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Modulation of the orientational order profile of the lipid acyl chain in the L_α phase

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Abstract. The orientational order profile along the lipid acyl chain has been characterized under several different conditions of polar headgroup composition, temperature, and cholesterol content. Despite the different nature of these factors, the variation of the order is governed by two common trends. First, the relative change of order induced by the variation of these factors is always more pronounced towards the end of the chain than for the methylene groups near the interface. Second, there is, to a first approximation, a distinct correlation between the magnitude of the order parameters and the shape of the order profile. For example when the chain is highly ordered, the relative width of the order distribution is narrow indicating that the plateau region is longer. These conclusions suggest that the orientational order profile depends on only a small number of parameters and demonstrate clearly that the correlation length for changes in orientational order is much greater than one C–C bond length. Our results also show that the *reduced temperature* is not related in simple terms to orientational order and probably has little theoretical significance. The orientational order profiles of POPC and POPE bilayers are significantly different even when expressed in terms of reduced temperature. The behavior of POPC/cholesterol systems also indicates that the orientational order of the lipid chain and the gel-to-liquid crystalline phase transition temperature are not related in a straightforward manner.

Key words: Orientational order – ^{2}H-NMR – Phospholipid – Hydrocarbon chain

Abbreviations: POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid

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Introduction

Because of their amphiphilic nature, phospholipids aggregate to form bilayers in an aqueous environment in order to minimize unfavorable hydrophobic interactions between their acyl chains and water molecules. This aggregation leads to the formation of a hydrophobic core shielded from water by the polar headgroups. The hydrocarbon region of the bilayer is not an isotropic milieu. It has been well established by ^{2}H-NMR (Seelig and Seelig 1977, 1980) that the order distribution along the lipid chain in the liquid crystalline (L_α) phase varies in a characteristic manner. Aside from small systematic oscillations associated with local chain geometry, the order parameter of the segment near the interface is relatively constant: this part is usually referred as the *plateau* region. The chain order decreases more rapidly towards the middle of the bilayer.

As shown by several ^{2}H-NMR studies, many factors modulate the lipid chain order. Indeed, the structure of the acyl chain itself is a primary determinant of the orientational order of the methylene groups. For example, it has been shown that the presence of an unsaturated bond (Seelig and Seelig 1977) or a cyclopropane substituent (Perly et al. 1985) dramatically affects the order measured along the lipid acyl chain. The molecular structure of the lipid polar headgroup also influences the chain orientational order, as demonstrated by the different acyl chain order profiles observed for PC and PE bilayers (Perly et al. 1985; Cullis et al. 1986). Increased temperatures increase the motional averaging of the quadrupolar interactions and cause the chain order to decrease (Davis 1983). The orientational order is also sensitive to the symmetry of the phase adopted by the lipids. For example the lipid chains experience more motional averaging in the H_{II} phase than in the L_α phase (Perly et al. 1985; Sternin et al. 1988; Lafleur et al. 1990b). The lipid chain order may also be influenced by the presence of other lipophilic molecules. The presence of cholesterol inside the bilayer, for example, stiffens the lipid chains (Stockton and Smith 1976; Oldfield et al. 1978; Dufourc et al. 1984). Con-

versely the presence of even a very large proportion of proteins incorporated in the bilayer does not modify appreciably the orientational order of the lipid chains (Bloom and Smith 1985). These facts have been rationalized by a qualitative description of the elastic properties of the surface of the embedded molecules: cholesterol has a rigid ring network which obliges the lipid chains to straighten (Stockton and Smith 1976; Dufourc et al. 1984) while the fluid-like surface of proteins embedded in bilayers with matching thickness (Mouritsen and Bloom 1984), does not influence the motional freedom of the lipid chain (Bloom 1979).

Most previous studies of hydrocarbon order have been limited to the description of the effects of various factors on the motional properties at a few specific positions along the acyl chain and have not examined the distribution of order along the whole chain. This is mainly due to the method normally employed for obtaining the whole order profile. Generally, a set of specifically deuteriated lipids have been synthesized and then the profile has been determined by a set of discrete values of quadrupolar splittings. This approach requires considerable effort in lipid synthesis. Recently a new method has been developed to extract the order profile from a single ^{2}H-NMR spectrum of a lipid bearing a perdeuteriated saturated acyl chain (Sternin et al. 1988; Lafleur et al. 1989). It has been shown that the general shape of the order profile can be successfully reproduced by this method.

In this paper, we have investigated the dependence of the shape of the order profile in the L_α phase on factors known to affect hydrocarbon order (temperature, cholesterol content and polar headgroup composition). General trends of the modulation of the shape of the order distribution by these factors are reported here. We have employed 1-palmitoyl-d_{31}-2-oleoyl-phosphatidylcholine (POPC-d_{31}) in this study for several reasons. First, POPC bilayers are in the L_α phase over a wide range of temperature above 0 °C. Secondly the combination of one saturated and one unsaturated chains is representative of the lipids found in the plasma membrane of eukaryotic cells. Finally, the use of a lipid bearing a saturated perdeuteriated chain provides a straightforward and efficient method of determining the *smoothed* order profile.

Materials and methods

1 - Palmitoyl - d_{31} - 2 - oleoyl - phosphatidylethanolamine (POPE-d_{31}), 1-palmitoyl-d_{31}-2-oleoyl-phosphatidylcholine (POPC-d_{31}) and all other lipids were obtained from Avanti Polar Lipids Inc. (Birmingham, USA). The lipids were pure as ascertained by thin layer chromatography. For the deuteriated lipids, a chain analysis revealed an equimolar mixture of palmitoyl and oleoyl chains. As discussed previously (Lafleur et al. 1989), approximately 20% of acyl chain transmigration, occuring during the lipid synthesis, is suspected. This is the most likely explanation for the origin of the small peaks which are observed in some spectra.

The lipid mixtures were first dissolved in a benzene : methanol 96 : 04 (v/v) mixture and then freeze-dried. The lipid was then hydrated in a 20 m*M* HEPES buffer, 300 m*M* NaCl and 5 m*M* EDTA, pH = 7.4. The final phospholipid concentration was approximately 60 mg/ml.

A homebuilt 46 MHz ^{2}H-NMR spectrometer described previously (Davis 1979; Sternin 1985) was used. The powder pattern spectra were produced using a quadrupolar echo pulse sequence with a τ value equals to 50 μs. The 90° pulse length was 4 μs and the free induction decays were acquired in quadrature collecting 4096 points with a dwell time of 5 μs. The time between two successive pulse sequences was at least 300 ms. The number of scans was at least 25 000. The dePaked spectra were obtained using the iterative method presented elsewhere (Bloom et al. 1981; Sternin et al. 1983) and were smoothed on 5 points. The *smoothed* order profiles have been determined from the powder pattern spectra using the method previously discussed (Lafleur et al. 1989).

Results

Effect of temperature

Temperature has a pronounced effect on the order parameters of the lipid chains. The decrease in order induced by increased temperature is illustrated in Fig. 1. The powder pattern and dePaked spectra of POPC-d_{31} are presented for the various temperatures. Each methylene group and the terminal methyl group give rise to a powder pattern with a characteristic quadrupolar splitting; the spectra obtained from the lipids with a perdeuteriated acyl chain are the superpositions of these powder patterns. The spectra obtained over the whole range of temperature (0 to 70 °C) are characteristic of the L_α phase. The change in shape of the spectra as a function of temperature is similar to that previously observed for phosphatidylcholine with two perdeuteriated saturated chains (Davis 1979, 1983); a decrease of the width of the quadrupolar splittings is observed with increasing temperature. The probability distribution of order parameters along the acyl chain is directly described by the dePaked spectra (Fig. 1 b). In addition to causing a decrease of the individual quadrupolar splittings, increasing temperature also results in an increase in the number of resolved doublets. In other words, a larger proportion of the signal contributes to the doublets exhibiting the largest splittings (associated with the plateau region) at low temperatures. Thus not only are the absolute values of quadrupolar splittings affected by temperature variation but the distribution of order along the lipid chain itself is also modified.

The smoothed order profile along the palmitoyl chain of POPC has been characterized for the different temperatures (Fig. 2 a). The reduction in the order parameters with increasing temperature is clearly illustrated on these profiles, and this increase of motional averaging is observed all along the lipid chains. As previously mentioned, the dePaked spectra show, in addition to the change in

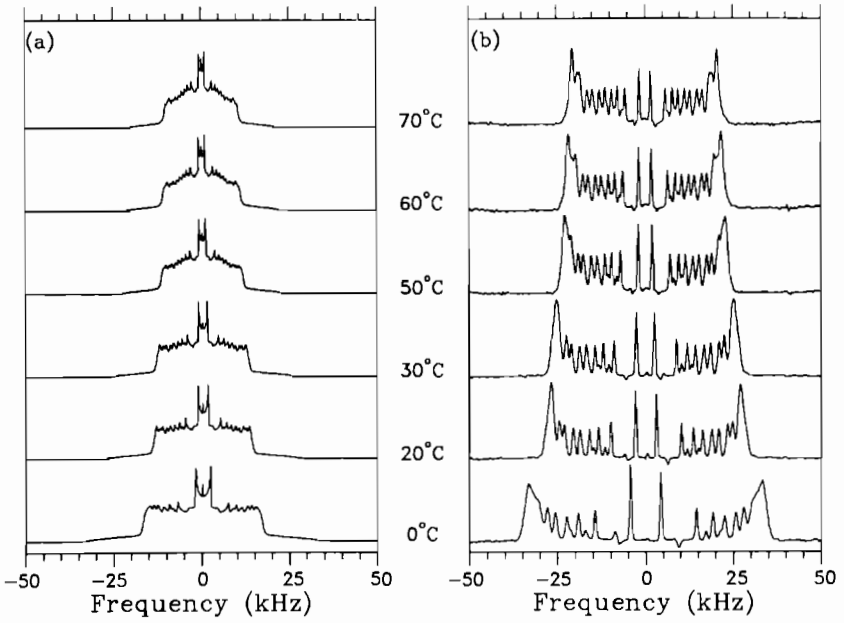

Fig. 1. **a** Powder patterns and **b** dePaked spectra of POPC-d_{31} at various temperatures

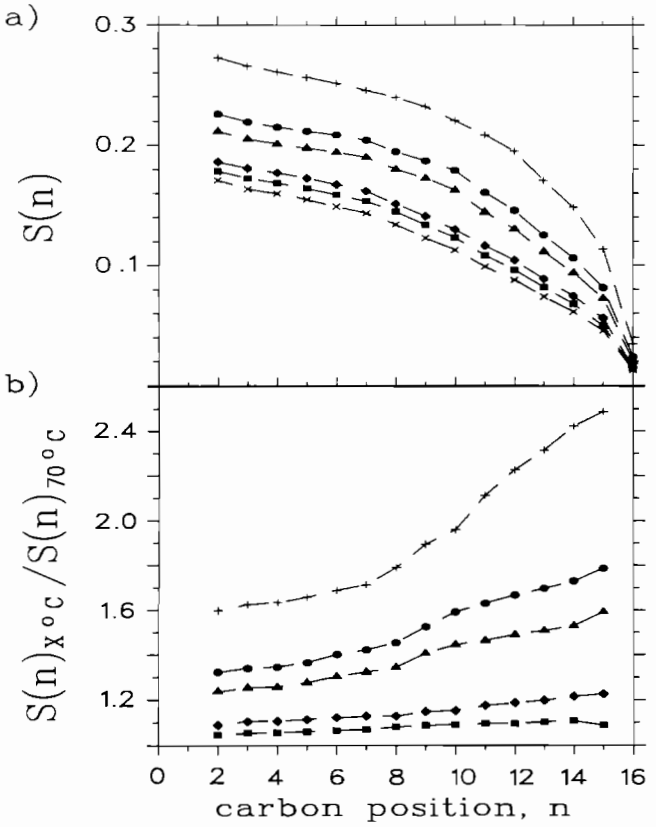

Fig. 2. **a** Order profile of POPC-d_{31} derived from the spectra in Fig. 1. (+) 0 °C, (●) 20 °C, (△) 30 °C, (◇) 50 °C, (□) 60 °C and (×) 70 °C. **b** Ratios of $S(n)$ at (+) 0 °C, (●) 20 °C, (▲) 30 °C, (◆) 50 °C or (■) 60 °C over $S(n)$ at 70 °C

order, a modification of the distribution of order along the acyl chains. In order to highlight the change in distribution, the ratios of different order profiles have been plotted in Fig. 2b. The common denominator for these ratios is the $S(n)$ profile obtained for POPC-d_{31} at 70 °C, the highest temperature employed, which exhibits the smallest order parameters. The ratios therefore represent the relative increase of $S(n)$ versus n induced by cooling. As seen in Fig. 2b, no single scaling factor can express the change of $S(n)$ induced by the temperature variation. For the segment near the interface, the ratio is quite constant; this is not surprising considering this corresponds to the ratio of the plateau segments typical of the L_α phase. Subsequently the diminution in order becomes more pronounced towards the end of the chain. For example the ratio of $\{S(n)$ at 0 °C$\}/\{S(n)$ at 70 °C$\}$ equals 1.6 for the first carbon positions and reaches 2.5 near the end of the chain. This indicates that for a given increase of temperature, the increase of motional averaging is not uniform along the chain, but is larger near the end.

Effect of the polar headgroup composition

The chemical composition at the bilayer interface can also modulate the order of the acyl chain. It has been shown that, in the L_α phase, the acyl chain order observed for lipids with different headgroups may be different (Perly et al. 1985) and it has been reported that lipid mixtures exhibit order parameters intermediate between those observed for the pure species (Cullis et al. 1986). In order to obtain insight into the influence of the headgroup composition on the order profile, POPE, POPC and various mixtures of these two lipids have been studied. Figure 3 shows the powder patterns and the dePaked spectra of several mixtures of POPE and POPC, recorded at 30 °C. For these different mixtures, the palmitoyl chain of POPE or POPC was labelled. The order detected by the palmitoyl chain is practically independent of the lipid bearing it. This is demonstrated by the very similar spectra of the equimolar mixture of POPE/POPC obtained from POPE-d_{31} or POPC-d_{31}. A linear decrease of the quadrupolar splittings is observed as the proportion of POPC is increased in the

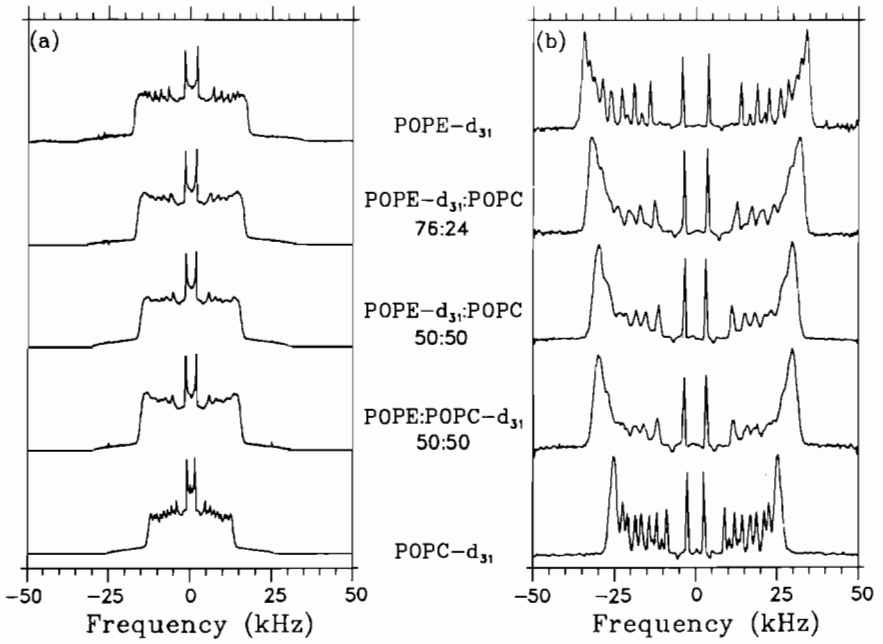

Fig. 3. **a** Powder patterns and **b** dePaked spectra obtained for different mixtures of POPC and POPE at 30 °C

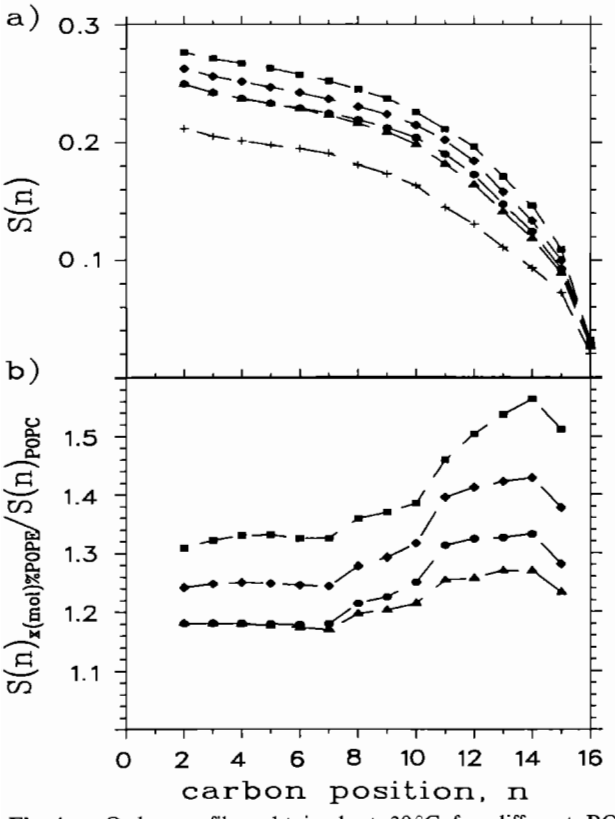

Fig. 4. **a** Order profiles obtained at 30 °C for different POPC/POPE mixtures derived from the spectra in Fig. 3. (+) pure POPC-d_{31}, (▲) POPC-d_{31} : POPE 50 : 50, (●) POPC : POPE-d_{31} 50 : 50, (◆) POPC : POPE-d_{31} 76 : 24 and (■) pure POPE-d_{31}. **b** Ratios at 30 °C of $S(n)$ of (▲) POPC-d_{31} : POPE 50 : 50, (●) POPC : POPE-d_{31} 50 : 50, (◆) POPC : POPE-d_{31} 76 : 24 or (■) pure POPE-d_{31} over $S(n)$ of pure POPC-d_{31}

mixture. A reduction by 26% of the mean quadrupolar splitting is observed between pure POPE and pure POPC. It may also be observed that the width of the peaks is markedly broader for the lipid mixtures than for the pure lipid species. The origin of this broadening will be discussed in the next section. As can be seen in Fig. 4, changes similar to those presented for the temperature variation can be observed. The order profiles are plotted in Fig. 4a. The values obtained for the pure lipids are in good agreement with the order parameters obtained previously using specifically labelled lipids (Perly et al. 1985). Ratios of order profiles are also displayed (Fig. 4b) where the order profile of pure POPC (the one with the smallest quadrupolar splittings for this set of samples) is used as the common denominator. The change of the chemical composition at the interface affects the order of the whole chain; for an increasing proportion of POPE, $S(n)$ is increased for every value of n. Based on the ratios of order parameters, a larger effect is observed at the end of the chain than for the methylene groups near the interface.

Effect of cholesterol

As mentioned in the Introduction, cholesterol has a dramatic ordering effect on the lipid chains. Here the effect of cholesterol on the shape of the order profile of the acyl chains of POPC has been investigated; spectra of POPC-d_{31} for cholesterol content varying from 0 to 45(mol)% were recorded at 30 °C (Fig. 5). All the spectra are characteristic of the liquid crystalline phase but as the proportion of cholesterol is increased, the quadrupolar splittings become progressively larger. The addition of 45(mol)% of cholesterol in a POPC bilayer leads to a remarkable 80% increase in order for the methylene groups near the interface. As observed for changes of temperature and headgroup composition, the change in the absolute values of $S(n)$ induced by cholesterol is accompanied by a change in the order distribution along the lipid chain as indicated by the number of resolved doublets observed on the dePaked spectra. The order profile as well as the order parameter ratios are shown in Fig. 6. The ratios have been calculated using the values

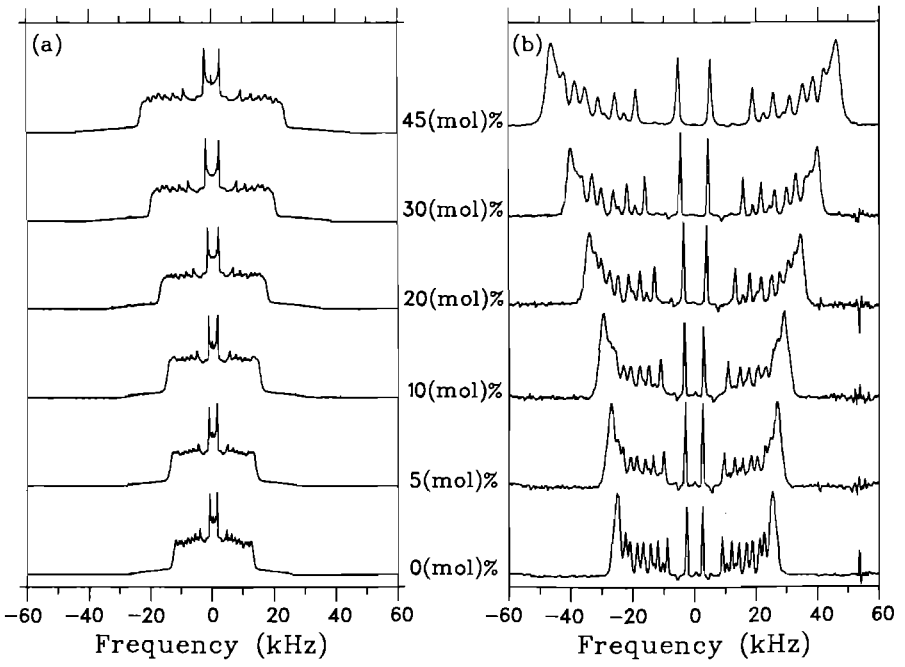

Fig. 5. **a** Powder patterns and **b** dePaked spectra of POPC-d_{31} for various cholesterol content (in mol%) obtained at 30 °C

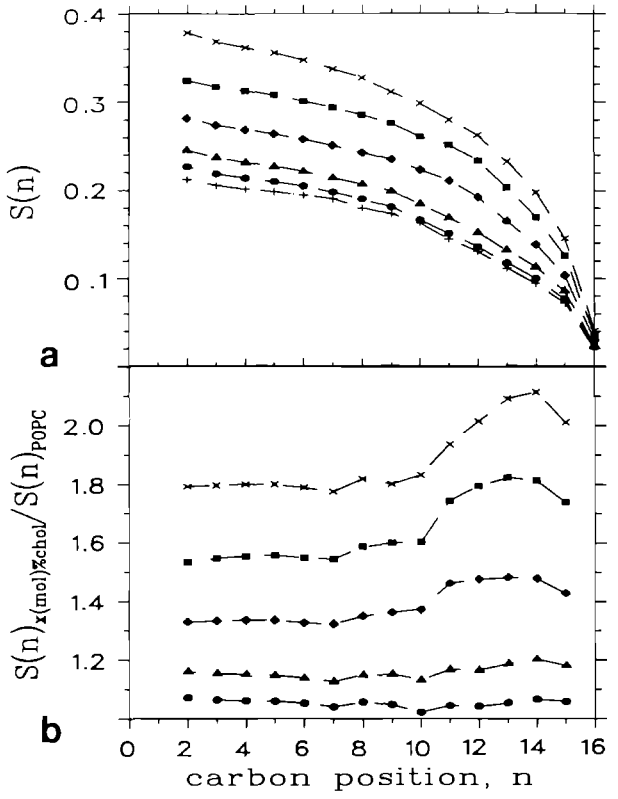

Fig. 6. **a** Order profiles of POPC-d_{31}/cholesterol system at 30 °C derived from the spectra in Fig. 5. (+) pure POPC, (●) 5%, (▲) 10%, (◆) 20%, (■) 30% and (×) 45% (in mol%) of cholesterol. **b** Ratios at 30 °C of $S(n)$ of POPC bilayer containing (●) 5%, (▲) 10%, (◆) 20%, (■) 30% or (×) 45% of cholesterol over $S(n)$ of pure POPC

obtained for pure POPC at 30 °C as the common denominator. As can be seen the ordering effect of cholesterol is observed along the entire lipid chain and again this effect is relatively more pronounced near the end of the chain as compared with the plateau section.

Discussion

All the order profiles presented here exhibit the bilayer *signature*, however two features of the orientational order profile have been shown to be affected by variations in temperature, headgroup composition and cholesterol content. First, the magnitude of the order parameters can be modulated, and second, as shown here, the shape of the order distribution itself can also be modified. The first point of interest is whether there is a relationship between these two changes. The magnitude of the order can be expressed as the average value calculated from the $S(n)$, $\langle S \rangle$. Analogously the distribution of order along the lipid chain can be characterized by the relative mean squared width of the order parameter distribution, Δ_2, defined as

$$\Delta_2 = \frac{\langle S^2 \rangle - \langle S \rangle^2}{\langle S \rangle^2}$$

The width primarily reflects the length of the plateau; the longer the plateau, the narrower the width of the distribution. The contribution of the terminal methyl groups has been omitted from these calculations because their different symmetry leads to a very small quadrupolar splitting and a variation in order distribution along the methylene groups is de-emphasized.

Figure 7 represents the variation of Δ_2 *versus* $\langle S \rangle$ for the methylene groups of the lipid palmitoyl chain observed for different polar headgroup compositions, temperatures and cholesterol contents. Each set of symbols corresponds to the variation of temperature for a given sample. As can be seen, this covers a wide range of order parameters and leads to a variation of $\langle S \rangle$ by a factor 3. The largest value is 0.35. This average value over the whole chain (the value at the plateau is 0.42) corresponds to a very ordered system since the value calculated for a *trans* methylene group rotating about the bilayer normal

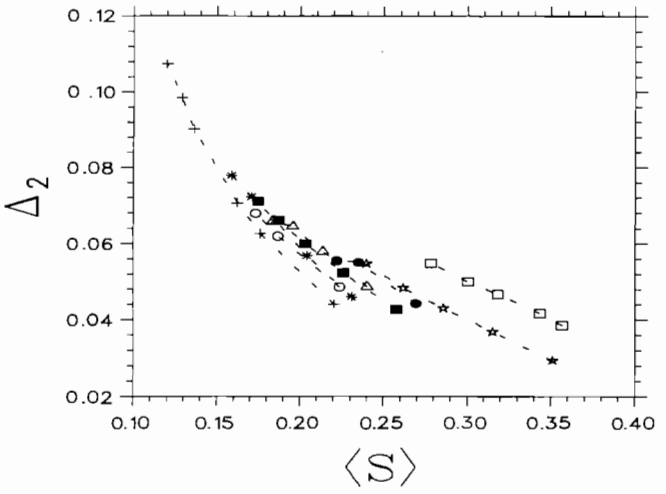

Fig. 7. Variation of the relative width of the order distribution, Δ_2, as a function of the mean order parameter, $\langle S \rangle$; these parameters have been calculated for the methylene groups only. (+) pure POPC-d_{31}, (○) pure POPE-d_{31} and POPC-d_{31} containing (*) 5%, (■) 10%, (△) 15%, (●) 20%, (☆) 30% and (■) 45(mol)% cholesterol

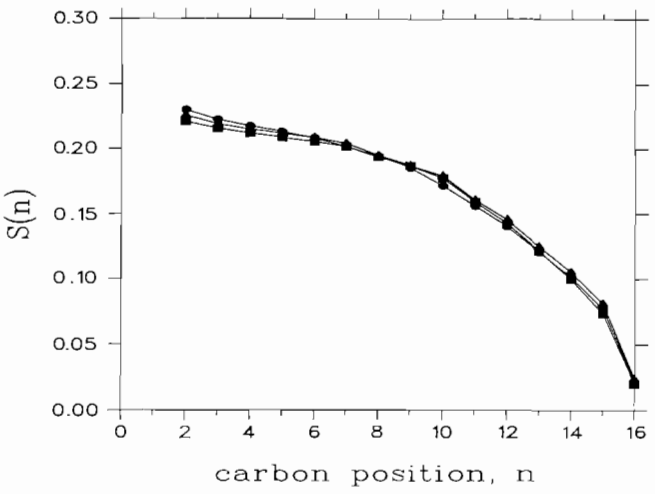

Fig. 8. Order profile of (■) POPE-d_{31} at 60 °C, (▲) POPC-d_{31} at 20 °C and (●) POPC-d_{31} : cholesterol 90 : 10 at 40 °C

is 0.5 (Seelig and Seelig 1980; Ipsen et al. 1990). Over this wide spread of order a general trend can be inferred: Δ_2 decreases as $\langle S \rangle$ increases. This indicates that the shape of the order distribution is correlated with the mean order parameter. The plateau length is longer when the acyl chains are more ordered.

This behavior is likely to be a general feature of bilayer membranes. Similar conclusions have been drawn from a study on biological membranes. For example, in isolated membranes of *Acholeplasma laidlawii* labelled with palmitate-d_{31} an increased order observed at lower temperatures is accompanied by an augmentation of the length of the plateau region (Davis et al. 1980).

The correlation between the absolute values of order parameters and the shape of the order profiles is perhaps surprising since factors which can influence order are intrinsically different. Temperature affects the motions responsible for the averaging of the quadrupolar interactions. The polar headgroup composition influences the acyl chain order in an indirect way by modulating the behavior at the water-lipid interface. It has been proposed, for example, that the smaller size of the POPE headgroup compared to POPC leads to a lateral compression of the lipid acyl chains restricting the motional freedom (Lafleur et al. 1990a). Finally, the presence of cholesterol modulates the lipid chain order directly through intermolecular interactions. However, all of these factors influence the orientational order profile in a similar manner. This can be illustrated by the fact that different combinations of experimental conditions leading to similar $S(n)$ for a given n give, to a first approximation, similar order all along the chain. An illustration of this behavior is shown in Fig. 8 where for different phospholipid compositions, temperature and cholesterol contents, three bilayer systems exhibit a very similar order profile. This suggests that the magnitude of the order parameters and the distribution of order along the acyl chain are dictated by the same forces and implies that the order profile can be described by very few parameters. A variety of different parameters have already been suggested. Using the van der Waals model, Meraldi and Schlitter (1981) have used the lateral pressure as the crucial parameter to simulate the order distribution. The absolute value of order parameters as well as the plateau length are both affected by a change of lateral pressure and their calculations predict that the plateau should be longer for more ordered chains. Alternatively, using a statistical approach, Dill and Flory (1980) have simulated the order profile considering as determinant parameters the surface density and the chain length and assuming that the density is conserved throughout the lipid layer. For increasing surface density, an increase of both the plateau length and the order parameters is predicted. Similar conclusions have been obtained using the bilayer thickness as the crucial constraint determining the whole $S(n)$ curve (Ipsen et al. 1990). It should be emphasized that these approaches are intimately related. Surface density and bilayer thickness are interdependent (assuming lipids form an incompressible fluid), and the lateral pressure exerted on the lipid acyl chains essentially modulates these two parameters of the bilayer. The ^{2}H-NMR measurements presented here indicate that the coupling between methylene groups of lipid acyl chains gives rise to a correlation length for local changes in orientational order of much greater than one C–C bond length. Under these circumstances, local perturbations at specific positions are not expected to produce localized changes in $S(n)$, but to affect the entire $S(n)$ versus n curve.

Lipids may be characterized by the temperature (T_m) at which they undergo a transition from a gel phase to a liquid crystalline phase. Differences in T_m have been tentatively proposed to account for the different chain order observed for different lipids (Seelig and Browning 1978). This had led to the concept of reduced temperature defined as $\theta = \dfrac{T - T_m}{T_m}$; for a given reduced temperature the orientational order is proposed to be equivalent (Seelig and Browning 1978). In this regard the gel to liquid

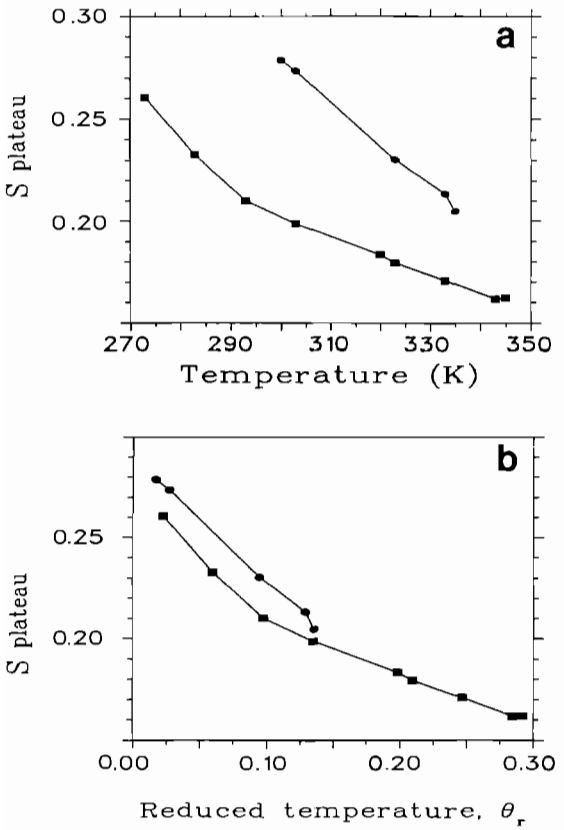

Fig. 9a, b. Variation of the order parameter at the plateau as a function of **a** temperature (K) or **b** reduced temperature for (●) POPE-d_{31} and (■) POPC-d_{31}

crystalline phase transition of POPE and POPC is different; it is approximately 22 °C for POPE-d_{31} (as determined by calorimetry) and −6 °C for POPC-d_{31} (J. Thewalt, personal communication). As shown in Fig. 9, the order parameters in the plateau region of the acyl chain for POPC and POPE compared on a reduced temperature scale are closer than if the comparison is made on the absolute temperature scale but are still different by 5–10%. The validity of the reduced temperature normalization has been also challenged by the fact that the order parameters of the plateau for DMPE have been found to be larger by 15% than those for DMPC when the comparison was made on a reduced temperature scale (Marsh et al. 1983). For more complex systems, the correlation between reduced temperature and $S(n)$ is even more problematic. For example, recent studies (Vist and Davis 1990) show clearly that a new type of fluid phase is observed in DPPC/cholesterol mixtures having more than about 20% cholesterol. These mixtures exhibit no gel to liquid crystalline phase transition and have large values of $S(n)$. So, based on the comparison of the head group effect and that of cholesterol, it appears that the correlation between reduced T_m and $S(n)$ is not straightforward and should be used with care.

The variation of the shape of the distribution as a function of the average order behaves to a first approximation in a universal manner but a detailed analysis shows that distinctions can be made. For example, the same average order of the acyl chain of POPC can be obtained when the cholesterol content and the temperature are increased (changes in the two factors "cancel out" each other). However for a given mean order parameter, the value of Δ_2 is significantly larger for the sample with high cholesterol content and high temperature. A similar observation can be made from the ratios of order parameters. The ratios of order parameters for POPC-d_{31} at 0 °C over those at 30 °C varied from 1.29 at the beginning of the chain to 1.58 for the last positions. For the addition of cholesterol, the ratios at 30 °C obtained for the order parameters of POPC-d_{31} : cholesterol 80 : 20 over those of pure POPC-d_{31} vary from 1.32 to 1.48. So for a given change in the plateau region, cholesterol is relatively less effective near the end of the lipid chain than a decrease of temperature. These observations can be related to the molecular shape of cholesterol itself. The rigid ring system of cholesterol which is proposed as the origin of the straightening effect is not long enough to go through a whole lipid layer. This arrangement may lead to a less constrained area under the bulky ring system and then the end of the lipid acyl chain experiences less restrictions for the motional averaging. It should be stressed however that our results show clearly that the order parameters *all along* POPC chain increase in the presence of cholesterol. A comparison of the effect of cholesterol on DMPC labelled at various positions along the acyl chain has led to the proposal that the stiffening effect of cholesterol is generalized throughout the bilayer (Oldfield et al. 1978; Dufourc et al. 1984). The order profiles obtained for POPC : cholesterol system presented here are in good agreement with that conclusion. Even more, they show that the relative order of the end part is more affected by the addition of cholesterol than at the plateau level.

Finally, we would like to comment briefly on the interesting variation in the linewidth in the spectra of POPE/POPC mixtures. So far, in our study, the focus has been on the average values of order parameters and on the order distribution along the phospholipid chain. The ^{2}H-NMR measurements on POPE/POPC systems provide additional information. The doublets of POPE/POPC mixtures containing 24 or 50% of POPC show an extra broadening compared to those observed for the pure lipid spectra. It is a general observation for the whole acyl chain. This extra broadening may be related to a probability distribution of order probed by every carbon position. In bilayers containing only one lipid component, the environment of every lipid is equivalent. However in a lipid mixture, the quadrupolar splittings of each deuteriated lipid molecule are averages over an ensemble of local lipid composition because of lateral diffusion in the bilayer plane. Since the average orientational order is sensitive to the average POPE/POPC proportion, this sampling may contribute to the extra broadening observed in phospholipids mixtures. The broadening may also include a T_2 effect associated with the randomly fluctuating time dependence of the quadrupolar splittings as the molecules diffuse through regions of different lipid compositions. A more detailed investigation is necessary to establish the relative importance of these different mechanisms of line broadening.

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