solution [18] containing 50 mM CaCl_2 . The reaction was stopped as soon as the first traces of phosphatidic acid were formed. The diethyl ether was removed by evaporating under reduced pressure. The lipids were isolated from the resulting aqueous phase by Bligh and Dyer extraction [17]. Phosphatidylethanolamine was isolated by column chromatography (column length 30 cm, width 3 cm) over silica gel (Merck, Darmstadt, Germany) by first eluting stepwise with 100-ml portions of chloroform containing 0, 1, 2, 3, 4, 5 and 6% (v/v) methanol. Thereafter the column was eluted with a linear gradient of 6–30% (v/v) methanol in chloroform. Chromatographically pure phosphatidylethanolamine was obtained from the fractions containing 10–15% (v/v) methanol. The final yield of phosphatidylethanolamine was 40–60%.

(iii) *Phosphatidylglycerols*. 1,2-Dilauroyl-*sn*-glycero-3-phosphatidyl-1-*sn*-glycerol (12 : 0/12 : 0 phosphatidylglycerol), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-1-*sn*-glycerol (14 : 0/14 : 0 phosphatidylglycerol), 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-1-*sn*-glycerol (18 : 1c/18 : 1c phosphatidylglycerol) and egg phosphatidylglycerol were prepared from the corresponding phosphatidylcholines by treatment with partially purified phospholipase D [16] in the presence of glycerol as described by Papahadjopoulos et al. [19]. The purified phosphatidylglycerols were finally converted to their sodium salts [20].

(iv) *Phosphatidic acid*. 1,2-Dimyristoyl-*sn*-glycerol-3-phosphate (14 : 0/14 : 0 phosphatidic acid) and egg phosphatidic acid were prepared from the corresponding phosphatidylcholines by the action of partially purified phospholipase D from cabbage [16, 18]. Phosphatidic acid was converted to its disodium salt by Bligh and Dyer extraction [17] of the lipids with 100 mM $\text{Na}_2\text{-EDTA}$ and 1 M NaCl present in the aqueous phase. All lipids were pure as evidenced by thin-layer chromatography and were stored as dry materials at -20°C (under a N_2 atmosphere). The synthetic phosphatidylcholines and phosphatidylglycerols were a kind gift of the Biomembranes group of the Department of Biochemistry, Utrecht.

Chemicals

$^2\text{H}_2\text{O}$ was obtained from Ryvan, Southampton, U.K. All other chemicals were analytical grade.

Methods

Preparation of lipid dispersions. A chloroform solution containing 50–100 μmol phospholipid was dried down under a stream of N_2 such that a film of lipid was formed on the wall of the glass vial. Residual traces of chloroform were removed by storing the sample overnight under high vacuum. The lipids were dispersed in 1.0 ml $^2\text{H}_2\text{O}$ containing 0.2 mM EDTA and 25 mM Tris/acetic acid buffer at the desired $p^2\text{H}$ by agitating for 5 min on a Vortex mixer at a temperature approx. 5°C above the lipid phase transition temperature. In some cases varying quantities of NaCl , CaCl_2 or MgCl_2 were added to the buffer prior to dispersing the lipids.

Nuclear Magnetic Resonance. Two ^{31}P NMR spectrometers operating in the Fourier transform mode were used in this study. Both spectrometers were interfaced with Nicolet B-NC-12 computers and were equipped with temperature control and field stabilisation via a deuterium lock. The higher frequency (129 MHz) spectrometer was constructed in this laboratory [21] and had quadrature detection facilities but no proton decoupling. The lower frequency (36.4 MHz) machine was a Bruker WH-90 which was equipped with broad band proton decoupling (maximum power input 20 W). Accumulated free induction decays were obtained from 5000 to 50 000 transients with an interpulse time of 0.2–0.5 s and a pulse angle of 45° . On the 129 MHz spectrometer a delay time of 20 μs was employed, whereas a delay time of 100 μs was used on the 36.4 MHz spectrometer.

Two parameters may be obtained from the "solid state" ^{31}P NMR spectra observed for phospholipid liposomes, the effective chemical shift anisotropy $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ (the frequency separation between the main peak and the low field shoulder) and the width at half height $\Delta\nu_{\frac{1}{2}}$. As explained previously [6], it is often difficult to measure $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ in situations where proton decoupling is not employed. The parameter $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ may be accurately measured from proton-decoupled spectra, however, as is indicated in Fig. 3 for proton-decoupled spectra of 18 : 1c/18 : 1c phosphatidylethanolamine. As proton decoupling was not available on the 129 MHz instrument, but was on the 36.4 MHz spectrometer, we have therefore obtained most measures of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ from 36.4 MHz proton-decoupled ^{31}P NMR spectra. Alternatively, as the 129 MHz spectrometer was of significantly greater sensitivity than the lower frequency machine, all measures of $\Delta\nu_{\frac{1}{2}}$ were obtained from non-decoupled 129 MHz ^{31}P NMR spectra.

RESULTS

Phosphatidylcholines

The ^{31}P NMR spectra of unsonicated aqueous dispersions (liposomes) of phosphatidylcholines have a characteristic "solid state" lineshape with a low field shoulder, which arises because of the chemical shift anisotropy of the phosphate phosphorus [5]. The two parameters which may be measured from these spectra, the effective chemical shift anisotropy $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ and the width at half height $\Delta\nu_{\frac{1}{2}}$, may be used to obtain the chemical shift anisotropy order parameter S_{CSA} [7] and the dipolar order parameter S_{DIP} [6], respectively. The temperature dependence of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ and $\Delta\nu_{\frac{1}{2}}$ for

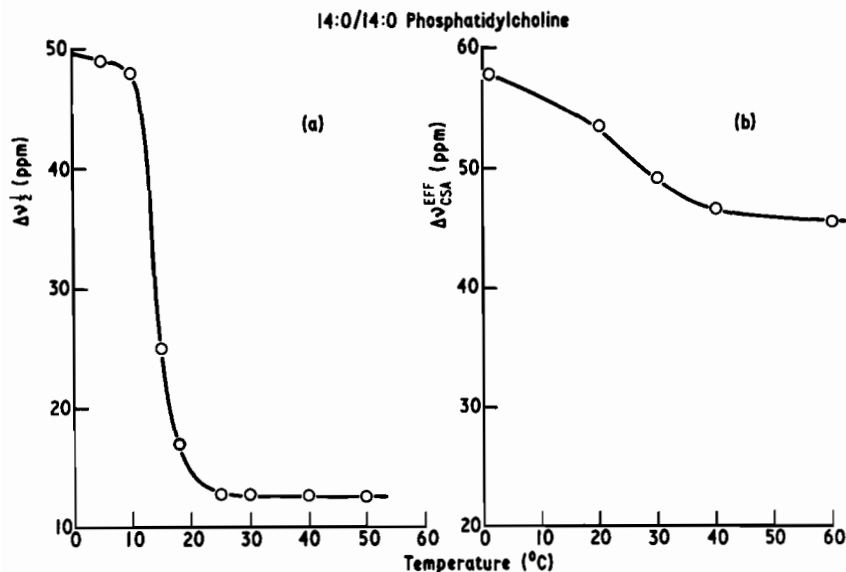


Fig. 1. Temperature dependence of ^{31}P NMR spectral parameters for 14 : 0/14 : 0 phosphatidylcholine. a, width at half height (obtained from non-decoupled 129 MHz spectra) and b, effective chemical shift anisotropy (obtained from 36.4 MHz proton-decoupled spectra).

for 14 : 0/14 : 0 phosphatidylcholine is shown in Fig. 1. It is observed that $\Delta\nu_{CSA}^{EFF}$ increases gradually at lower temperatures and no marked effect is observed at or near the gel-liquid crystalline phase transition temperature of 24 °C [22]. This may be contrasted with the temperature dependence of the width at half height $\Delta\nu_{\frac{1}{2}}$, which increases strongly below 24 °C.

In order to ascertain whether $\Delta\nu_{CSA}^{EFF}$ for phosphatidylcholine was dependent on the $p^2\text{H}$ of the aqueous medium, proton-decoupled spectra at 50 °C of 12 : 0/12 : 0 phosphatidylcholine liposomes were obtained at $p^2\text{H} = 7.0$ and 1.5, for which it was found that $\Delta\nu_{CSA}^{EFF} = 43$ and 44 ppm, respectively. Since the pK of the phosphate group for phosphatidylcholine is approx. 2 [11], this result demonstrates that $\Delta\nu_{CSA}^{EFF}$ is not affected by the presence or absence of a deuterium atom on the phosphate group.

The reader is referred to ref. 6 for an extended study of the effects of chain length and degree of unsaturation on the ^{31}P NMR liposome spectra of phosphatidylcholine.

Phosphatidylethanolamines

Saturated molecular species. The ^{31}P NMR spectra obtained from 12 : 0/12 : 0 and 14 : 0/14 : 0 phosphatidylethanolamine show similar lineshapes and temperature-dependent behaviour as observed for corresponding species of phosphatidylcholine. In the liquid crystalline state both molecular species exhibit very similar lineshapes and linewidth. The values of $\Delta\nu_{CSA}^{EFF}$ obtained for 12 : 0/12 : 0 and 14 : 0/14 : 0 phosphatidylethanolamine in the liquid crystalline state are approx. 5 ppm smaller than $\Delta\nu_{CSA}^{EFF}$ for liquid crystalline phosphatidylcholines (see Figs. 1 and 2) whereas $\Delta\nu_{\frac{1}{2}}$ is approximately the same for both phospholipid species (see Fig. 1 and ref. 6).

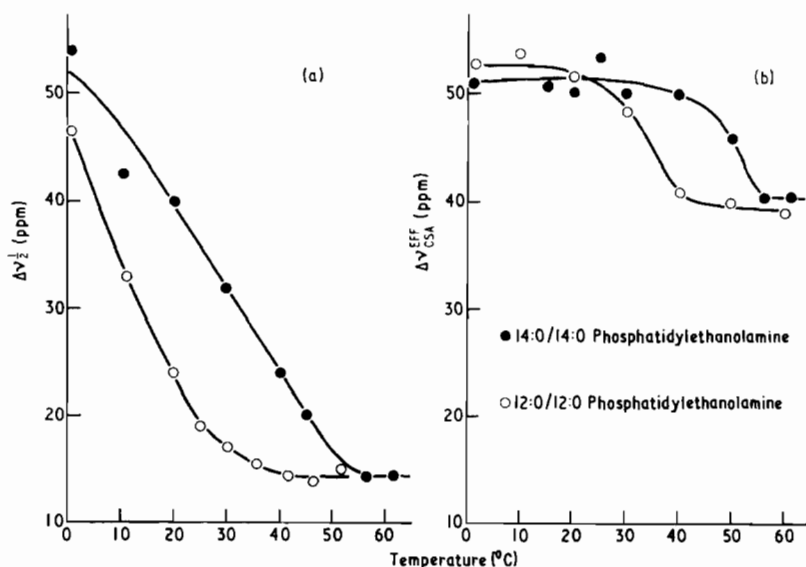


Fig. 2. Temperature dependence of ^{31}P NMR spectral parameters for 12:0/12:0 and 14:0/14:0 phosphatidylethanolamine. a, width at half height (obtained from 129 MHz non-decoupled spectra) and b, effective chemical shift anisotropy (obtained from 36.4 MHz proton-decoupled spectra).

Calorimetric studies on aqueous dispersions of 14:0/14:0 and 16:0/16:0 phosphatidylethanolamine reveal transition temperatures of 50 [27] and 64 $^{\circ}\text{C}$ [25], respectively, which are approx. 23 $^{\circ}\text{C}$ higher than the transition temperatures of the corresponding phosphatidylcholines [22, 24, 25]. Thus the transition temperature of 12:0/12:0 phosphatidylethanolamine may be estimated to be approx. 30 $^{\circ}\text{C}$.

The ^{31}P NMR spectra obtained reflect the gel-liquid crystalline phase transitions in a fashion similar to that observed for 14:0/14:0 phosphatidylcholine. Below the respective transition temperatures the linewidths ($\Delta\nu_{\frac{1}{2}}$) increase strongly for 12:0/12:0 and 14:0/14:0 phosphatidylethanolamine, whereas $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ is much less sensitive to temperature (see Fig. 2). It is interesting to note that the temperature interval ΔT over which line-narrowing effects are observed for $\Delta\nu_{\frac{1}{2}}$ is much smaller (approx. 15 $^{\circ}\text{C}$) for 14:0/14:0 phosphatidylcholine than for 12:0/12:0 and 14:0/14:0 phosphatidylethanolamine, where $\Delta T \geq 50$ $^{\circ}\text{C}$.

Unsaturated molecular species. As shown in Fig. 3, the 36.4 MHz proton-decoupled ^{31}P NMR spectra of 18:1c/18:1c phosphatidylethanolamine show several interesting features. Below 5 $^{\circ}\text{C}$ the spectra is similar to that obtained for the saturated phosphatidylethanolamines in the liquid crystalline state. As the temperature is raised to 10 $^{\circ}\text{C}$ a peak midway between the original main peak and low field shoulder is observed, the relative intensity of which increases as the temperature is raised further. Finally, above 20 $^{\circ}\text{C}$ a single spectral feature is observed which has the characteristic solid state shape associated with chemical shift anisotropy, but where $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ has a different sign and is reduced by a factor of 2 as compared to the lower temperature (< 5 $^{\circ}\text{C}$) situation. Identical effects were observed for the 129 MHz ^{31}P NMR spectra of 18:1c/18:1c phosphatidylethanolamine.

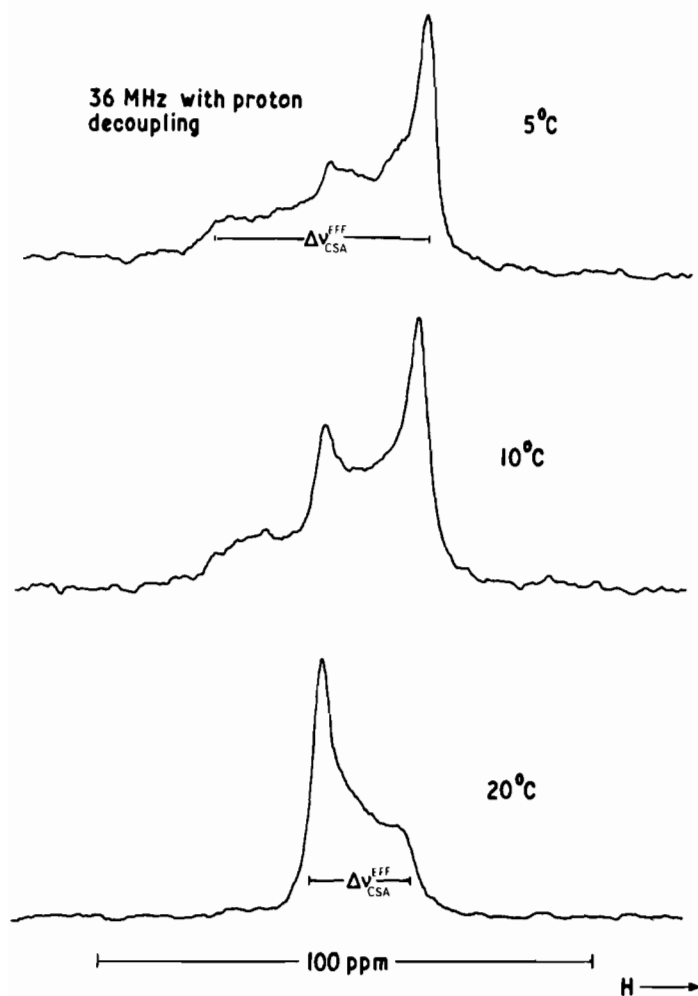


Fig. 3. 36.4 MHz proton-decoupled ^{31}P NMR spectra obtained from aqueous dispersions of 18 : 1c/18 : 1c phosphatidylethanolamine.

X-ray diffraction experiments [26] indicate that dispersions of unsaturated phosphatidylethanolamines in excess water undergo a transition at approx 10°C from a lamellar to an hexagonal H_{II} phase which consists of long "pipes" of phospholipid where the polar headgroups surround an inner aqueous channel. The ^{31}P NMR results obtained for 18 : 1c/18 : 1c phosphatidylethanolamine are fully consistent with such a phase change. In the liquid crystalline lamellar phase the rapid rotation of the phospholipid molecule about its long axis produces an axially symmetric chemical shift anisotropy tensor, where the axis of symmetry is perpendicular to the plane of the bilayer (ref. 7 and Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., unpublished). If an additional mechanism allowing averaging about an axis perpendicular to this axis of rotation is present, it is easy to show that a change in sign and reduction by a factor of 2 of $\Delta\nu_{CSA}^{EFF}$ would be expected. Such an additional

averaging mechanism would be supplied by rapid lateral diffusion of the phospholipid around the long pipes of the hexagonal phase.

Aqueous dispersions of 16 : 1c/16 : 1c phosphatidylethanolamine show quite different ^{31}P NMR spectra. Below 50 °C a spectrum was observed with a broad solid state component and a narrow component similar to that shown in Fig. 8 for 18 : 1c-18 : 1c phosphatidylglycerol in the presence of CaCl_2 . Above 50 °C only the narrow component is observed. The broad component is interpreted as due to a fraction of the sample in a lamellar phase, whereas the narrow line reflects a non-lamellar phase where the lateral diffusion of the phospholipid produces fast isotropic averaging. It should be noted that the narrow line does not arise from a component of the phosphatidylethanolamine in small vesicular or micellar forms free in solution as the lipid: $^2\text{H}_2\text{O}$ dispersion formed a visible two-phase system consisting of the hydrated lipid and aqueous phase, respectively. Vesicles, on the other hand, form a homogeneous translucent mixture.

Phosphatidic acid. The phosphate group of phosphatidic acid has two pK values which are given by $pK_1 = 3$ and $pK_2 = 8.5$ [28]. It has been shown that the gel-

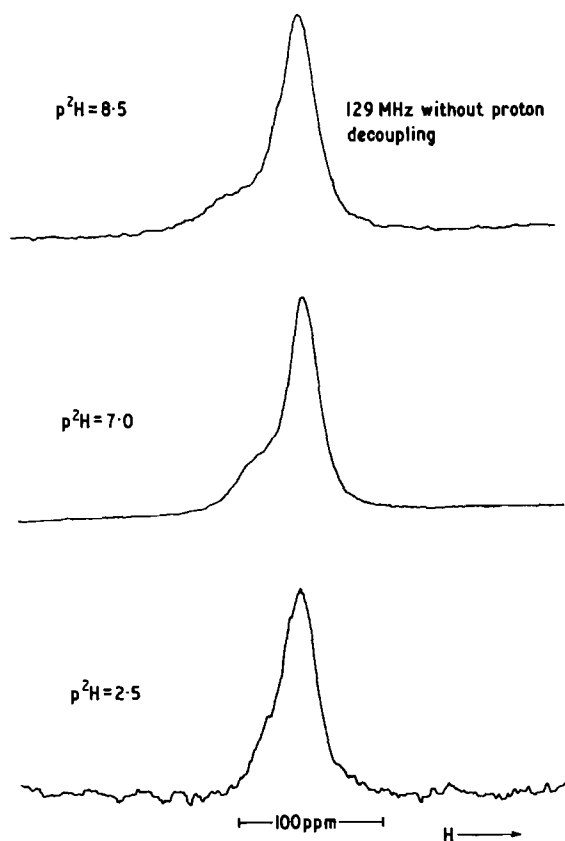


Fig. 4. 129 MHz ^{31}P NMR spectra obtained from aqueous dispersions of 14 : 0/14 : 0 phosphatidic acid (at 5 °C) for various $p^2\text{H}$ values.

liquid crystalline phase transition temperature for 14 : 0/14 : 0 phosphatidic acid is 30 °C for the singly protonated form and 50 °C for the doubly protonated form [11]. At 5 °C it might therefore be expected that the ^{31}P NMR spectra of aqueous dispersions of 14 : 0/14 : 0 phosphatidic acid would progressively broaden as the p^2H is lowered (reflecting more restricted motion in the phosphate group region for lipids further below the phase transition temperature). As shown in Fig. 4, however, such broadening is not observed. A marked decrease of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ is apparent on reducing the p^2H from 8.5 to 2.5, but it may be calculated that the dipolar order parameter S_{DIP} [6] does not change significantly as the values of $\Delta\nu_{\frac{1}{2}}$ are approximately the same at all p^2H values. It may therefore be concluded that $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ is very sensitive to the state of protonation of the phosphate group in phosphatidic acid.

As shown in Fig. 5 the ^{31}P NMR spectra of 14 : 0/14 : 0 phosphatidic acid at various p^2H values show anomalous temperature dependences over the interval 0–60 °C. In particular $\Delta\nu_{\frac{1}{2}}$ decreases only slightly as the temperature is raised to 40 °C and, at p^2H 8.5 and 7.0, $\Delta\nu_{\frac{1}{2}}$ subsequently increases at higher temperatures. This behaviour may be contrasted to that observed for $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ (Fig. 5) which decreases strongly as the temperature is raised for p^2H 8.5 and 7.0. At p^2H 2.5 both $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ and $\Delta\nu_{\frac{1}{2}}$ are relatively insensitive to temperature. This behaviour is in strong contrast to that observed for saturated species of phosphatidylethanolamine (Fig. 2) and phosphatidylcholine (Fig. 1 and ref. 6).

It may be calculated that S_{DIP} increases at temperatures higher than 40 °C for 14 : 0/14 : 0 phosphatidic acid at $\text{p}^2\text{H} = 8.5$ and 7.0. This indicates that the motion in

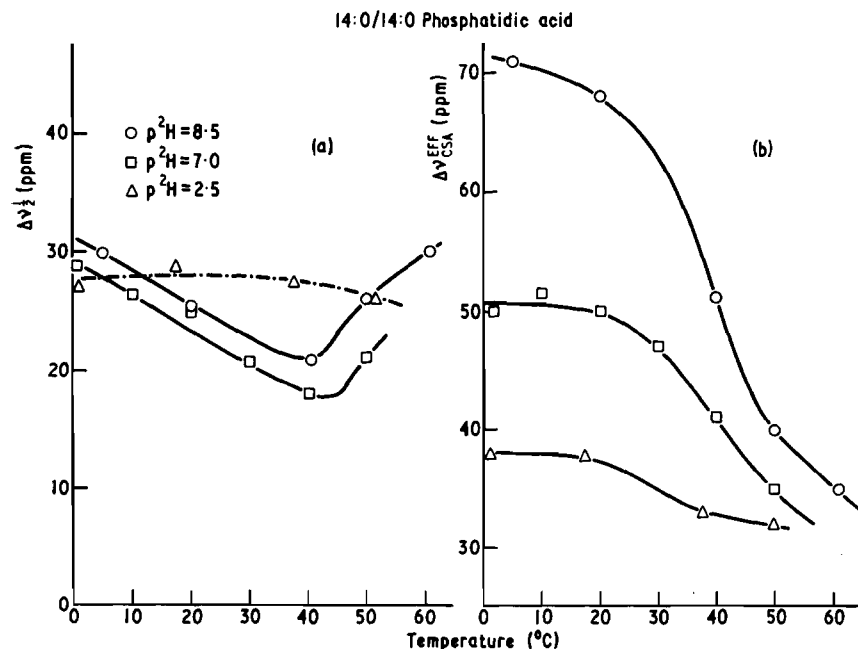


Fig. 5. Temperature dependence of ^{31}P NMR spectral parameters obtained from 129 MHz ^{31}P NMR spectra of aqueous dispersions of 14 : 0/14 : 0 phosphatidic acid at various p^2H values; a, width at half height and b, effective chemical shift anisotropy.

the phosphate group region actually decreases as the temperature is increased above the phase transition temperature, which is again in strong contrast to the behaviour of other saturated phospholipids. Further, at $p^2H = 2.5$ S_{DIP} changes by less than 5% over the temperature interval 0–50 °C.

There are a variety of possible explanations for the above observations. Firstly, it should be noted that the spectra of Fig. 4 strongly resemble spectra obtained for other species of liquid crystalline phospholipids, i.e. where rapid rotation about the long axis of the phospholipid molecule is allowed [6]. In the case of 14 : 0/14 : 0 phosphatidic acid in the gel state similar lineshapes would arise from (1) rotation of the whole lipid molecule, or (2) rapid rotation about the C-C bond between the nearest methylene group to the phosphate group and the rest of the glycerol backbone. Both of these effects would be consistent with the small size of the polar headgroup for phosphatidic acid and consequently reduced steric hindrances to motion. The large changes in Δv_{CSA}^{EFF} as the p^2H is changed would then correspond to changes in the angle between the director of the chemical shift anisotropy tensor and the axis of rotation (i.e. a change in orientation of the phosphate group region with respect to the rest of the molecule). If explanation (2) is more correct, the large decrease in Δv_{CSA}^{EFF} for 14 : 0/14 : 0 phosphatidic acid at $p^2H = 8.5$ on heating from 0 to 50 °C (while S_{DIP} remains relatively constant) would correspond to a reorientation of the phosphate group toward that obtained at lower pH values.

Alternatively, the large changes in Δv_{CSA}^{EFF} may arise from changes in Δv_{CSA} , the rigid lattice chemical shift anisotropy, due to changes in the chemical shielding on changing the net charge of the phosphate group.

Phosphatidylglycerol

The effects of the ionic environment upon the packing properties of phosphatidylglycerol in monolayers and liposomes have been well studied [10, 12–14, 29]. The mean molecular area is reduced on decreasing the pH below the pK value of approx. 4.5 [10] and a corresponding small increase in the hydrocarbon phase transition temperature has been observed [12]. The addition of up to 50 mol % Ca^{2+} or Mg^{2+} brings about a strong condensation of the phosphatidylglycerol molecules which leads to an increase of approx. 20 °C in the gel-liquid crystalline phase transition temperature [10, 12–14, 29]. At higher concentrations of Ca^{2+} or Mg^{2+} the bilayers precipitate and form cylindrical structures with very tight lipid packing where the transition temperature is increased by about 70 °C [12, 13, 29].

The effects of changing the p^2H and adding various concentrations of Ca^{2+} on the ^{31}P NMR spectra of 14 : 0/14 : 0 phosphatidylglycerol in the temperature range 0–60 °C are summarized in Figs. 6 and 7. It has been shown that in the presence of 100 mM NaCl the gel-liquid crystalline phase transition for 14 : 0/14 : 0 phosphatidylglycerol occurs at 23 °C when the $p^2H = 8.0$ [29]. As noted in Fig. 6 a corresponding increase in both $\Delta v_{\frac{1}{2}}$ and Δv_{CSA}^{EFF} occurs below this temperature, indicating that the motion in the phosphate region of the polar headgroup is reduced in the gel state. This is in accord with results obtained for phosphatidylcholine and saturated species of phosphatidylethanolamine. On decreasing the p^2H to 3 however $\Delta v_{\frac{1}{2}}$ is significantly decreased below the phase transition whereas Δv_{CSA}^{EFF} is not much affected.

On adding 40 mol % Ca^{2+} (or Mg^{2+}) to 14 : 0/14 : 0 phosphatidylglycerol the phase transition temperature is increased to 37 °C [29]. This increase in the tran-

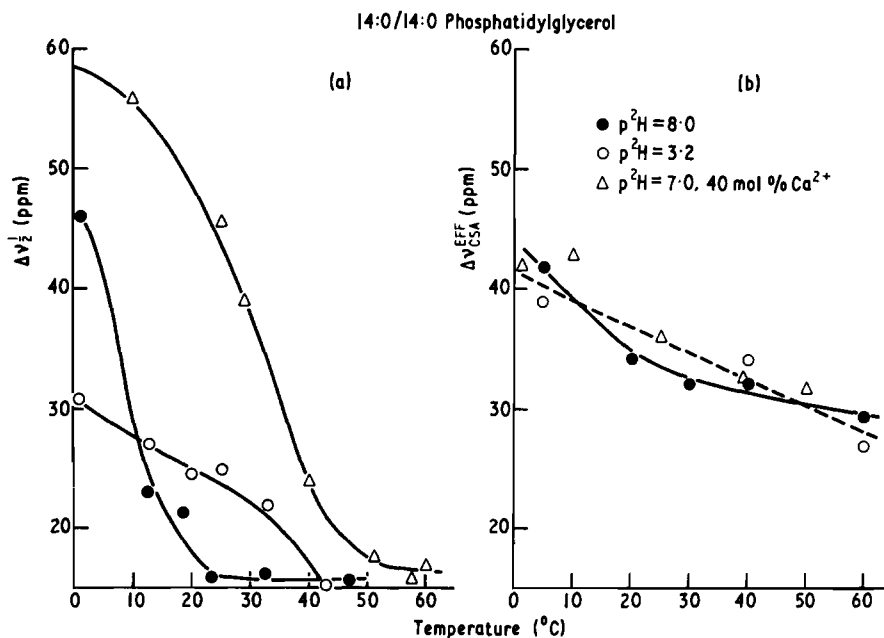


Fig. 6. Temperature dependence of the ^{31}P NMR spectral parameters for 14 : 0/14 : 0 phosphatidylglycerol at different p^2H values and in the presence and absence of 40 mol % Ca^{2+} . a, the width at half height (obtained from non-decoupled 129 MHz spectra) and b, the effective chemical shift anisotropy (obtained from proton-decoupled 36.4 MHz spectra).

sition temperature on adding Ca^{2+} is also observable by ^{31}P NMR, as shown in Fig. 6. Below approx. 45°C $\Delta\nu_{\frac{1}{2}}$ and $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ increase, in accord with the usual phase transition behaviour of the ^{31}P NMR liposome spectra. It is surprising that in the liquid crystalline state where the presence of the divalent cation still strongly affects the molecular packing [10], the effects on $\Delta\nu_{\frac{1}{2}}$ and $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ are negligibly small. It may be noted that completely equivalent effects were obtained on the addition of 40 mol % MgCl_2 to the 14 : 0/14 : 0 phosphatidylglycerol preparations.

The addition of higher concentrations of divalent cations results in the precipitation of the 14 : 0/14 : 0 phosphatidylglycerol and the disappearance of the ^{31}P NMR signal (see Fig. 7). As the high frequency (129 MHz) spectrometer is capable of detecting signals from similar quantities of anhydrous 16 : 0/16 : 0 phosphatidylcholine ($\Delta\nu_{\text{CSA}} = 170$ ppm) [5], it may be concluded that for the Ca^{2+} -phosphatidylglycerol precipitate $\Delta\nu_{\text{CSA}}^{\text{EFF}} \geq 170$ ppm. No signal could be observed even on heating the sample to 70°C (20°C below the phase transition temperature [29]).

The effects of p^2H and divalent cations on the ^{31}P NMR spectra of aqueous dispersions of 12 : 0/12 : 0 phosphatidylglycerol were very similar to those observed for 14 : 0/14 : 0 phosphatidylglycerol. The only major differences were caused by the 20°C lower phase transition temperature of 12 : 0/12 : 0 phosphatidylglycerol [12, 13]. In particular, the cylindrical bilayer structures formed on the addition of equimolar quantities of Ca^{2+} melt at 70°C [12, 13]. At this temperature a ^{31}P NMR signal was observed which corresponded very well with the 14 : 0/14 : 0 phosphatidylglycerol spectra shown in Fig. 7 for $R = 0.4$. At lower temperatures no signal could

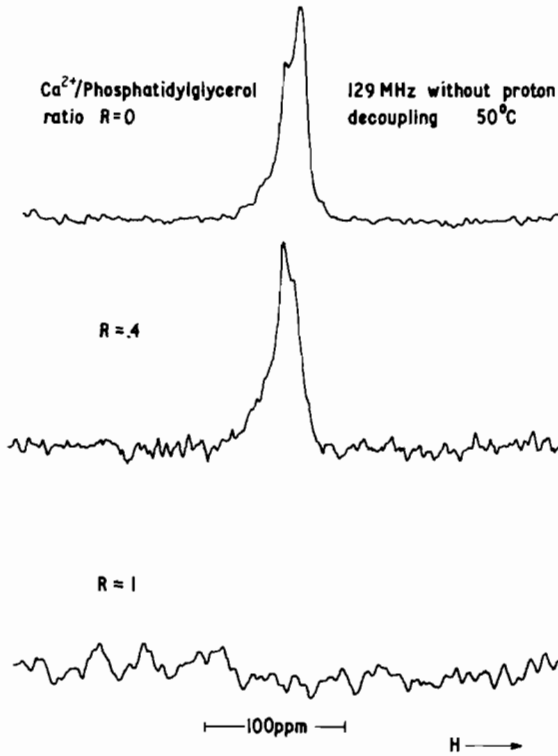


Fig. 7. 129 MHz ^{31}P NMR spectra obtained from aqueous dispersions of 14 : 0/14 : 0 phosphatidylglycerol ($\text{p}^2\text{H} = 8.0$) at 50°C for varying concentrations of Ca^{2+} . The spectra shown for Ca^{2+} /phosphatidylglycerol ratios $R = 0$ and 0.4 were obtained from 40 000 transients whereas the spectrum shown for $R = 1$ was obtained from 1000 000 transients.

be obtained. Again, this result strongly suggests that $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ is very markedly increased in the cylindrical gel phase formed by the Ca^{2+} (or Mg^{2+})-phosphatidylglycerol complex.

The 36.4 MHz proton-decoupled spectra of liquid crystalline 12 : 0/12 : 0 and 14 : 0/14 : 0 phosphatidylglycerol liposomes showed a narrower line at the position of sonicated vesicles on top of the normal chemical shift anisotropy solid state spectrum. The fact that a similar narrow line was not observed on the higher frequency 129 MHz machine indicated that this effect is due to the smaller value of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ relative to the dipolar coupling at 36.4 MHz. It may also be noted that the 129 MHz ^{31}P NMR spectra of egg phosphatidylglycerol in water at $\text{pH} 8.0$ and 3.0 showed identical spectra to those observed in $^2\text{H}_2\text{O}$. This indicates that the protonation of the phosphatidylglycerol phosphate has no effect on the static dipolar interactions experienced by the phosphate phosphorus.

The 129 MHz ^{31}P NMR spectra of aqueous dispersions of 18 : 1c/18 : 1c phosphatidylglycerol showed very different characteristics from the saturated phosphatidylglycerol species. A single narrow ($\Delta\nu_{\frac{1}{2}}$ approx. 600 Hz) symmetrical line was observed (see Fig. 8) both at $\text{pH} 3.0$ and 8.0 over the entire temperature range 0 – 60°C . Similar narrow lines ($\Delta\nu_{\frac{1}{2}} = 80$ Hz) were obtained at 36.4 MHz in the presence of

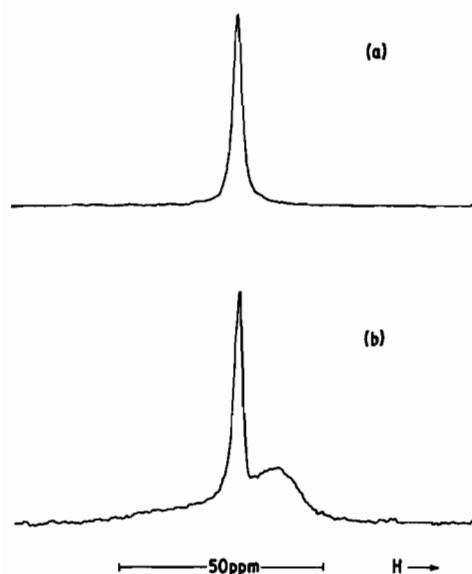


Fig. 8. 129 MHz ^{31}P NMR spectra of aqueous dispersions of 18 : 1c/18 : 1c phosphatidylglycerol ($\text{p}^2\text{H} = 8.0$) at 50 °C. a, in the absence of Ca^{2+} and b, in the presence of 40 mol % Ca^{2+} .

proton decoupling. On the addition of 40 mol % Ca^{2+} , however, a broad solid state component appeared, as shown in Fig. 8. The narrow lines would be consistent with a non-lamellar phase in which the lateral diffusion of the phospholipids produced isotropic averaging. The addition of Ca^{2+} may then be thought to decrease the charge density and induce a bilayer configuration. Alternatively, these results would be consistent with the formation of 18 : 1c/18 : 1c phosphatidylglycerol into large vesicles, which are aggregated on addition of Ca^{2+} .

DISCUSSION

As shown in this and other recent work [5–7] the large phospholipid liposomes (which consist of many layers of concentric bilayers) exhibit characteristic “solid state” ^{31}P NMR spectra, which have a low field shoulder. For phospholipids in the liquid crystalline state, this type of lineshape is associated with lipids which experience rapid rotation about the long axis of the molecule, but which have relatively restricted motion about axes perpendicular to this axis of rotation (ref. 7 and Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., unpublished). Obviously, such motional characteristics are consistent with phospholipids in a bilayer configuration, and the observation of the solid state type of ^{31}P NMR lineshape may therefore be taken to indicate bilayer structure. It should be noted, however, that the dimensions of the liposomes must be large enough to make isotropic averaging mechanisms such as liposome tumbling and lateral diffusion of the phospholipid ineffective. In the case of the much smaller “vesicles” obtained by sonicating liposomes for example, fast isotropic tumbling produces high resolution ^{31}P NMR spectra [5].

It is interesting to extend these considerations to phospholipids which may

exhibit hexagonal or other non-lamellar phases in addition to the standard bilayer phase. The clearest effect observed concerns the transition of 18 : 1c/18 : 1c phosphatidylethanolamine from a liquid crystalline lamellar phase to a hexagonal phase consisting of long "pipes" of phospholipid, as shown in Fig. 3. The small diameter of the pipes [30] and the fast lateral diffusion of the liquid crystalline phosphatidylethanolamine combine to introduce averaging over a direction perpendicular to the long axis of the molecule, i.e. around the pipe. This is reflected by the change in sign and reduction by a factor of 2 of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$. The fact that no significant averaging along the length of the phospholipid pipe is observed implies that the constituent pipes are fairly long ($\geq 500 \text{ \AA}$ if it is assumed that the lateral diffusion rate $D_{\text{t}} = 1 \cdot 10^{-8} \text{ cm}^2/\text{s}$). In the case of 16 : 1c/16 : 1c phosphatidylethanolamine the ^{31}P NMR spectra reflect a phase characterized by a very narrow ^{31}P NMR line ($\Delta\nu = 400 \text{ Hz}$) which is produced by an isotropic averaging mechanism due to lateral diffusion of the phospholipid. If the (unknown) phase observed for 16 : 1c/16 : 1c phosphatidylethanolamine is also hexagonal, the ^{31}P NMR results show that the constituent pipes are appreciably shorter than in the case of 18 : 1c/18 : 1c phosphatidylethanolamine ($\leq 100 \text{ \AA}$ for $D_{\text{t}} = 1 \cdot 10^{-8} \text{ cm}^2/\text{s}$). In general it should be noted that ^{31}P NMR is a very sensitive indicator for phases where fast isotropic averaging by tumbling or lateral diffusion is possible.

The effective chemical shift anisotropy ($\Delta\nu_{\text{CSA}}^{\text{EFF}}$ and the width at half height ($\Delta\nu_{\frac{1}{2}}$) of the normal "solid state" ^{31}P NMR liposome spectra may be employed to obtain the chemical shift anisotropy order parameter $S_{\text{CSA}} = \Delta\nu_{\text{CSA}}^{\text{EFF}}/\Delta\nu_{\text{CSA}}$ [7] (where $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ is the rigid lattice chemical shift anisotropy) and the dipolar order parameter S_{DIP} [6], respectively. Unfortunately it is difficult to relate these order parameters to the detailed motion in the methylene-phosphate-methylene region of the polar headgroup as such details are sensitive to the particular model of molecular motion chosen. In the case of S_{CSA} the situation is complicated by the fact that the orientation of the main director of the rigid lattice chemical shift anisotropy tensor is not as yet known. ^{31}P NMR studies of anhydrous 16 : 0/16 : 0 phosphatidylcholine suggest that the rigid lattice chemical shift tensor is approximately axially symmetric, and that $\Delta\nu_{\text{CSA}} \approx 170 \text{ ppm}$, but also show that the sign of $\Delta\nu_{\text{CSA}}$ is different to that observed for $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ in hydrated phosphatidylcholine bilayers [5]. There is also a possibility (as indicated in this study for charged phospholipids) that $\Delta\nu_{\text{CSA}}$ may be sensitive to the pH or the presence of divalent cations. In this work we have therefore only reported the values of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ obtained, and the changes in $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ observed may arise from changes in motion, orientation, or $\Delta\nu_{\text{CSA}}$ itself.

The dipolar order parameter S_{DIP} is calculated from the width at half height ($\Delta\nu_{\frac{1}{2}}$) of the non-decoupled ^{31}P NMR spectra under the assumption that only the two nearest neighbour methylene groups contribute to the observed dipolar broadening and that the contribution of these two groups is equal [6]. Interpretation of S_{DIP} in terms of local motion is complicated by the fact that S_{DIP} is then the square root of the sum of squares of four separate order parameters, and is therefore sensitive to a wide variety of local motions and orientations. Thus the fact that the number obtained for S_{DIP} for liquid crystalline 16 : 0/16 : 0 phosphatidylcholine ($S_{\text{DIP}} = 0.13$ [6]) is similar to values obtained for ^2H order parameters [27] of the two nearest neighbour methylene groups is probably fortuitous. S_{DIP} is most validly employed as an indicator of changes, in that changes in S_{DIP} show changes in either the local

TABLE I

DIPOLAR ORDER PARAMETERS FOR LIQUID CRYSTALLINE PHOSPHATIDYLCHOLINES, PHOSPHATIDYLETHANOLAMINES AND PHOSPHATIDYLGLYCEROLS

Dipolar order parameters for 14 : 0/14 : 0 phosphatidic acid in the gel state are included to illustrate the strong p²H dependence of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ for constant values of S_{DIP} .

Lipid	Temperature (°C)	p ² H	$\Delta\nu_{\text{CSA}}^{\text{EFF}}$ (ppm)	S_{DIP}
14 : 0/14 : 0 phosphatidylcholine	60	7.2	45.5	0.13
12 : 0/12 : 0 phosphatidylethanolamine	52	7.0	39	0.145
14 : 0/14 : 0 phosphatidylethanolamine	61	7.0	41	0.145
12 : 0/12 : 0 phosphatidylglycerol	20	8.0	31	0.16
14 : 0/14 : 0 phosphatidylglycerol	47.5	8.0	30.5	0.15
14 : 0/14 : 0 phosphatidylglycerol + 40 mol % Ca ²⁺	60	7.0	30	0.16
14 : 0/14 : 0 phosphatidic acid	5	8.5	71	0.40
14 : 0/14 : 0 phosphatidic acid	5	7.0	50.5	0.40
14 : 0/14 : 0 phosphatidic acid	5	2.5	38	0.40

motion in the methylene-phosphate-methylene region and/or changes in the local orientation of the methylene group(s) with respect to the phosphorus atom.

Table I shows representative values of $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ and S_{DIP} for various saturated phospholipids in the liquid crystalline state. There are several important conclusions which may be drawn from this data. Firstly, in accord with previous work [6] similar values of S_{DIP} and $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ are observed for the phospholipids which differ only in chain length. Thus the motional properties observed in the phosphate region of the polar headgroup are independent of chain length in the liquid crystalline state. Secondly, the values obtained for S_{DIP} are remarkably similar for the saturated species of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol. Only 14 : 0/14 : 0 phosphatidic acid forms an exception. These results show that the motion in the methylene-phosphate-methylene region of the polar headgroup is very similar for these different liquid crystalline phospholipid species. It is to be noted that $\Delta\nu_{\text{CSA}}^{\text{EFF}}$ varies slightly between the different species, but, as previously indicated, this may arise from different orientations of the polar headgroup in the phosphate group region, or from different values of the rigid lattice chemical shift anisotropy tensor. A third conclusion is that the motion of the phosphatidylglycerol polar headgroup is not affected by the presence of divalent cations, as long as the phospholipid is in the liquid crystalline state. The only effect of Ca²⁺ observed in this study is that the phase transition temperature as detected by the phosphate group is increased by 20 °C in the presence of approx. 50 mol % Ca²⁺, and by about 70 °C for Ca²⁺/phosphatidylglycerol ratios of approx. 1, in complete agreement with previous differential scanning calorimetry experiments [29]. This result is also in full accord with previous results obtained for isolated membranes and derived liposomes of *Acholeplasma laidlawii* B, (for which 40 % of the lipid is phosphatidylglycerol) where it was observed that the addition of equimolar quantities of Ca²⁺ does not materially affect the motional properties of the polar headgroup in the phosphate group region [8]. It may thus be concluded that the decrease in the available area per molecule on the addition of Ca²⁺ [10] does not affect this motion. This result is in agreement with a previous study [6] which shows that

changes in the available area per liquid crystalline phosphatidylcholine molecule also had little effect on the motion in the phosphate group region.

The effects of changes in p^2H on the ^{31}P NMR spectra of acidic phospholipids are very marked. In the case of 14 : 0/14 : 0 phosphatidic acid the large changes in $\Delta\nu_{CSA}^{EFF}$ (but constant values of S_{DIP}) on changing the p^2H suggest changes in the orientation of the phosphate group, or that the rigid lattice chemical shift anisotropy tensor is sensitive to the protonation of the phosphate group. The fact that a similar p^2H dependence of $\Delta\nu_{CSA}^{EFF}$ is not observed for 14 : 0/14 : 0 phosphatidylglycerol (see Fig. 6) or phosphatidylcholine implies that a change in the orientation may be the more correct interpretation. Further work on orientated systems is in progress to clarify this point. It may also be observed for 14 : 0/14 : 0 phosphatidic acid that the motion in the phosphate region is much less sensitive to the gel or liquid crystalline nature of the hydrocarbon chains than for other saturated phospholipids, and furthermore, that this motion is increasingly restricted above the transition temperature. These effects are not as yet understood, but could possibly arise due to different hydration characteristics of phosphatidic acid. In the case of 14 : 0/14 : 0 phosphatidylglycerol the large decrease in $\Delta\nu_3$ on changing the p^2H from 8.0 to 3.2 shows a corresponding increase in the motion available to the methylene-phosphate-methylene region of the polar headgroup when the polar headgroup is uncharged. This could be accounted for by a change in the hydration shell, as a negatively charged phosphate group must have considerably more "bound" water than a neutral phosphate group. At lower pH values the size of the hydration shell might be reduced enough so that the motion in the phosphate group region increases, even though there is a reduction in the mean molecular area due to the charge neutralization.

In the gel phase the behaviour of the ^{31}P NMR liposome spectra of saturated species of phosphatidylethanolamine and phosphatidylglycerol is similar to that observed for various species of phosphatidylcholine. As the temperature is increased toward the transition temperature the spectra become progressively narrower, reflecting less restricted motion in the phosphate group region until at, or a few degrees below the transition temperature the spectrum is similar to that observed in the liquid crystalline state. This is in accord with previous work, where it is shown that the main cause of the narrowing is the onset of rapid axial rotation of the phospholipid molecule (ref. 7 and Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., unpublished). The fact that most of the observed narrowing occurs well below the phase transition temperature shows that relatively fast rotation ($\tau_R \approx 10^{-4}$ s) may occur well below the transition temperature. It should be noted that there are quantitative differences between the observed narrowing for the different phospholipids however. In particular, the temperature interval over which the narrowing takes place is much less ($\Delta T \approx 15^\circ C$) for 14 : 0/14 : 0 and 16 : 0/16 : 0 phosphatidylcholine [6] than for 12 : 0/12 : 0 and 14 : 0/14 : 0 phosphatidylethanolamine ($\Delta T \geq 50^\circ C$) and 14 : 0/14 : 0 phosphatidylglycerol ($\Delta T \approx 30^\circ C$). In the case of 14 : 0/14 : 0 and 16 : 0/16 : 0 phosphatidylcholine most of the narrowing takes place in the region of the "pretransition" often noted in differential scanning calorimetry studies [31], and it is therefore tempting to associate this pretransition with the onset of rapid axial rotation, as has been previously suggested [32]. The relatively small values of ΔT observed for the phosphatidylcholines (with the exception of 18 : 0/18 : 0 phosphatidylcholine [6]) might be attributed to the formation of the $p\beta'$ phase [33] at the pretransition

[34] in which the molecules become rapidly immobilized due to strong intermolecular interactions between the polar headgroups. The saturated phosphatidylethanolamines and phosphatidylglycerols do not form a $p\beta'$ phase but remain in the normal lamellar gel phase below the hydrocarbon phase transition temperature (ref. 23 and Verkley, A. J., unpublished) and have no detectable pretransition [24, 25, 29]. The large ΔT values obtained for these lipids may then reflect (1) that no strong intermolecular interactions (such as may be obtained in the $p\beta'$ phase) occur, and thus the motion is more gradually restricted below the phase transition or (2) that the pretransition of phosphatidylethanolamines and phosphatidylglycerols is much broader than for phosphatidylcholines and thus cannot be resolved by calorimetric techniques.

ACKNOWLEDGEMENTS

We would like to thank Drs. R. E. Richards F.R.S. and G. K. Radda for their interest and the provision of laboratory facilities. We also thank Dr. A. C. McLaughlin for helpful discussions, Dr. D. I. Hoult for valuable assistance and Mrs. E. Richards for the provision of 36.4 MHz facilities. P.R.C. acknowledges support from the Medical Research Council (Canada) and B. de K. is the recipient of a stipend from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). Financial support was also supplied by the S.R.C.

REFERENCES

- 1 Michaelson, D. M., Horwitz, A. F. and Klein, M. P. (1973) *Biochemistry* 12, 2637-2645
- 2 Berden, J. A., Barker, R. W. and Radda, G. K. (1975) *Biochim. Biophys. Acta* 375, 186-208
- 3 de Kruyff, B., Cullis, P. R. and Radda, G. K. (1975) *Biochim. Biophys. Acta* 406, 6-20
- 4 Berden, J. A., Cullis, P. R., Hoult, D. I., McLaughlin, A. C., Radda, G. K. and Richards, R. E. (1974) *FEBS Lett.* 46, 55-58
- 5 McLaughlin, A. C., Cullis, P. R., Berden, J. A. and Richards, R. E. (1975) *J. Magn. Res.*, 20, 146-165
- 6 Cullis, P. R., de Kruyff, B. and Richards, R. E. (1976) *Biochim. Biophys. Acta* 426, 433-446
- 7 McLaughlin, A. C., Cullis, P. R., Hemminga, M. A., Hoult, D. I., Seeley, P. J., Radda, G. K. and Richards, R. E. (1975) *FEBS Lett.* 57, 213-218
- 8 de Kruyff, B., Cullis, P. R., Radda, G. K. and Richards, R. E. (1976) *Biochim. Biophys. Acta* 419, 411-424
- 9 Ohki, S. and Papahadjopoulos, D. (1970) *Surface chemistry of biological systems*, p. 155, Plenum Press
- 10 Tocanne, J. F., Ververgaert, P. H. J. Th., Verkley, A. J. and van Deenen, L. L. M. (1974) *Chem. Phys. Lipids* 12, 201-219
- 11 Träuble, H. and Eible, H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 214-219
- 12 Verkley, A. J., de Kruyff, B., Ververgaert, P. H. J. Th., Tocanne, J. F. and van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta* 339, 432-437
- 13 Ververgaert, P. H. J. Th., de Kruyff, B., Verkley, A. J., Tocanne, J. F. and van Deenen, L. L. M. (1975) *Chem. Phys. Lipids* 14, 97-101
- 14 Jacobson, K. and Papahadjopoulos, D. (1975) *Biochemistry* 14, 152-161
- 15 van Deenen, L. L. M. and de Haas, G. H. (1964) *Adv. Lipids Res.* 2, 168-229
- 16 Dawson, R. M. C. and Hemington, N. (1967) *Biochem. J.* 102, 76-86
- 17 Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* 37, 911-918
- 18 Davidson, F. M. and Long, C. (1958) *Biochem. J.* 69, 458-466
- 19 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) *Biochim. Biophys. Acta* 311, 330-348
- 20 Tocanne, J. F., Vechey, H. M., Opdenkamp, J. A. F. and van Deenen, L. L. M. (1974) *Chem. Phys. Lipids* 12, 389-403

- 21 Houlton, D. I. and Richards, R. D. (1975) *Proc. R. Soc. Lond.* 344, 311-340
- 22 Ladbroke, B. D. and Chapman, D. (1969) *Chem. Phys. Lipids* 3, 304-367
- 23 Verkley, A. J. and Ververgaert, P. H. J. Th. (1975) *Ann. Rev. Phys. Chem.* 26, 101-122
- 24 Chapman, D., Williams, R. M. and Ladbroke, B. D. (1967) *Chem. Phys. Lipids* 1, 445-475
- 25 Vaughan, D. J. and Keough, K. M. (1974) *FEBS Lett.* 47, 158-161
- 26 Rand, R. P., Tinker, D. O. and Fast, P. G. (1971) *Chem. Phys. Lipids* 6, 333-342
- 27 Gally, H. U., Niederberger, W. and Seelig, J. (1975) *Biochemistry* 14, 3647-3652
- 28 Träuble, H. and Eibl, H. (1975) in *Functional Linkage in Biomolecular Systems* (Schmitt, F. O., Schneider, D. M. and Crothers, D. M., eds.), p. 59-90, Raven Press, New York
- 29 Van Dyck, P. W. M., Ververgaert, P. H. J. Th., Verkley, A. J., van Deenen, L. L. M. and de Gier, J. (1975) *Biochim. Biophys. Acta* 406, 465-479
- 30 Reiss-Husson, F. (1967) *J. Mol. Biol.* 25, 363-372
- 31 Hinz, H. J. and Sturtevant, J. M. (1972) *J. Biol. Chem.* 19, 6071-6075
- 32 Lee, A. G. (1975) *Prog. Biophys. Mol. Biol.* 29, 3-56
- 33 Tardieu, A., Luzzati, V. and Reman, F. C. (1973) *J. Mol. Biol.* 75, 711-720
- 34 Jeniak, M. J., Shipley, G. G. and Small, D. M. (1975) *Proceedings of the 5th International Biophysics Conference*, Copenhagen