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A ^{1}H NMR STUDY OF THE EFFECTS OF METAL IONS, CHOLESTEROL AND *n*-ALKANES ON PHASE TRANSITIONS IN THE INNER AND OUTER MONOLAYERS OF PHOSPHOLIPID VESICULAR MEMBRANES

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Summary

A novel method is described which allows study of the effect of various asymmetries across a phospholipid bilayer. High resolution, ^{1}H NMR spectra of dipalmitoyl phosphatidylcholine single-bilayer vesicles are obtained at various temperatures in the presence of the lanthanide ions Pr^{3+} and Gd^{3+}. The spectra are used to detect separately the phase transitions which occur in each monolayer of the bilayer.

1. The separate ^{1}H NMR signals from the inner and outer head-groups of the vesicles are obtained using a concentration of 6 mM Pr^{3+} in the extravesicular solution. The effect of addition of other ions, Ca^{2+}, Mg^{2+} and K^+, to the extra- or intravesicular solution is studied. It is found that interaction of metal ions with the head-groups of one monolayer can be transmitted to the other monolayer and alters the temperature and extent of the phase transitions and pre-transitions observed in each layer. If a suitable metal ion concentration gradient is set up across the bilayer then the two monolayers can be in different phases at temperatures near the phase transition.

2. Using 0.2 mM Gd^{3+} in the extra- or intravesicular solutions indicates that there are differences between the phase behaviour and mobility of the lipid in the inner and outer monolayers, which can be attributed to their difference in curvature.

3. Dipalmitoyl phosphatidylcholine vesicles containing 0—50 mol% cholesterol are studied using the same techniques and the effect of increasing concentration of cholesterol on each monolayer is observed. The data obtained indicate that even for a symmetrical distribution of cholesterol its presence can reverse the effect of a metal ion concentration gradient on the relative fluidity of the two monolayers. At 15 mol% cholesterol the bilayer is very susceptible to lysis at temperatures near the phase transition.

4. When *n*-alkanes are incorporated into dipalmitoyl phosphatidylcholine

bilayers they affect the temperature of the phase transitions, depending on their chain length. The stability of the bilayer is also affected, but only at temperatures near T_c, and by alkanes of similar hydrocarbon chain length to those in the acyl chains of the lipid. There is thus a striking correlation between the chain length-dependent effect of alkanes as promotors of chemical carcinogenesis and their ability to de-stabilise the phospholipid bilayer.

Introduction

The study of molecular motion and phase transitions in model phospholipid systems [4—7,12] has given insight into molecular organisation and function in biological membranes. However, the typical physical methods used in these studies do not as yet allow the behaviour of the individual monolayers in the lipid bilayer to be separately studied. A separation of signals from each monolayer is necessary if asymmetric properties of the bilayer are to be detected directly and the possible functional significance of these properties recognised [2,3].

To monitor each monolayer separately and simultaneously we have combined variable temperature, high resolution ^1H NMR spectroscopy of single-bilayer phospholipid vesicles [8], together with the use of the lanthanide ions Pr^{3+} and Gd^{3+} to give the required separation of signals from the inner and outer monolayers [9]. The method depends on measuring the line width of the ^1H signals arising from the choline head-groups, $N^+(CH_3)_3$ forming the boundaries of the intra- and extravesicular surfaces. When the temperature is varied, the width of the signal at half height ($\nu_{1/2}$) changes, since it is governed by the motion of the choline protons. There has been controversy over the extent of line narrowing due to (a) the tumbling rate of the vesicles, and (b) intra- and inter-molecular motions of the phospholipid molecules in the bilayer [10,11]. We observe different variations in $\nu_{1/2}$ for the inner and outer head-group signals over the temperature range studied (typically 60—25°C), indicating that the changes in $\nu_{1/2}$ are not due solely to alterations in the tumbling rate of the whole vesicle, but also reflect changes in intra- and inter-molecular motions which occur during phase transitions. Our observations also confirm and extend a number of studies [8,16] which suggest that the motion experienced by the polar head-group of the phospholipids is sensitive to the phase transition of the hydrocarbon chains. Thus the temperatures we record for the gel to liquid-crystal transitions (T_c) and pre-transitions (T_p) correspond to those determined by methods which monitor the hydrocarbon regions, e.g. calorimetry [6], fluorescence [14] and Raman spectroscopy [13].

Despite the existence of metal ion concentration gradients across cellular membranes, very little is known about the effects of such asymmetry on membrane properties [22]. The method described here is well suited to the investigation of asymmetry in bilayer properties resulting from differences in membrane curvature [20] and from concentration gradients across the bilayer. The results obtained using the neutral dipalmitoyl phosphatidylcholine bilayer begin to reveal the extent of the influence of each monolayer on the other, and form the basis upon which further experiments using negatively charged lipids

and vesicles of known compositional asymmetry [27] can be interpreted.

To investigate a possible selective interaction between hydrophobic substances and each monolayer, we have used cholesterol and *n*-alkanes. The most intensively studied lipid-lipid interaction is that between cholesterol and phospholipids (for reviews, see refs. 23 and 24) but many of the details are still obscure. *n*-Alkanes have been extensively used in the formation of planar bilayer membranes [25] and can have physiological effects on membranes in the promotion of cancer [26] but no previous attempt seems to have been made to study the effects of *n*-alkanes on membrane properties in bilayer vesicles.

Materials and Methods

Chemicals. DL-α-Dipalmitoyl phosphatidylcholine, cholesterol (puriss grade), gadolinium and praeseodmium chlorides were obtained from Koch-Light. The purity of the dipalmitoyl phosphatidylcholine was checked by thin-layer chromatography; no traces of lysophosphatidylcholine were seen after sonication procedures. *n*-Octane, *n*-decane and *n*-dodecane were obtained from B.D.H., while tetradecane, hexadecane and octadecane from Sigma. A further sample of hexadecane was obtained from Hopkin and Williams, Essex (standard for gas chromatography), and gave the same results as the sample from Sigma. Gas-liquid chromatography of the *n*-alkanes showed an absence of substantial cross-contamination with the other *n*-alkanes. ^2H_2O (99.8 atom % ^2H) was obtained from Prochem.

Preparation of vesicles. The vesicles were prepared by sonicating 50 mg of dipalmitoyl phosphatidylcholine in 2 ml ^2H_2O for 10 min at 50—60°C, using a Dawe Soniprobe Type 573A fitted with a microtip, at a setting delivering approx. 25 W. Under these conditions it has been shown that a homogeneous population of small vesicles is produced, each containing a single lipid bilayer [27]. The dipalmitoyl phosphatidylcholine vesicles so formed have consistent average diameters of 26 nm, as deduced from the ratio of the signal areas from the outer and inner phospholipids, which is 1.8 in a large number of our recorded samples. The homogeneity of the vesicles was also checked using electron microscopy of freeze-fractured samples. In order to ensure that the condition of each vesicle sample was similar, they were not allowed to fall below 50°C before commencing the recording of NMR spectra. For the same reason they were not centrifuged, but the sonicator probe tip was repolished after every few sonications, a procedure which avoided contamination by Ti particles from the microtip.

The concentration of metal ions in the extravesicular solution is adjusted by adding calculated volumes of stock solutions to the sonicated lipid. When ions are to be introduced into the intravesicular solution the dipalmitoyl phosphatidylcholine is shaken with ^2H_2O solutions of the ions at the desired concentration for 1 h at 60°C. The liposomes formed are then sonicated as above. Gd^{3+} ($2 \cdot 10^{-4}$ M) is removed from the extravesicular solution by adding 10^{-3} M EDTA or by ion-exchange chromatography using a small column of Bio-rad Chelex resin in the $^2H^+$ form. The line-width of the signal obtained from the outer monolayer by both methods of removing the extravesicular Gd^{3+} is

identical from 60 to 40°C. Cholesterol or *n*-alkanes were incorporated into the dipalmitoyl phosphatidylcholine by dissolving weighed amounts of dipalmitoyl phosphatidylcholine, cholesterol or hydrocarbon in chloroform, then removing the solvent under a stream of nitrogen, followed by evacuation. No loss of weight occurred during this process for cholesterol or *n*-alkanes above C_{12}, but for shorter chain lengths some loss of hydrocarbon took place. The accurate mol% was then based on the final weight of material. The dipalmitoyl phosphatidylcholine cholesterol and dipalmitoyl phosphatidylcholine alkane mixtures were then sonicated as above except that those containing more than 25 mol% cholesterol were sonicated at 70°C for 15 min. Vesicles containing *n*-alkanes of longer chain length than C_{12} tend to precipitate as a gel on cooling from 60°C, but this tendency is considerably reduced in the presence of the 6 mM Pr^{3+} used in the experiments described.

NMR experiments. The ^1H NMR spectra were recorded using a JEOL C-60HL spectrometer at 60 MHz, fitted with a calibrated temperature control. Spectra were recorded at decreasing temperatures, typically over the range 60—30°C, always commencing above the transition temperature. The recorded line widths at half-height ($\nu_{1/2}$) are the average obtained from between two and four separate experiments using different samples of vesicles of the same composition.

Results and Discussion

Effect of metal ions on the ^1H NMR spectra of dipalmitoyl phosphatidylcholine vesicles

Both ^1H NMR [8] and ^{31}P NMR [16] can be used to detect the gel to liquid-crystal transition temperature (T_c) in phospholipid bilayers. This is illustrated in Fig. 1. The upper inset shows the overlapping ^1H NMR signals from the $N(CH_3)_3^+$ head-groups on the outer (O) and inner (I) surfaces of the dipalmitoyl phosphatidylcholine vesicles at 42°C. The width at half-height ($\nu_{1/2}$) of the combined head-group signal varies with temperature as shown in Fig. 1(a). On lowering the temperature from 60°C, $\nu_{1/2}$ undergoes a large increase at 40—41°C. This represents the upper limit of the main phase transition and is close to the values of T_c for dipalmitoyl phosphatidylcholine liposomes and vesicles as determined by a number of methods [13,14]. These comparative studies of liposomes and vesicles have shown that the width of the phase transition is greater in vesicles, this probably representing a lower cooperativity or smaller size of the cooperative unit in the vesicle bilayers [5]. In the ^1H NMR data of Fig. 1, the separation between the signals O and I (the chemical shift difference, $\Delta\nu$) and the width at half-height ($\nu_{1/2}$) of each signal O and I, all vary with temperature. So the combined width of the overlapping signals cannot be used to give any detailed information on the temperature range or degree of cooperativity of the phase transition.

However, as is now well documented [9], in the presence of millimolar concentrations of suitable lanthanide ions, the signals O and I can be separated. Thus on adjusting the extravesicular solution to 6 mM Pr^{3+} the separation is as shown in Fig. 1(b) (inset). The paramagnetic Pr^{3+} is in rapid equilibrium with the external phosphate sites only, which results in a down-field shift of the outer head-group signal O with respect to the inner head-group signal I. The

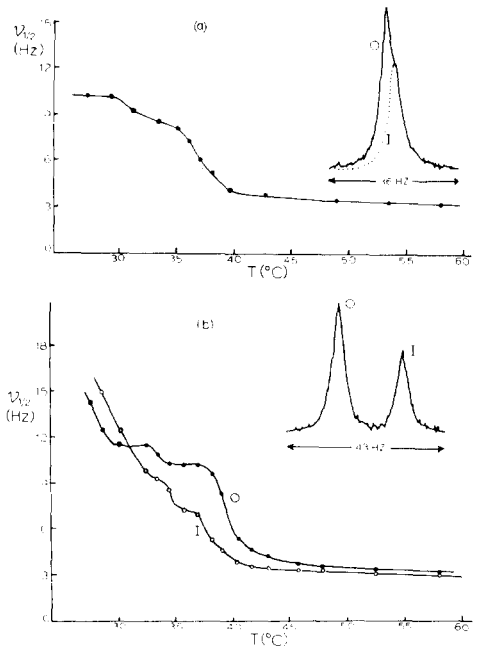

Fig. 1. Temperature dependence of the width at half-height ($\nu_{1/2}$) of ^{1}H NMR signals from the head-groups (-N(CH_3)$_3$) of dipalmitoyl phosphatidylcholine vesicles: (a) ●———●, values of $\nu_{1/2}$ for the total head-group signal; inset shows the total unsplit signal obtained at 42°C. (b) Separate head-group signals obtained using 6 mM Pr^{3+} in the extravesicular solution; ●———●, values of $\nu_{1/2}$ for the outer head-groups signal (O); ○———○, values of $\nu_{1/2}$ for the inner head-groups signal (I); inset shows the separated outer head-group signal (O) and inner head-group signal (I), recorded at 58°C. Experiments carried out as in Materials and Methods.

temperature variation of $\nu_{1/2}$ for these two well-separated signals can then easily be followed. The results are shown in Fig. 1(b). The variation of $\nu_{1/2}$(I) and $\nu_{1/2}$(O) is seen to be different, which implies immediately that they are not due to a change in overall vesicle tumbling rates as the temperature is altered. The upper limit of T_c for the outer monolayer (signal O) is 44—46°C, while that for the inner monolayer (signal I) is 42—43°C. Both signals also now show well-defined lower limits to T_c (37°C (outer and inner)) and pronounced pre-transitions centred at temperatures (T_p) of 33°C (outer layer) and 34°C (inner layer). If the temperature is increased to 60°C again, following a cooling scan, then $\nu_{1/2}$ for O and I return to the original values and there is no change in the separation between the signals or their area ratios. There is also no change in sample turbidity. These observations imply that the vesicles are stable and remain impermeable under the conditions applied [28].

Experiments using differential scanning calorimetry (DSC) of liposomal preparations have shown that the presence of metal ions increases the values of T_c (and T_p for cholines), even though the effect is considerably less for neutral lipids (phosphatidylcholine or sphingomyelin) than for negatively charged lipids such as phosphatidylserine [29,30]. The increased value of T_c for the outer layer (O) as observed in Fig. 1(b) could then be explained by the interaction of the Pr^{3+} with the outer layer. A small transmembrane effect is also implied by Fig. 1(b) since the upper limit of T_c for the inner layer is also

increased to 43°C compared to that for the unsplit peak (no ions in contact with vesicles), shown in Fig. 1(a), where the upper limit to T_c is 40—41°C.

The temperatures of the pre-transitions seen in Fig. 1(b) correspond with those determined for liposomes of dipalmitoyl phosphatidylcholine using DSC and X-ray methods [6,31]. Recently Brady and Fein [12] also report pre-transitions occurring in vesicles. The pre-transitions have been associated with hydrocarbon chain tilting and change in head-group conformation [17–19,31]. These two events may occur together and it is therefore not surprising that since our ^1H NMR method is monitoring head-group signals, the method should be sensitive to the pre-transition changes. The particularly strong pre-transitions seen in the outer head-group signal in Figs. 1(b), 2(a), 3(a) and 4(a) would imply that the presence of Pr^{3+} enhances the conformation change of the outer head-groups which accompanies a change in the tilt of the acyl chains.

At this stage we cannot rule out the possibility that the difference in the two monolayer behaviours as seen in Fig. 1(b) may be due in part to their difference in both curvature and the packing of the lipid molecules. This possibility can be investigated using very low concentrations of Gd^{3+} as described below. Meanwhile the phase transition curves of Fig. 1(b) (6 mM Pr^{3+} outside vesicles) can be used as a control with which to compare the effects of addition of other ions to the extra- and intravesicular aqueous space. This method has the advantage that the signal from both outer and inner monolayers can be observed simultaneously from the same sample of vesicles under the same conditions.

Figs. 2(a) and 2(b) show the result of increasing the extravesicular Pr^{3+} concentration to 12 and 21 mM compared with 6 mM. In Fig. 2(a) the effect on the outer monolayer can be seen to be 3-fold: (i) $\nu_{1/2}$ is broadened at all temperatures compared to 6 mM, this is evidently a direct result of increased interaction of Pr^{3+} with the head-groups causing a decrease in their mobility; (ii) an increase in sharpness of upper limit of the phase transition; (iii) the mid-points of T_c and T_p occur at higher temperatures. The effect on the inner monolayer is seen in Fig. 2(b): (i) $\nu_{1/2}(I)$ is much less increased than $\nu_{1/2}(O)$, which is as expected since the inner head-groups are not in contact with increased concentration of Pr^{3+}; (ii) the upper limit of T_c is increased in sharpness; (iii) the mid-point of T_c is raised in temperature but less so than for the outer layer; (iv) $\nu_{1/2}(I)$ can be discerned to decrease just above T_c in the case of 21 mM Pr^{3+} (this feature will become more pronounced under the conditions described below). These observations suggest that the changes in $\nu_{1/2}$ monitor both the local mobility of the $\overset{+}{N}(CH_3)_3$ head-groups and reflect changes in motion and orientation of the hydrocarbon chains in each monolayer. The changes in $\nu_{1/2}$ for the inner monolayer (ii), (iii) and (iv), consequent on alterations in extravesicular Pr^{3+} concentration, strongly imply that there are transmembrane effects mediated from the outer monolayer to the inner, but these are small compared to the magnitude of the effect on the outer layer itself [22].

The effects of additional 6 mM Mg^{2+} and 6 mM Ca^{2+} to the extravesicular solution are compared in Figs. 3(a) and 3(b). The 6 mM Mg^{2+} (plus 6 mM Pr^{3+}) is very similar to the 12 mM Pr^{3+}. However, the addition of 6 mM Ca^{2+} produces some interesting differences: (i) T_p is elevated into T_c for the outer monolayer. Chapman et al. [30] recently reported a similar effect on the whole

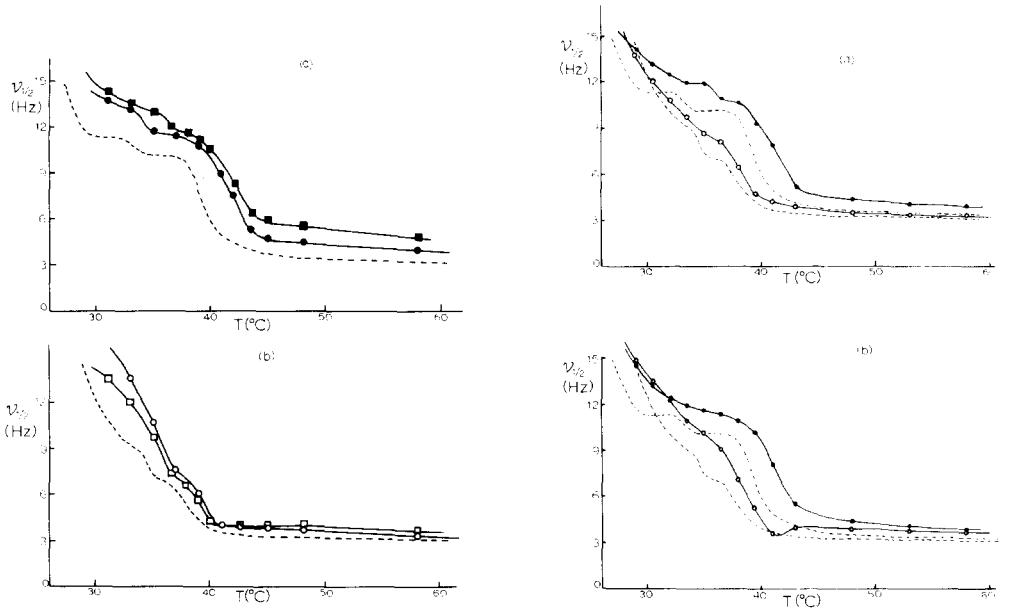

Fig. 2. The effect of different concentrations of Pr^{3+} in the extravesicular solution on the temperature dependence of the width at half-height ($\nu_{1/2}$) of the head-group ^1H NMR signals from dipalmitoyl phosphatidylcholine vesicles. (a) Values of $\nu_{1/2}$ for the outer head-group signal (O): ■———■, 21 mM Pr^{3+}; ●———●, 12 mM Pr^{3+}; - - - - - -, 6 mM Pr^{3+} (data points as in Fig. 1(b)). (b) Values of $\nu_{1/2}$ for the inner head-groups signal (I): □———□, 21 mM Pr^{3+}; ○———○, 12 mM Pr^{3+}; - - - - - -, 6 mM Pr^{3+} (data points as in Fig. 1(b)).

Fig. 3. (a) The effect of addition of 6 mM Mg^{2+} plus 6 mM Pr^{3+} to the extravesicular solution, on the temperature dependence of the width at half-height ($\nu_{1/2}$) of the head-group ^1H NMR signals: ●———●, outer head-group; ○———○, inner head-group; - - - - - -, controls (6 mM Pr^{3+} only, data points as in Fig. 1(b)). (b) As in (a), except using 6 mM Ca^{2+} plus 6 mM Pr^{3+} in the extravesicular solution.

bilayer using DSC of liposomes. (ii) $\nu_{1/2}$(I) is broader at all temperatures using Ca^{2+} than Mg^{2+}, and there is a much more noticeable narrowing of signal I just at the upper limit of T_c than in the case of 21 mM Pr^{3+}. Both (i) and (ii) imply a stronger transmembrane effect by calcium compared with that of magnesium. In the presence of 6 mM Ca^{2+} plus 6 mM Pr^{3+} at 40°C the inner monolayer can be seen to be still fluid $\{\nu_{1/2}(40°C) = \nu_{1/2}(60°C)\}$. Whereas $\nu_{1/2}$ for the outer monolayer at 40°C is much broader and corresponds to a state well into the liquid-crystal to gel phase change. This observation would seem to be a confirmation of the mechanism proposed by Papahadjopoulos et al. [21] for calcium-dependent fusion of negatively charged phospholipid vesicles. They propose that a de-stabilisation of the phosphatidylserine vesicles is mediated by an interaction of Ca^{2+} with the outer layer, rendering it gel-like, leaving the inner layer still fluid. Fusion is not observed in the case of dipalmitoyl phosphatidylcholine vesicles, presumably because the interaction between Ca^{2+} and the zwitter-ionic phosphatidylcholine is much less than for phosphatidylserine [32].

Since concentrations of 50 or 100 mM KCl are often used in the preparation of vesicles [14,33] we tested its effect on the phase transition curves obtained as above. Using 150 mM KCl in addition to 6 mM Pr^{3+} outside the vesicles, the result can be seen in Fig. 4(a). When both the intra- and extravesicular spaces

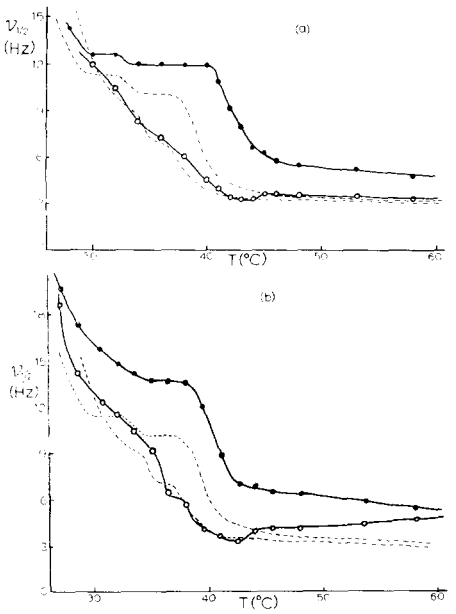

Fig. 4. The effect of addition of 150 mM KCl plus 6 mM Pr^{3+} to the extravesicular solution, on the temperature dependence of the width at half-height ($\nu_{1/2}$) of the head-group ^1H NMR signals: •———•, outer head-group; ○———○, inner head-group; - - - - - -, control (6 mM Pr^{3+} only, data points as in Fig. 1(b)). (b) As in (a) except using 150 mM KCl in the intra- and extravesicular solution.

contain 150 mM KCl the result is shown in Fig. 4(b). Both sets of data reveal that the inner and outer monolayers behave differently, but mutually influence each other. In Fig. 4(a) the overall broadening of signal O can be associated with decreased mobility of the outer head-groups; however, the main transition is clearly raised in temperature compared to the 6 mM Pr^{3+} control. The value of $T_p(O)$ remains unchanged. The inner monolayer signal is only slightly broadened from 60 to 45°C, but the narrowing effect is pronounced between 45 and 42°C, again indicating a transmembrane effect of the extravesicular KCl. In Fig. 4(b) the added influence of the extravesicular KCl can be seen. Thus $\nu_{1/2}(I)$ is much greater at 60°C than in Fig. 4(a), but the narrowing of the signal not continues from 60°C down to 42°C. T_p is raised for the inner monolayer also and this clearly influences the T_p value for the outer monolayer so that $T_p(O)$ and $T_c(O)$ merge. It should be noted that in Fig. 4(b) the strong narrowing of the inner signal produces values of $\nu_{1/2}(I)$ considerably smaller than $\nu_{1/2}(O)$, indicating that at 39°C the inner monolayer is as fluid as it is at 60°C while the outer monolayer at 39°C is in the gel phase.

It is significant that the change in $\nu_{1/2}$ corresponding to the main phase transition of the outer monolayers is seen from Figs. 1(b), 2(a), 3(a) and 4(a) to be constant at about 6 Hz. This implies a quantitative relationship between $\nu_{1/2}$ and the change on head-group mobility.

The effect of curvature of the bilayer

The reasonably sharp increase in $\nu_{1/2}$ at 40—41°C seen in Fig. 1(a) for vesicles with no added ions, suggests that the upper limit of the gel to liquid-crystal transition occurs at about the same temperature for both monolayers. As

indicated above, vesicles which have metal ions in the extravesicular aqueous medium have upper limits to T_c about 42—43°C for the inner layers, but for the outer layer at considerably higher temperatures. There remains some doubt whether part of the distinction in behaviour between the outer and inner layers may be due to their difference in sign and radii of curvature. (For dipalmitoyl phosphatidylcholine vesicles having signal ratio O : I = 1.8, the calculated radii are r_i = 9.3 nm and r_o = 13 nm, based on a bilayer thickness of 3.7 nm [34].) In order to clarify this we have used Gd^{3+}, which at concentrations as low as 10^{-4} M enhance the relaxation of the choline protons and so broaden the choline ^1H NMR signals into the base-line [9].

The addition of $2 \cdot 10^{-4}$ M Gd^{3+} to the outside of the vesicles then allows us to observe the inside signal I alone. At this concentration the outside signal is removed and any transmembrane effect of the Gd^{3+} is likely to be minimal. The temperature variation for the inside signal I so obtained is shown in the upper curve of Fig. 5(a). The upper limit of T_c is seen to be 41—42°C. A weak second inflection is seen at 35—36°C. When Gd^{3+} ($2 \cdot 10^{-4}$ M) is allowed to interact with the inner head-groups only (see Materials and Methods) the outer signal O is observable. The variation of $\nu_{1/2}$ of this signal O is shown in the lower curve of Fig. 5(a); the upper limit of T_c is again seen to be 41—42°C, but comparison of the two curves suggests that the phase transition extends over a

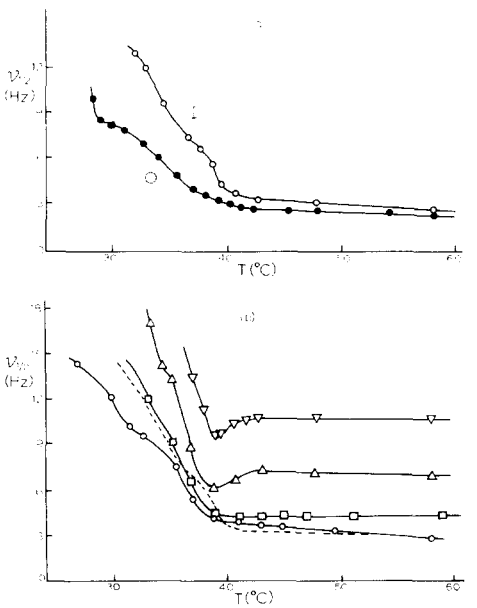

Fig. 5. (a) Upper curve (○———○) shows the variation with temperature of the ^1H NMR signal width at half-height ($\nu_{1/2}$) for the inner head-group signal I obtained by adding $2 \cdot 10^{-4}$ M Gd^{3+} to the extravesicular solution. Lower curve (●———●) shows the variation in $\nu_{1/2}$ for the outer head-group signal O, obtained by having $2 \cdot 10^{-4}$ M Gd^{3+} in the extra- and intravesicular solution plus 10^{-3} M EDTA in the extravesicular solution. (b) Variation with temperature of the ^1H NMR signal width at half-height ($\nu_{1/2}$) for the inner head-group signals, all values obtained using $2 \cdot 10^{-4}$ M Gd^{3+} in the extravesicular solution: - - - - - -, control (data points as (a), upper curve I); ○———○, 150 mM KCl in extra- and intravesicular solutions; □———□, 150 mM KCl in extra- and intravesicular solutions, plus 6 mM Pr^{3+} in extravesicular solution; △———△, 6 mM Pr^{3+} in the extra- and intravesicular solution; ▽———▽, 21 mM Pr^{3+} in the extra- and intravesicular solution.

greater temperature range (from 40°C down to 30°C) for the outer monolayer than the inner. It should be noted that the change in $\nu_{1/2}$ for the transition in the outer layer (lower curve) corresponds to that observed in Figs. 1(b), 2(a), 3(a) and 4(a), i.e. 6 Hz. It is also important to note that in Fig. 5(a), i.e. in conditions where the head-groups are ion free, the outer signal O is narrower than the inner signal I at all temperatures. This implies that the outer layer is more mobile than the inner, which could be the result of looser packing in the outer monolayer. It will be important to bear in mind this difference between the two layers when considering the effects of cholesterol on the bilayer as described below.

The phase transition curves in Fig. 5(b) all present the changes in $\nu_{1/2}$ for the inner monolayer under various ionic conditions, observed by having $2 \cdot 10^{-4}$ M Gd^{3+} outside the vesicles. When the vesicles are prepared by sonicating in 150 mM KCl the inside signal is seen not to narrow near T_c; this should be contrasted with Fig. 3(b). However, when the same procedure is followed but having 6 mM Pr^{3+} also in the extravesicular solution, then narrowing is seen. It would thus appear to be essential for the narrowing of the inner layer signal to have the polyvalent cation interacting with the outer layer and so is a transmembrane effect. We have seen in Fig. 4(b) that the narrowing is enhanced by KCl in the intravesicular space. When the vesicles are prepared by sonication in 6 mM Pr^{3+} solution so that both inner and outer layer head-groups are directly affected by Pr^{3+}, then further addition of Gd^{3+} ($2 \cdot 10^{-4}$ M) again allows the inner signal to be monitored alone. The two upper curves in Fig. 5(b) show the effect on $\nu_{1/2}$(I) of 6 and 21 mM Pr^{3+}, and the narrowing of signal I near T_c is striking. A comparison of these latter $\nu_{1/2}$(I) curves with that of Fig. 1(b), particularly between 40 and 60°C shows that direct contact with Pr^{3+} broadens the inner signal considerably more than the outer. This confirms that the narrowing effect of signal I described in Figs. 2(b), 3(b) and 4(a) and 4(b) cannot be due to penetration of Pr^{3+} into the intravesicular space.

Summarising the results of these experiments with various ionic concentration gradients across the bilayer we can conclude: (i) The difference in curvature between the outer and inner monolayers of dipalmitoyl phosphatidylcholine vesicles causes differences in the cooperativity and temperature range of the main transition; however, the upper temperature limit of the phase change is similar in both layers. (ii) The temperature range of the phase transition is more extended in the outer layer. (iii) The outer layer is at all temperatures through the transition region more mobile than the inner layer. (iv) This greater mobility of the outer over the inner layer can be reversed by interaction of ions with the outer layer, so much so that the difference in fluidity of the two layers can be extreme near T_c. (v) Interaction with metal ions can enhance and alter the pre-transitions. (vi) The interaction of metal ions with the head-groups of one monolayer can be transmitted to the other monolayer.

The most striking example of the last point would appear to be the narrowing of the signal from the inner monolayer when certain metals interact with the outer head-groups. The decrease observed in $\nu_{1/2}$(I) implies an increase in fluidity of the inner layer just above T_c. Although the observation will require further investigation, it is possible to speculate on the molecular events causing the change. In a bilayer vesicle the radii of curvature are of different sign on the

two monolayers, i.e. the outer head-groups lie on a convex surface but the inner head-groups on a concave surface. Also, the radius of curvature of the outer monolayer decreases from head-groups to the terminal methyl groups of the acyl chains, while the radius of curvature increases for the lipid molecules of the inner layer when measured from head-groups to terminal methyl groups. When metal ions interact with the outer head-groups the lipid molecules are probably cross-linked through $PO_4^- - - - M^{n+} - - - PO_4^-$ links [35] binding the molecules closer and so raising T_c of the outer layer by several degrees, through a cooperative effect. If the temperature is such that the outer layer is predominantly in the gel phase (acyl chains in the 'all-trans' extended form) while the inner layer is still fluid, the interpenetration (registry) of the two layers will be less and this will allow greater freedom of movement for the acyl chains of the inner layer. If metal ions also interact with the inner head-groups, the parts of the inner layer acyl chains nearer the centre of the bilayer, because of the increasing radius of curvature, will be even more mobile. There would thus seem to be a reasonable explanation of the extra fluidity of the inner monolayer just above T_c and hence the narrowing of the NMR signal observed.

The effect of cholesterol on the two monolayers

Figs. 6(a) and 6(b) are the phase transition curves for the outer and inner

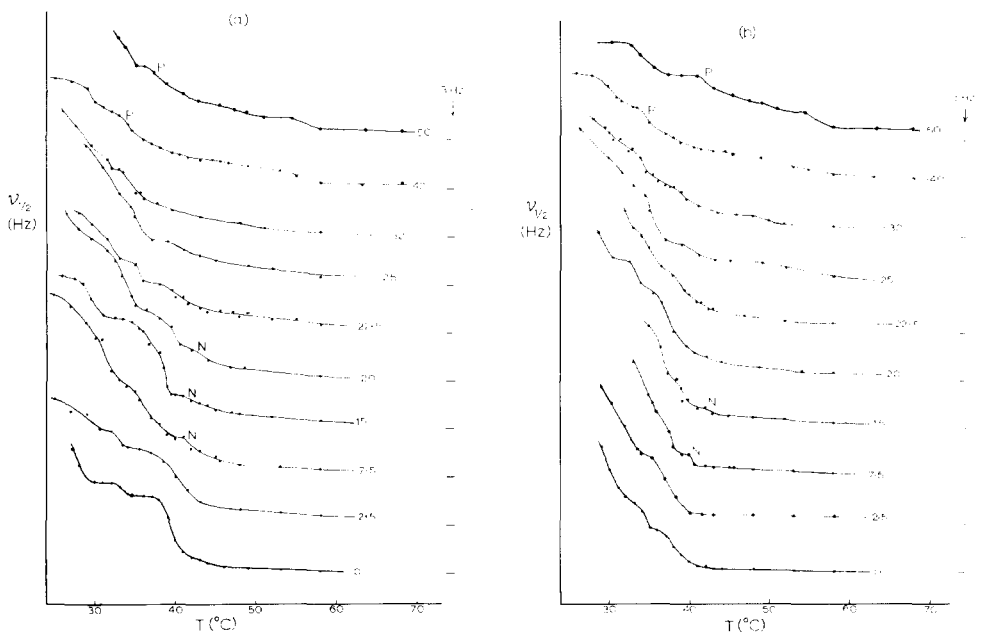

Fig. 6. The effect of varying concentrations of cholesterol on the phase transitions of the outer monolayer (a) and inner monolayer (b) of dipalmitoyl phosphatidylcholine vesicles containing 6 mM Pr^{3+} in the extravesicular region, as indicated by the temperature dependence of the outer and inner head-group ^1H NMR signal widths at half-height $\nu_{1/2}$. The numbers at the end of each phase transition curve indicate the mol% of cholesterol incorporated in the dipalmitoyl phosphatidylcholine vesicles. The dashed lines under the heading '3 Hz' correspond to $\nu_{1/2}$ = 3 Hz for each phase transition curve. Experiments were carried out as described in Materials and Methods, except that at above 25 mol% cholesterol, Gd^{3+} was added to the extravesicular solution of some samples. This enabled inner monolayer signal-widths to be measured at temperatures below 35°C without overlap with the outer monolayer signal.

monolayers, respectively, of dipalmitoyl phosphatidylcholine vesicles containing increasing mol percentages of cholesterol. The curves are obtained as above by plotting $\nu_{1/2}$(O) and $\nu_{1/2}$(I) against temperature, using 6 mM Pr^{3+} in the extravesicular aqueous medium to obtain separation of the signals from the outer (O) and inner (I) head-groups. The values of $\nu_{1/2}$ are shown with respect to the 3 Hz width in each case. On inspection of the two figures it is clear that both halves of the bilayer present a similar pattern of behaviour on increasing the cholesterol content. Addition of small amounts of cholesterol cause $\nu_{1/2}$(O) and $\nu_{1/2}$(I) to increase, but above 15 mol% there is a gradual decrease in $\nu_{1/2}$(O) and $\nu_{1/2}$(I) for temperatures below T_c, but a further increase in line-widths above T_c.

From Figs. 7(a) to 7(d) it can be seen that as the percentage of cholesterol increases there is a leveling of the phase transition curves for both outer and inner monolayers. Our data would then indicate that for both halves of the bilayer a general decrease in fluidity occurs from 0 to 15 mol% cholesterol (above and below T_c) but an increase in fluidity occurs above 15 mol% for temperatures below T_c. This is in keeping with the generally accepted view of the effect of cholesterol, that it acts to produce a condition of intermediate fluidity in the bilayer [24] at temperatures near that of the phase transition. Thus these ^{1}H NMR results again show that the mobility of the head-groups reflects that of the acyl hydrocarbon chains as they are affected by the presence of cholesterol.

The general trend for both layers suggests an even distribution of cholesterol across the bilayer and this is confirmed by the fact that we observe no change

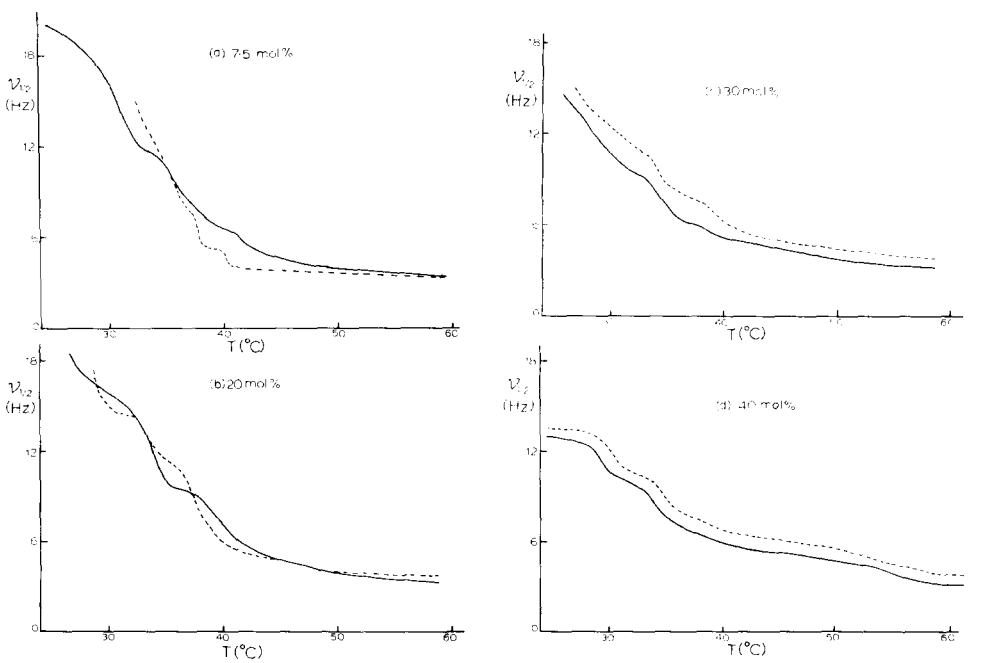

Fig. 7. ^{1}H NMR indications of the phase transitions for the outer (———) and inner (- - - - -) monolayers of dipalmitoyl phosphatidylcholine/cholesterol vesicles with 6 mM Pr^{3+} in the extravesicular region. The experiments were carried out as described in Materials and Methods with vesicles containing (a) 7.5, (b) 20, (c) 30 and (d) 40 mol% cholesterol.

in the ratio of the areas of the signals O to I ($R_{O/I}$) or change in the chemical shift difference between signals O and I ($\Delta\nu$), until above 30 mol% cholesterol. De Kruijff et al. [15] draw the same conclusions using ^{31}P NMR.

Examination of Figs. 7(a) to 7(d) also reveals that as the proportion of cholesterol is increased to 30 mol%, the width at half-height of the inside signal, $\nu_{1/2}(I)$, increases with respect to that of the outside signal $\nu_{1/2}(O)$. Since there is no asymmetry of cholesterol across the bilayer up to this concentration there must be a selective interaction of cholesterol within each monolayer producing a relative change in fluidity depending on the sign and radius of curvature (and possibly the packing) of the two monolayers. It should be noted that at concentrations of 30—40 mol% cholesterol (Figs. 7(c) and 7(d)), the value of $\nu_{1/2}(I)$ is at all temperatures greater than that of $\nu_{1/2}(O)$, which is the reverse of the situation in the control (0% cholesterol) shown in Fig. 1(b). Since these results are all obtained using a 6 mM Pr^{3+} concentration gradient across the bilayer, it would seem that the presence of 30 mol% cholesterol restores the relative values of $\nu_{1/2}(O)$ and $\nu_{1/2}(I)$ to those of Fig. 5(a), where there are no ions interacting with either monolayer. This observation suggests that cholesterol acts as a moderator of fluidity changes brought about by metal ion concentration gradients, under conditions of membrane curvature, and could have relevance to the role of cholesterol in plasma or vesicular membranes in cells.

Further inspection of Figs. 6(a) and 6(b) reveals finer details of changes in the phase behaviour of the monolayers than yet discussed. But before interpreting these changes it is clearly necessary to establish whether the vesicles remain impermeable under these conditions of variable cholesterol content and temperature. This can be done conveniently using the NMR signals as follows. Fig. 8(a) is the spectrum obtained at 58°C from dipalmitoyl phosphatidylcholine vesicles containing 15 mol% cholesterol which have been held within the main phase transition (37°C) for 6 h. The ratio of signals O to I ($R_{O/I}$) has increased from 1.8 to 4.9 during this time. On increasing the concentration of Pr^{3+} in the extravesicular solution the spectrum (b) is obtained, containing three signals O, I′ and I, with the new signal I′ having the same chemical shift as O in spectrum (a). On addition of further Pr^{3+} spectrum (c) is obtained in which I and I′ have not changed their chemical shift, but O is moved further downfield. Signals I′

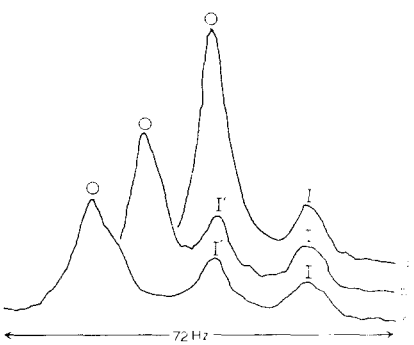

Fig. 8. ^1H NMR signals of the outer (O) and inner (I and I′) choline head-groups from vesicles containing dipalmitoyl phosphatidylcholine and 15 mol% cholesterol, with 6 mM Pr^{3+} in the extravesicular region. Spectrum (a) was recorded at 58°C after holding the vesicles for 6 h at 37°C. Spectra (b) and (c) show the result of adding increasing amounts of Pr^{3+} to the extravesicular solution.

are thus revealed as originating from the inner head-groups of vesicles which have lysed, allowing the Pr^{3+} concentration to equilibrate between outside and inside the vesicles, and so shifting some of the original signal I downfield to join signal O as in (a). Using this method we tested all the concentrations of cholesterol used in Fig. 6 for possible lysis. At temperatures above T_c (50—60°C) all vesicles were stable. At temperatures within the gel to liquid-crystal transition (36—38°C) only the 15 mol% cholesterol vesicles were appreciably lysed within 1 h, and a much smaller change was also observed for 7.5 mol%.

The effect of a small amount of lysis on the phase transition curves at 15 and 7.5 mol% cholesterol, can in fact be seen in Figs. 6(a) and 6(b). The lysis is restricted to the time during which spectra are being obtained in the 33—37°C range. An extra broadening of the outer signal O (Fig. 6(a)) is observed between 34 and 37°C for both these concentrations. Also the signals I from the inner layer head-groups diminish in size so that below 33—34°C their line widths cannot be accurately measured. It should be noted however, that even in the 15 and 7.5 mol% cases, the signals from the inner head-groups (Fig. 6(b)) are being obtained from the unlysed vesicles, and the widths at half-height are therefore comparable with the other concentrations for as low a temperature as they can be measured. De Kruijff et al. [15] also record that lecithin vesicles containing 15 mol% cholesterol are unstable and permeable to ions.

Having established that only the outer head-group signals within the main phase transition for 15 and 7.5 mol% cholesterol are artificially broadened by lysis, Figs. 6(a) and 6(b) can be interpreted in more detail. The most important observable features are as follows: (i) A break in the curves appears on the high temperature end of the main phase transition (marked N) at cholesterol concentrations of 7.5—20% in the outer monolayer (Fig. 6(a)) and 7.5—15% in the inner monolayer (Fig. 6(b)). (ii) N merges with the main transition at 22.5 mol% cholesterol for the outer monolayer and at 20 mol% for the inner. (iii) The upper temperature limit of the main phase transition combined with the N gradually rises with increasing cholesterol concentration to reach a maximum of 55—57°C at 40% cholesterol (for both monolayers). (iv) Breaks in the curves can be seen from 20 to 50 mol% cholesterol in both monolayers in the region of the pre-transition temperature of the control (0% cholesterol) i.e. 33°C, or even below this temperature, as especially noticeable at 40 mol%. (v) The change in $\nu_{1/2}$(O) and $\nu_{1/2}$(I) for 40 and 50 mol% cholesterol occurs over a wide temperature range from at least 25 to 55°C. (vi) These changes at 40 and 50 mol% still show some fine detail, and give a similar overall shape to the curves at both these concentrations for both monolayers. The most prominent feature of this detail (labelled P in Figs. 6(a) and 6(b)) is increased in temperature on going from 40 to 50 mol% from 34 to 36°C in the outer monolayer, but considerably more from 35 to 43°C, in the inner monolayer.

We feel that these data can be best interpreted in terms of the 1 : 1 cholesterol : lecithin complex formation proposed by Phillips and Finer [1] and supported recently by Lee [36]. The former workers base their interpretation on NMR data from vesicles and liposomes, and propose that regions of a 1 : 1 lecithin : cholesterol complex are separated from free lecithin by 'boundary layers'. Instead Lee [36], using chlorophyll fluorescence, in dipalmitoyl phosphatidylcholine liposomes, proposed full or partial miscibility between the

1 : 1 complex and free phosphatidylcholine. However, the two ideas appear to be compatible. Thus observations (i) and (ii) above, of the new break N, could correspond to the upper limit of a broad transition for the boundary layer lipid, expected to be prominent between 10 and 20 mol% cholesterol, but less so as the percentage of 1 : 1 complex increases. The observed maximum of 55—57°C at 40 mol% cholesterol, for the upper limit of the observed transitions agrees with Lee's observations and is indicative of partial miscibility of the 1 : 1 complex and free phosphatidylcholine in the fluid phase, up to neat 40 mol% cholesterol. Above this concentration the two are immiscible. Observations (iv) and (v) indicate that changes in the monolayer properties continue up to 50 mol% and this argues for a 1 : 1 rather than 2 : 1 phosphatidylcholine : cholesterol interaction. Lee [36] also reports the existence of pre-transitions observable in phosphatidylcholine/cholesterol liposomes up to at least 20 mol% cholesterol, but whether the breaks in the curves referred to in (iv) correspond to pre-transitions can be decided definitely only by X-ray diffraction. As already noted, Brady and Fein [12] have detected pre-transitions in diplamitoyl phosphatidylcholine vesicles by this method and it would be most interesting to see similar experiments on vesicles containing 30—50 mol% cholesterol.

Both spin-label [37] and Laser-Raman [38] studies agree with observation (v), suggesting that even in the presence of 40—50 mol% cholesterol the dipalmitoyl phosphatidylcholine bilayer still undergoes a broad phase transition. Since it is known that cholesterol primarily affects the mobility of the first 10 carbon atoms in the lipid acyl chain [39,40], then the fine detail observed as in (iv) and (vi) could be due to a residual cooperativity in the methyl ends of the acyl chains of the 1 : 1 complex.

The fact that we observe in (vi) a much greater increase in temperature of feature P for the inner monolayer suggests a possible asymmetric distribution of cholesterol. We observe a decrease in $R_{O/I}$ to 1.7 (40 mol% cholesterol) and 1.6 (50 mol%) indicating an increase in the size of the vesicles, as also deduced by de Kruijff et al. [15] using ^{31}P NMR. We further observe a change in $\Delta\nu$ from 0.29 ppm (0—30 mol% cholesterol) to 0.28 ppm (40 mol%) and 0.24 ppm at 50 mol%, implying an alteration in the relative distribution of cholesterol between monolayers. Unfortunately, de Kruijff's estimate for the asymmetry of cholesterol between the two monolayers depended on measurement of ν at 45°C for dipalmitoyl phosphatidylcholine vesicles containing 50 mol%. Fig. 6 indicates that at this temperature ν is already being increased by the phase transition, rather than the tumbling rate of the vesicles and so the estimates of cholesterol asymmetry may be too high.

It may also be noted that Huang et al. [41] reported the same change in $\Delta\nu$ and a slight decrease in $R_{O/I}$ for dipalmitoyl phosphatidylcholine vesicles containing cholesterol but occurring between 10 and 20 mol% rather than between 40 and 50 mol%. The discrepancy may be due to the fact that these workers sonicated their mixed lipid (dipalmitoyl phosphatidylcholine/cholesterol) at 50°C, then stored and recorded the NMR spectra of the vesicles at 47°C. These temperatures are very close to the upper limit of the phase transitions even at 20 mol% cholesterol, as can be seen from Fig. 6. Lawaczeck et al. [28] have shown that uniformly small vesicles can only be obtained by sonicating well above the phase transition, and we have also noted that it is difficult to obtain

clear sonicated solutions of dipalmitoyl phosphatidylcholine vesicles containing more than a few percent cholesterol, unless the procedure is carried out well above the upper limit of phase transition, i.e. at least 70°C for the higher concentrations of cholesterol.

The effect of n-alkanes on the vesicle bilayer

Figs. 9(a) and 9(b) show the variation of $\nu_{1/2}$(O) and $\nu_{1/2}$(I) with temperature using dipalmitoyl phosphatidylcholine vesicles containing 25 and 35 mol% *n*-decane compared to the control (0% decane). The separation of signals O and I was obtained as above, using 6 mM Pr^{3+} in the extravesicular solution. It can be seen from Fig. 9 that in both monolayers T_c is lowered significantly and the breadth of the transition increased. This indicates that the shorter chain *n*-alkane (C_{10}) is capable of fluidising and decreasing the cooperativity of the longer chains (C_{16}) of the dipalmitoyl phosphatidylcholine molecules. The observation also argues for a significant degree of interdigitation of the alkane molecules between the lipid hydrocarbon chains, a point which has been questioned in the case of planar bilayers [42]. In contrast, when the alkane introduced into the bilayer is of the same chain length (*n*-hexadecane) as in dipalmitoyl phosphatidylcholine, T_c is found to be increased only slightly and the cooperativity little affected as can be seen from Figs. 10(a) and 10(b).

There is considerable evidence that the permeability of lipid bilayers can be strongly affected in the vicinity of the transition temperature T_c [43], so we

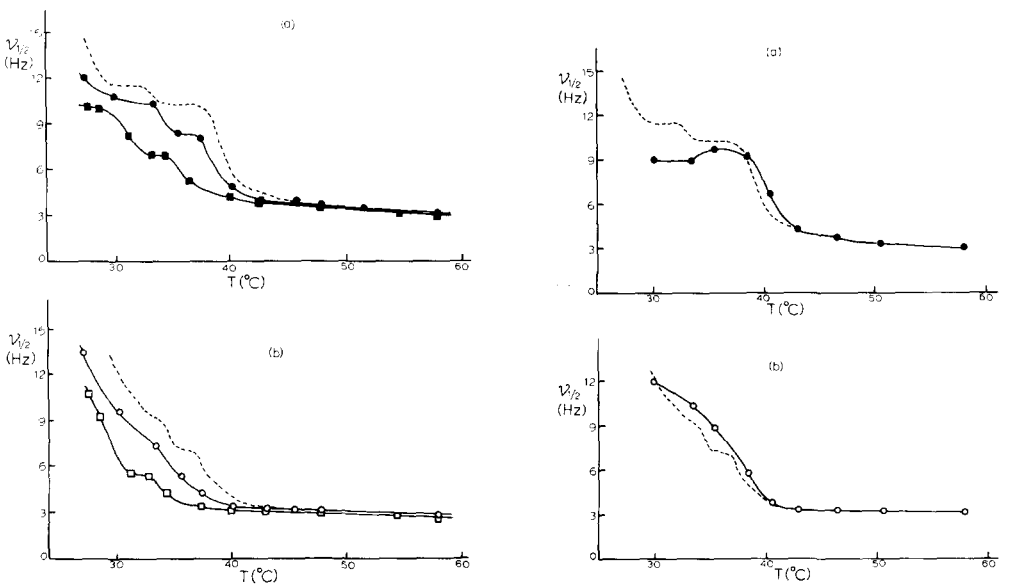

Fig. 9. ^{1}H NMR indications of the phase transitions for the outer monolayer (a) and inner monolayer (b) of dipalmitoyl phosphatidylcholine/decane vesicles with 6 mM Pr^{3+} in the extravesicular region. Experiments were carried out as described in Materials and Methods for vesicles containing 0 (- - - - - -), 25 (●———●, ○———○) and 35 (■———■, □———□) mol% decane.

Fig. 10. Phase transition curves for the outer monolayer (a) and inner monolayer (b) of dipalmitoyl phosphatidylcholine/hexadecane vesicles, with 6 mM Pr^{3+} in the extravesicular region. Experiments were carried out as described in Materials and Methods for vesicles containing 0 (- - - - - -) and 25 (●———●, ○———○) mol% hexadecane.

investigated this for dipalmitoyl phosphatidylcholine vesicles containing alkanes of various chain lengths. In Figs. 11(a) and 11(b) a comparison is made between the ^{1}H NMR spectra of pure dipalmitoyl phosphatidylcholine vesicles (50 mg per 2 ml sonicate) and dipalmitoyl phosphatidylcholine vesicles containing 25 mol% (7.7 wt.%) hexadecane but the same amount of dipalmitoyl phosphatidylcholine. The spectra were recorded at 58°C using 6 mM Pr^{3+} in the extravesicular solution. The ratio of the areas of the head-group signals ($R_{O/I}$) is the same in each case (1.8) but the intensity of the hydrocarbon chain signal H, can be seen to be increased for the hexadecane-containing vesicles (Fig. 11(b)). On slowly lowering the temperature of the vesicles containing 25 mol% hexadecane through the phase transition (41—35°C), head-group signals (on expanded scale) are obtained as shown in Figs. 11(c)—11(g). The ratio $R_{O/I}$ of the integrated signal areas is plotted against temperatures in the inset to Fig. 11. $R_{O/I}$ is seen to increase from 1.8 to 2.6.

Further experiments were performed in which dipalmitoyl phosphatidylcholine vesicles containing 25 mol% hexadecane were incubated in 6 mM Pr^{3+} at 37—38°C for increasing time intervals. After quickly warming the vesicles to 60°C the NMR spectra were recorded. It was found that the signal intensity rapidly transferred from peak I to peak 0, and that $R_{O/I}$ increased to very high values after several hours' incubation. Experiments performed at different incubation temperatures show that the change in signal ratio $R_{O/I}$ occurs at a measurable rate only between 40 and 36°C (as suggested by the results presented in Fig. 11 (inset)). In contrast, pure dipalmitoyl phosphatidylcholine vesicles in 6 mM Pr^{3+} solution do not show a change in $R_{O/I}$ on cooling through the phase transition or on being held for periods up to 24 h at 37—38°C.

As in the case of vesicles containing 15 mol% cholesterol, the increase observed in $R_{O/I}$ for hexadecane-containing vesicles can be explained by vesicle lysis which allows penetration of Pr^{3+} into the intravesicular aqueous space. Fig. 12(a) is the ^{1}H NMR spectrum, recorded at 60°C, from dipalmitoyl phosphatidylcholine vesicles containing 25 mol% hexadecane after being held at 37—38°C for 20 min with 6 mM Pr^{3+} in the extravesicular space. Spectrum (b) of Fig. 12 is recorded at 60°C immediately after (a) but with the addition of further Pr^{3+} to the extravesicular solution. The appearance of a signal I′ at the same chemical shift as O in spectrum (a) clearly indicates partial lysis of the vesicles.

The influence of the chain length of the *n*-alkanes on their ability to cause lysis in dipalmitoyl phosphatidylcholine vesicles was also investigated. We incorporated *n*-alkanes of chain lengths from C_8 to C_{18} into dipalmitoyl phosphatidylcholine vesicles and found that no change in $R_{O/I}$ (i.e. no lysis) occurs for *n*-octane, *n*-decane or *n*-dodecane on incubation of the vesicles at the phase transition temperature. Tetradecane and octadecane produce only a little lysis, but hexadecane, as shown above, is capable of causing considerable lysis.

These experiments with *n*-alkanes are made more significant in the light of their action as promotors of chemical carcinogenesis [26], i.e. though they have little carcinogenity in their own right they strongly increase the incidence of tumours in mouse skin when used in conjunction with a carcinogen. It has recently been shown that their effectiveness as promotors is strongly dependent on chain length, so that while *n*-decane is a weak promotor, the activity rises to

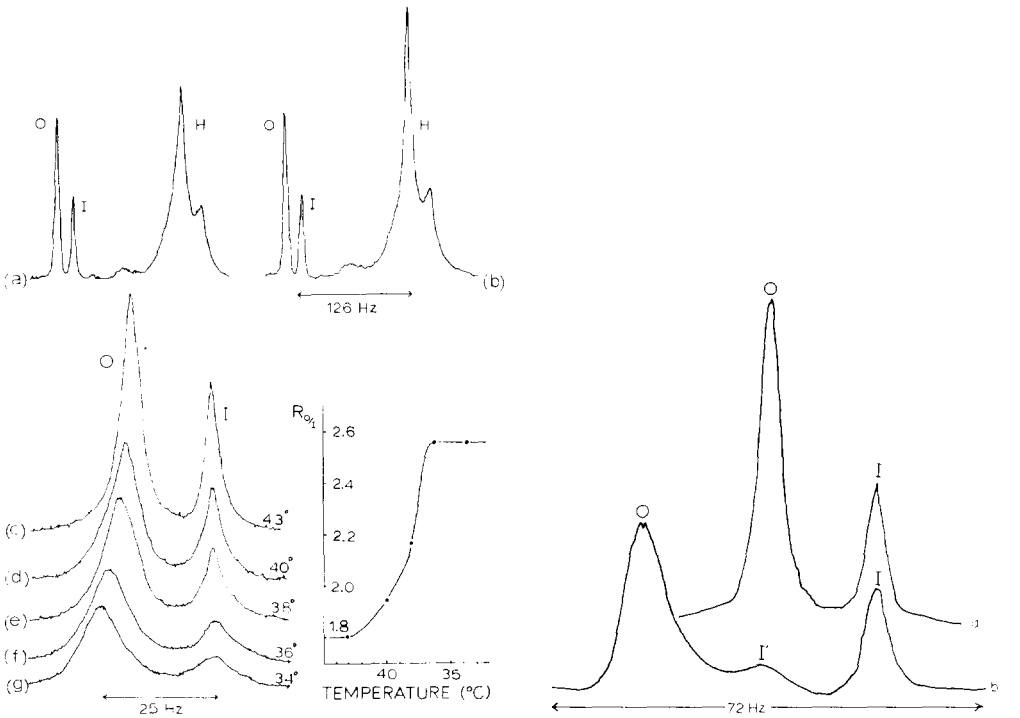

Fig. 11. (a) and (b) show the outer (O) and inner (I) choline ^{1}H NMR signals and the ^{1}H NMR signals due to the protons of the hydrocarbon chains (H) at 58°C for dipalmitoyl phosphatidylcholine vesicles containing 0 and 25 mol% hexadecane, respectively. Spectra (c)—(g) show the temperature dependence of the choline ^{1}H NMR signals. The inset shows the temperature dependence of the ratio of outer and inner choline ^{1}H NMR signals ($R_{O/I}$) for dipalmitoyl phosphatidylcholine vesicles containing 25 mol% hexadecane. The experiments were carried out as described in Materials and Methods with all vesicles containing 6 mM Pr^{3+} in the extravesicular region.

Fig. 12. ^{1}H NMR signals of the outer (O) and inner (I and I') choline head-groups from vesicles containing dipalmitoyl phosphatidylcholine and 25 mol% hexadecane, with 6 mM Pr^{3+} in the extravesicular region. Experiments were carried out as described in Materials and Methods. Spectrum (a) is recorded at 60°C after incubation of vesicles for 20 min at 37°C. Spectrum (b) shows the result of increasing the concentration of Pr^{3+} in the extravesicular solution.

a maximum at about *n*-octadecane [44]. Allowing for the fact that membrane lipids contain acyl chains principally from C_{14} to C_{20} [45] with varying degrees of unsaturation, the dependence of promotion on chain length strongly resembles that of the ability to cause lysis in dipalmitoyl phosphatidylcholine vesicles, as shown above. Further, the activity of another class of promotors found in croton oil, which are esters of phorbol, is also dependent on the length of alkyl chain in the ester [46].

While the lipid composition of most biological membranes is such that they are fluid at physiological temperatures, there is evidence that interaction with protein can alter the phase transitions [47] and the fluidity [7] of parts of the bilayer in contact with protein, Regions between this 'boundary lipid' and 'free lipid' could present a situation similar to that at the phase transition and so be susceptible to perturbations by alkanes of suitable chain length. Our experiments suggest that promotors could act selectively with membrane lipids to

produce changes at boundaries between lipid and protein, and the results may add to the significance of the altered lipid composition observed in cancer cells [48].

Acknowledgements

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