Lipid polymorphism and hydrocarbon order[1]

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The use of ^2H nuclear magnetic resonance for the characterization of the polymorphic behavior of lipids is illustrated. Different lipid phase preferences may be expected to influence the orientational order and its variation along the acyl chains. Several results are presented to support that view. An increase of motional freedom and a redistribution of the order along the acyl chains are observed during the lamellar-to-hexagonal phase transition, showing that the order profile is sensitive to the lipid phase symmetry. In addition, if the preferences for nonlamellar phases are not expressed explicitly, the presence of "nonbilayer" lipids constrained in bilayer environment induces increased hydrocarbon order. This suggests that order parameters of the acyl chains and lipid polymorphic tendencies are intimately related.

Key words: lipid, polymorphism, ^2H nuclear magnetic resonance, hydrocarbon order.

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L'utilisation de la resonance magnetique nucleaire du deuterium pour la caracterisation du comportement polymorphique des lipides est illustrée. Les preferences de phase des lipides influencent probablement I'ordre orientationnel ainsi que sa variation le long des chaines acyles. Des resultats sont présentés dans le but de supporter cette idee. Une augmentation de mobilite ainsi que la redistribution de l'ordre le long des chaines acyles sont observées durant la transition de phase cristal liquide - hexagonale, indiquant que le profil d'ordre est sensible a la symétrie de la phase adoptée. De plus, lorsque la preference pour des phases non-lamellaires ne se manifeste pas de façon concrete, la presence de lipides «non-lamellaires» contraints dans un environnement lamellaire entraîne une augmentation de l'ordre orientationnel des chaines acyles. 11 est donc suggéré que l'ordre orientationnel des chaines acyles et les tendances polymorphiques des lipides sont intimement lies.

Mots clés : lipide, polymorphisme, resonance magnttique nucléaire du deuttrium, ordre orientationnel des chaines acyles .

Introduction

Lipids may adopt different phases such as the micellar, liquid crystalline (L_α), or hexagonal (H_{II}) phases. The structure that lipids form spontaneously on hydration is dictated by the intrinsic tendency of the lipid system to adopt a certain phase and also by extrinsic factors (reviewed in Cullis ***et al.*** 1985, 1986). A major finding in recent years is that although the L_α phase is by far the most common phase adopted by biological membranes (Hui 1987), they contain significant proportions of lipids which, when isolated, form nonlamellar phases when rehydrated. In fact, under appropriate conditions, most lipid species can be induced to form nonlamellar phases. Our understanding of the role played by the different polymorphic tendencies of lipids in biological membranes is very limited. Some experimental results indicate that the polymorphic properties of lipids are tightly regulated. For example, certain organisms control the lipid composition of their membranes in such a way that a balance between lamellar and nonlamellar lipids is maintained (Wieslander et ***al.*** 1980, 1986; Goldfine et ***al.*** 1987). With regard to membrane function, a correlation has been established between the activity of the Ca^{2+}-ATPase and the proportion of "nonbilayer" lipids used for the reconstitution (Navarro ***et al.*** 1984). Local nonlamellar phases have been proposed as intermediate for certain phenomena that require the disruption of the lipid bilayer, such as membrane fusion or protein incorporation (Cullis ***et al.*** 1985; Batenburg and deKruijff 1988). These and other data have been used to suggest that the polymorphic preferences of lipids provide a basis for explaining the diversity of lipid found in biological membranes.

Until recently, most of the effort to investigate lipid polymorphism has been devoted to the study of the influence of various molecular features or exterior conditions on the phase preferences of lipid systems. This has led to the development of a simple shape concept (Israelachvili ***et al.*** 1980; Cullis ***et al.*** 1985; briefly reviewed in the next section) which suggests that phase preferences are dictated by the volumes occupied by polar and nonpolar regions. This has been successful in explaining qualitatively most of the experimental observations. Despite efforts to characterize more rigorously the factors that modulate lipid polymorphism, our under-

ABBREVIATIONS : NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine.

'This paper is dedicated to Dr. Morris Kates in honour of his valuable contributions to biochemistry in Canada.

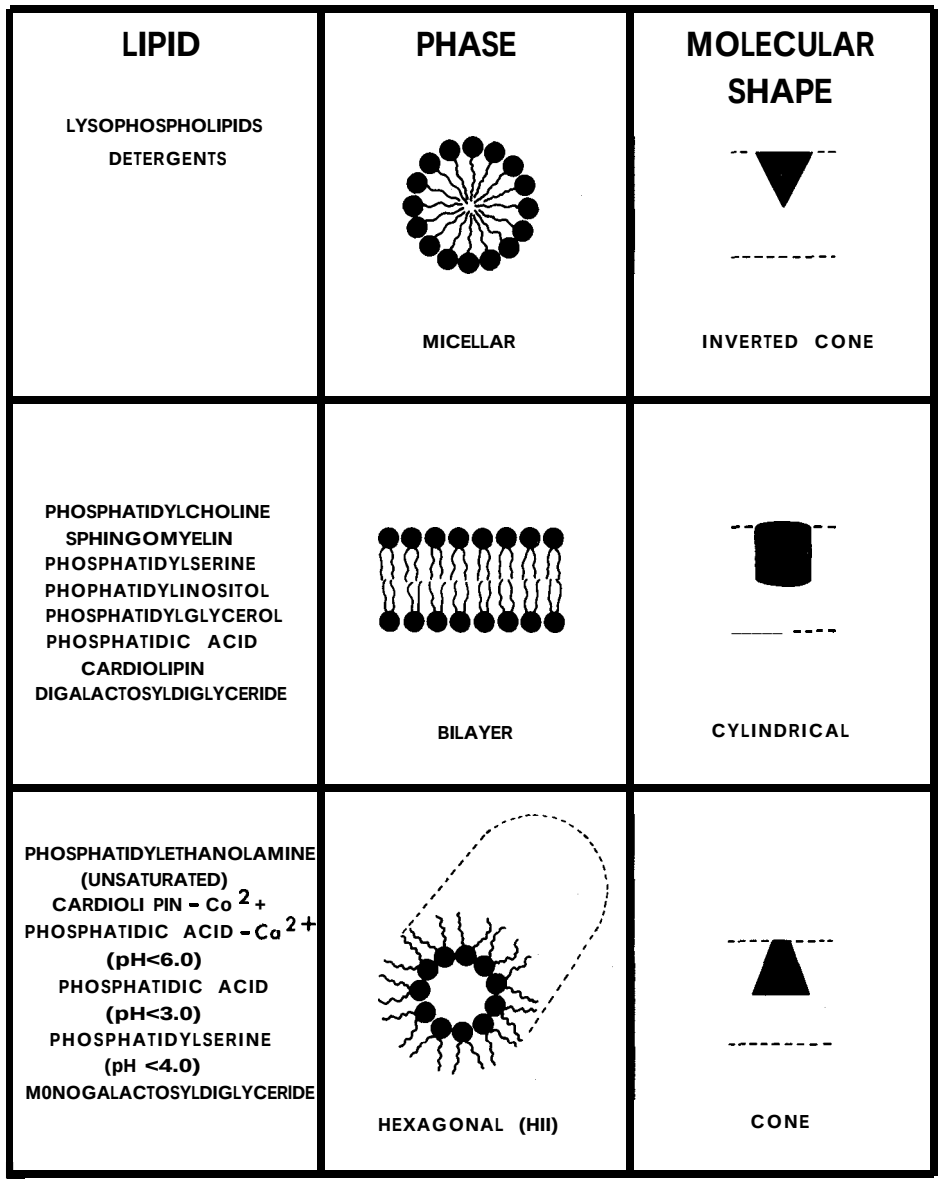

FIG. 1. Schematic description of the shape concept. The polymorphic phase is associated with the dynamic molecular shape of the component lipids. (From Cullis et ***al.*** 1985, with permission.)

standing of the physical basis of the different phases is still quite poor. This is essentially due to the fact that while the methods usually used may lead to the phase identification, they do not give insight into the factors leading to the phase adopted. An exception is X-ray diffraction from which the dimensions of the lattice unit can be determined. This quantitative information is extremely useful. For example, X-ray diffraction results have shown that the H_{II} phases adopted by various lipids are not all the same, but that the radius of the cylinders varies (Kirk and Gruner 1985). The "intrinsic" radius of curvature that a given lipid adopts in the absence of other constraints has been suggested to be a fundamental parameter dictating lipid polymorphism (Gruner 1985).

It is likely that ^{2}H-NMR will provide an additional rich source of information. ^{2}H-NMR is useful in the characterization of the orientational order of the lipid acyl chains (Mantsch ***et al.*** 1977; Seelig and Seelig 1980; Davis 1983); however, studies to date have been almost exclusively related to the L_α phase. A major finding has been the anisotropic nature of the order along the lipid chains (Seelig and Seelig 1977, 1980), which has been observed not only for model systems but also in biological membranes (Stockton ***et al.*** 1977; Gally ***et al.*** 1979). Theoretical models have been developed to reproduce the order profile in the L_α phase, suggesting that relatively few forces such as van der Waals interactions, lateral pressure, and steric hindrance dictate the order gradient (Marčelja 1974; Jähnig 1979; Dill and Flory 1980; Meraldi and Schlitter 1981). Little has been done so far to characterize the acyl chain order in the H_{II} phase or on the influence of "nonbilayer" lipids constrained in a bilayer environment. Such studies have been limited to some extent by the availability of deuteriated probes. In this paper, we present an overview of the possible applications of ^{2}H-NMR spectroscopy in the study of lipid polymorphism. The results already obtained indicate that the order parameters are sensitive to the polymorphic preferences of lipids.

Lipid polymorphism

The polymorphic preferences of lipid systems are dictated by the intrinsic tendency of lipids to adopt a particular phase and by extrinsic factors such as hydration and the presence of divalent cations. The wide variety of factors affecting the phase preferences of lipid systems have been reviewed extensively elsewhere (Cullis et *al.* 1985, 1986) and only the concepts necessary to unify these effects are presented here. The shape theory has been proposed to rationalize the polymorphic tendency of lipid systems. The basis of this theory is that the shape of the molecular species is a determining factor for the phase preference (Fig. 1). If the lipid is cone shaped (i.e., the surface occupied by the hydrophilic polar head group is small compared with the cross-sectional surface of the hydrophobic acyl chains), it shows a preference for the Hn phase. If the shape of the lipid is cylindrical, it prefers a lamellar phase, whereas if a lipid has an inverted cone shape, micellar structure is favored. The shape of the lipid is obviously directly related to its molecular structure. For example, the presence of unsaturation in the acyl chains favors the $\mathbf{H}_{II}$ phase, since it increases the space occupied by the hydrophobic part of the molecule. In a similar way, charges at the polar head group level diminish the tendency to form the $\mathbf{H}_{II}$ phase, since the electrostatic repulsion increases the effective area of the hydrophilic portion. This model is qualitative, as the molecular shape is not a rigorously defined concept. A related concept has been developed to describe the intrinsic tendency of aggregation of lipids to adopt different phases. In this formalism it is proposed that, in the absence of other constraints than elastic force, a lipid monolayer will curl to adopt a cylindrical shape with a certain spontaneous radius, R_0 (Kirk et *al.* 1984; Kirk and Gruner 1985). A system with a small radius of curvature has a greater tendency to form an $\mathbf{H}_{II}$ phase, while a system with an infinite R_0 forms a stable bilayer. The formation of the $\mathbf{H}_{II}$ phase induces defects in the chain packing in the hexagonally packed cylinders. In particular, the presence of intercylinder spaces is very unfavorable from a free energy point of view and thus inhibits the formation of $\mathbf{H}_{II}$ phase. The phase adopted by the lipid system thus corresponds to the one that minimizes the total free energy, taking in account the elastic curvature properties and the packing energies required to fill the interstitial spaces. According to this theory, the presence of hydrophobic agents such as alkane removes the packing constraint and allows the system to express its spontaneous radius of curvature. This is so far the only way to measure R_0 for systems normally constrained to the bilayer organization.

As previously indicated, many extrinsic factors can influence the polymorphic preference of lipid systems. Temperature, pressure, hydration, the presence of a wide variety of agents such as ions, amphiphiles, cholesterol, and proteins may favor a specific phase. According to the shape concept, a factor leading to an increase of the cross-sectional area of the hydrophilic volume or a decrease of the effective surface area sustended by the polar head groups will induce the formation of $\mathbf{H}_{II}$ phase. This prediction has considerable experimental support. For example, the increase of temperature, the neutralization of head group charges by ions, and the addition of alkane promote the formation of an $\mathbf{H}_{II}$ phase. Within the spontaneous radius of curvature formalism, two main classes of factors can favor $\mathbf{H}_{II}$ phase formation. First, a release of the packing problems resulting from the stacking of the cylinders promotes the $\mathbf{H}_{II}$ phase. This can be achieved by the addition of alkanes (Kirk and Gruner 1985), for example. The polymorphism can be also modulated by the variation of R_0 itself. Mixtures of molecular species with different radius of curvature have an intermediate value of R_0 and the proportions of the components can then control the phase preference of the system. In summary, a qualitative understanding of lipid polymorphism has been achieved and the polymorphic behavior of a system can be predicted in some ways. However, a detailed quantitative understanding of the phase preferences has not yet been obtained.

Orientational order profile

The quadrupolar splitting obtained from ^{2}H-NMR spectra is a direct measurement of the motional anisotropy of the C-D bond, since it is proportional to the order parameter S_{C-D}, defined by

$$[1] \quad S_{C-D}(n) = \frac{1}{2}\,(3\,\cos^2\theta_n - 1)$$

where θ_n is the angle formed by the C-D bond at the n^{th} carbon position and the effective axis of symmetry of the rapid motions of the acyl chain. The angular brackets represent an average over molecular conformation and orientation.

The order profile is a description of the variation of order parameters along the lipid chains. Two approaches have been used to determine the order profile using ^{2}H-NMR. First (Seelig and Seelig 1977, 1980), a set of lipids with deuterium nuclei at a single position on the chain can be synthesized. The quadrupolar splitting is then measured for each specifically deuteriated lipid and the order profile is described by this set of discrete values. This method reproduces the details of the order profile; since each position is probed independently, local variations of the order are detected. The main problem in this kind of study is the long lipid synthesis program involved.

A second approach is to use a lipid bearing a saturated perdeuteriated acyl chain. Since the signals arise from the deuterium nuclei uniformly distributed along the chain, it is possible to extract the information about the anisotropic nature of the chain order. Different methods have been proposed to determine the variation of S(n) versus n from the powder pattern 2H-NMR spectra obtained from a perdeuteriated chain. Since the deuterium nuclei of every methylene group exhibit a specific quadrupolar splitting, the powder spectrum of a lipid with a perdeuteriated chain of n carbons is actually the superposition of n powder patterns. This leads to a complex spectrum for which the assignment of specific peaks is very difficult. One way to extract the order profile from such a spectrum is to use the correlation that exists between the moments of the spectrum and the moments of the order distribution (Bloom *et al.* 1978; Davis *et al.* 1980). An expression for the moments of the spectrum is derived from an empirical function assumed to describe reasonably well the order profile, and the adjustable parameters of this function are determined by a least-squares fit of the calculated moments. The order profile corresponds in this case to the continuous function.

Recently a new method has been developed to determine the order profile based on the fact that the dePaked spectrum gives directly the distribution of order parameters

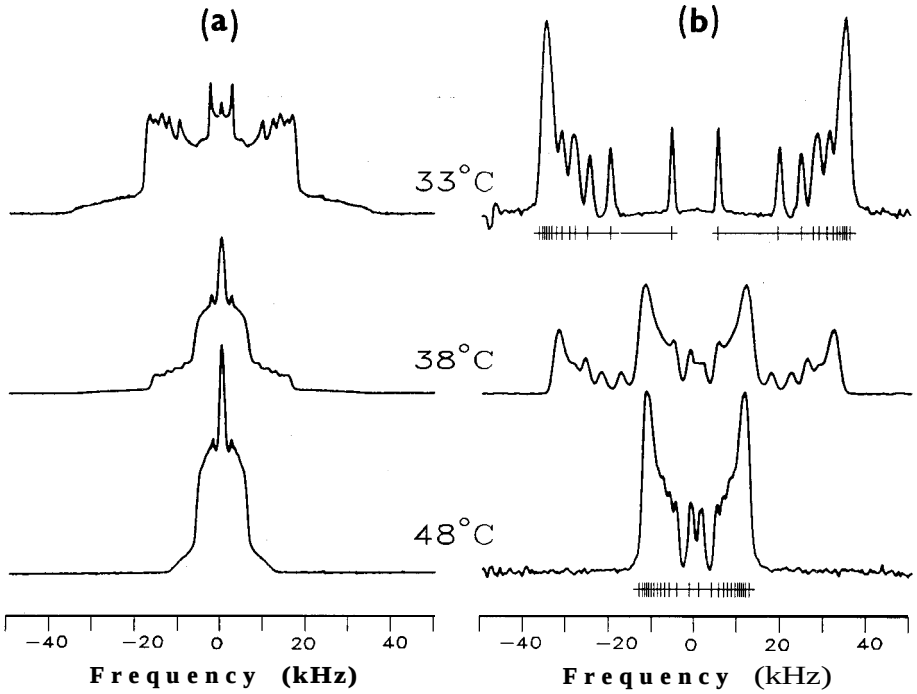

FIG. 2. ^{2}H-NMR powder pattern (a) and dePaked (b) spectra of deuteriated tetradecanol in a POPE matrix experiencing an L_α-to-H_{II} phase transition induced by a variation of temperature. At the bottom of the dePaked spectra, the tick marks represent the middle of an area associated with one CD, group (or one CD, aroun for the innermost doublet) determined by the integration method. (From Sternin ***et al.*** **1988, with permission.)**

(Sternin ***et al.*** 1988; Lafleur ***et al.*** 1989). The dePakeing procedure converts a powder spectrum into one representative of an oriented sample, assuming that the powder patterns composing the spectrum have the characteristic line shape of a randomly oriented sample (Bloom ***et al.*** 1981; Sternin ***et al.*** 1983). In the case of a chain with ***n*** carbons, the dePakeing procedure transforms the ***n*** superposed powder patterns into ***n*** doublets. The area of the dePaked spectrum is normalized in such a way that a mean value of order parameter is calculated for area portions associated to the contribution of one CD_2 group. Assuming a monotonic decrease of the order along the chain, the variation of S(n) versus ***n*** is obtained. This new method is based essentially on the order distribution given by the dePaked results, does not depend on the resolution obtained after dePakeing procedure, and does not assume a specific shape for the profile. The comparison of the profile obtained by this method with the order parameters obtained with specifically labelled lipids for POPC in the L_α phase shows that the general shape of the order profile can be accurately reproduced using the integration method (Lafleur ***et al.*** 1989). A very appealing aspect of this method is the use of perdeuteriated chains, which limits considerably the effort in lipid synthesis and data acquisition time. This is particularly true when the order profile of biological membranes are studied, as various organisms such as ***Acholeplasma laidlawii*** and ***Escherichia coli*** can incorporate in their membranes deuteriated fatty acid available in the growth medium.

Because the approach assumes a monotonic decreasing order, the order profile obtained is smoothed (i.e., local variations that may exist are not reproduced by the method). For example, there are oscillatory variations of the order in the segment near the interface in the L_α phase, which are not described by the integration method. The detailed structure of the order gradient is mainly related to local geometry and there is no indication that it is determinant for the polymorphic behavior. It is important to note that the general shape of the order profile appears to be sensitive to anisotropic forces exerted on lipid chains (Marčelja 1974; Jähnig 1979; Meraldi and Schlitter 1981). Because the polymorphism is likely modulated to some extent by the same forces, the determination of the order profile using perdeuteriated acyl chains is expected to reveal information about the influence of polymorphic tendencies of lipids on the acyl chain order.

Orientational order and the lamellar-to-hexagonal phase transition

The order profile describes the anisotropic nature of the order along the lipid chain and for the L_α phase the profile is now well characterized (Seelig and Seelig 1977, 1980). For the segment of the chain near the head group, the order does not vary considerably; this is usually referred to as the plateau region. This ordered segment is followed by a rapid decrease of the order towards the middle of the bilayer. This profile has been interpreted by several models, taking in account various factors such as ***trans-gauche*** isomerization, lateral pressure, van der Waals interactions, and steric hindrance (Marčelja 1974; Jähnig 1979; Dill and Flory 1980; Meraldi and Schlitter 1981). The L_α-to-H_{II} phase transition is likely to influence these factors and thus modify the order gradient.

This has been recently shown for a POPE matrix using perdeuteriated tetradecanol as a probe (Sternin ***et al.*** 1988). Because the polar hydroxyl group of tetradecanol is pinned down at the interface, the tetradecanol chains align with the lipid chains and can report the distribution of order across the bilayer. It has been shown that concentrations as high as 25 mol% of long chain alcohol do not perturb the chain packing (Thewalt ***et al.*** 1985, 1986) and thus tetradecanol

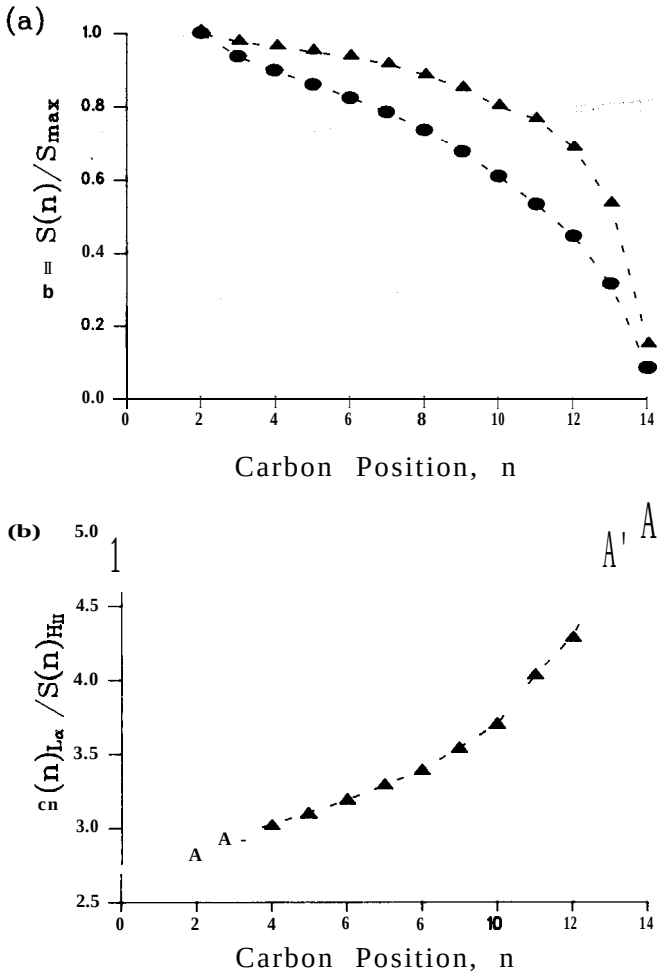

FIG. 3. **(a)** Normalized orientational order profile for the L_α (A) and the H_{II} (●) phase obtained from POPE + 20 mol% tetradecanol-d_{27} at 33°C for the L_α and 48°C for the H_{II} phase. The largest splitting has been normalized to **1.** (From Sternin *et al.* 1988, with permission.) (b) Ratio of the order parameters of the L_α phase over those of the H_{II} phase.

can be used to probe the order distribution. However, it should be noted that the presence of tetradecanol affects the polymorphism of POPE. A decrease of the L_α-to-H_{II} phase transition temperature is observed when 20 mol% of tetradecanol is added to POPE. Figure 2 shows the powder pattern and the dePaked spectra of perdeuteriated tetradecanol in POPE in the L_α phase **(33°C),** the H_{II} phase **(48°C),** and when both phases coexist (38°C). The integration method has been applied to obtain the order profiles displayed in Fig. **3*a*.** At low temperatures, the order parameter distribution along the tetradecanol chain is characteristic of the L_α phase as described previously. The L_α-to-H_{II} phase transition modifies the shape of the ^{2}H-NMR spectrum. Two main observations arise from the order profiles obtained for the L_α and the H_{II} phases. First, there is a drastic decrease of the absolute values of order parameters in the H_{II} phase. It has been shown that a reduction by a factor of two is expected from the extra motional averaging arising from the lipid diffusion around the H_{II} cylinders (Cullis and deKruijff 1979). In the L_α phase the symmetry axis of the rapid motion is perpendicular to the plane of the bilayer, since the rotation of the lipid around its axis is fast on the NMR time scale. However, lipid

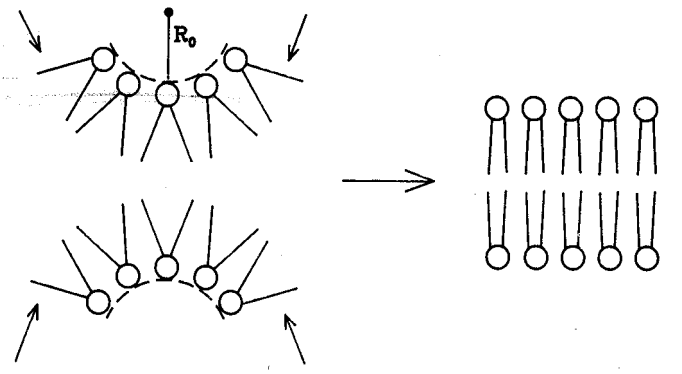

FIG. 4. The stacking of cone-shaped lipids leads to the formation of a cylinder characterized by the radius R_0. If the total free energy does not favor the expression of the curvature (see text), the lipid chains experience a lateral pressure leading to the formation of a bilayer.

diffusion in the bilayer plane does not modify the orientation of the lipid with the magnetic field. Because of the high curvature of the surface in the H_{II} phase, the diffusion around the cylinders causes extra motional averaging. The factor of – 2 has been observed experimentally during the L_α-to-H_{II} phase transition using ^{31}P-NMR (Cullis and deKruijff 1979). The ^{2}H-NMR results show that the lipid chains experience additional motional averaging in the H_{II} phase. The ratio of $S(n)_L / S(n)_{H_{II}}$ (Fig. 3b) is greater than 2 all along the acyl chain; it is approximately 2.8 at the beginning of the chain and reaches 5 near the end. This is in agreement with results obtained previously for some selected positions (Perly et *al.* 1985). The absence of a common scaling factor for every position is the second main feature observed during the L_α-to-H_{II} phase transition. That means that not only are the absolute values of order parameters different, but the order distribution along the acyl chain is also affected. As can be directly seen on the dePaked spectra or on the normalized order profile, the order decreases more uniformly along the chain in the H_{II} phase than in the L_α phase. A plateau region like the one observed for the bilayer structure is not detected in the H_{II} phase, but the whole chain shows a progressive decrease in the order along the chain. It should also be noted that the comparison of the order profile in the H_{II} and the L_α phases indicates an increase of motional averaging towards the end of the chain for the H_{II} phase. These observations can be qualitatively explained by the change of symmetry of the lipid phase. In the L_α phase the space available for chain motions is limited by the effective size of the polar head group, while in the H_{II} phase the volume accessible to the chain is conic, leading to an increase of motional freedom, particularly near the end of the chain. This shows that the orientational order profile is sensitive to the lipid phase symmetry. Very similar results are observed for the phospholipid chains themselves when using POPE bearing uniformly deuteriated palmitoyl chains (M. Lafleur, B. Fine, M. Bloom, and P.R. Cullis, unpublished results).

Polymorphic preferences and order parameters

The previous section demonstrates that the order parameters along the acyl chain are affected by the phase adopted by the lipids. The other question that can be addressed is whether the polymorphic preferences of lipids are also

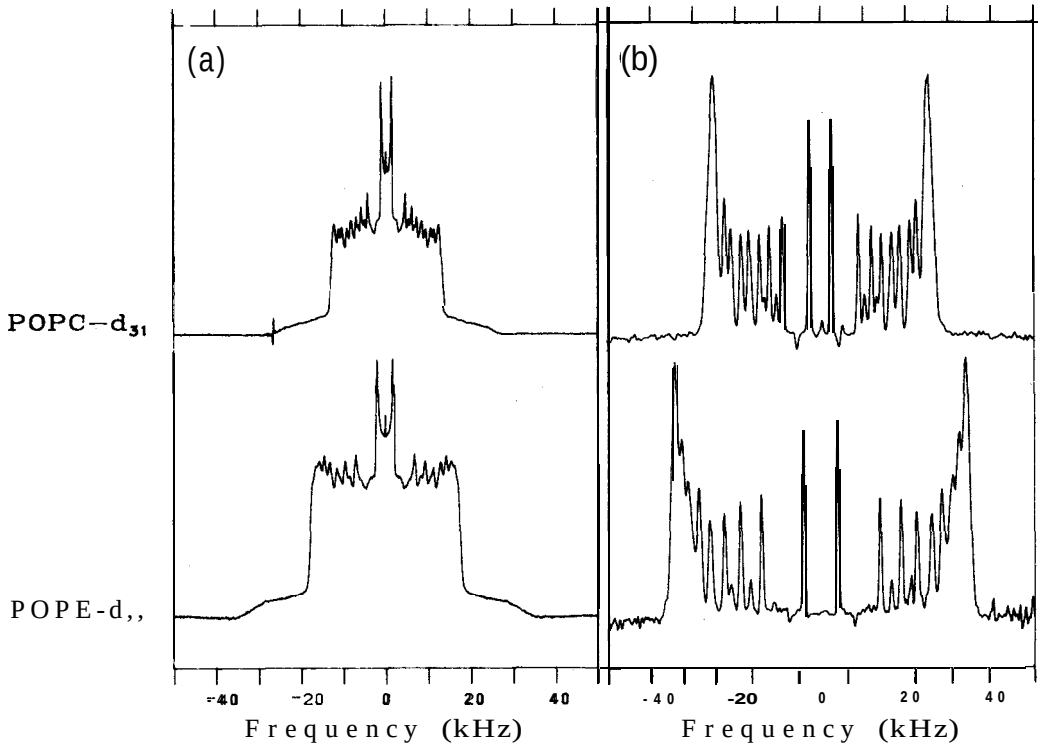

FIG. 5. Powder pattern (a) and dePaked (b) spectra of POPC and POPE, both having a perdeuteriated palmitoyl chain. The spectra are recorded at 30°C where both lipids are in the L_α phase.

expressed by the order parameters. As mentioned in the Introduction, the formation of nonbilayer phases in biological membranes may only be expected to occur infrequently, if at all. However, even if the preference for nonbilayer phases is not manifested explicitly, it may still modulate the order of the bilayer. This concept is schematically presented in Fig. 4. The stacking of cone-shaped lipids leads to the formation of a curved surface with a radius of curvature, R_0, if only the elastic properties are expressed. If conditions do not allow the expression of this curvature, a lateral compression has to be exerted on the acyl chains to optimize the matching between the hydrophilic and hydrophobic cross-section areas necessary for the bilayer. This lateral compression would be expected to increase the hydrocarbon order. Using a similar argument, the shape of the molecular species should influence the order parameters of the acyl chains.

So far, experimental results support that view. POPE is more cone shaped or has a smaller R_0 than POPC, as illustrated by the formation of H_{II} above 70°C. At 30°C, both lipids are in the L_α phase. The powder spectra (a) and the dePaked results (b) obtained for POPE and POPC where the palmitoyl chain is fully deuteriated are shown in the Fig. 5. Both display a typical spectrum characteristic of a perdeuteriated chain in a L_α phase. However, the values of the order parameters are different. The order profiles obtained using the integration method are shown in Fig. 6. The order parameters of POPE are larger than those of POPC all along the lipid chain. Similar conclusions have been reported for selected positions of the palmitoyl chains (Perly *et al.* 1985). This is proposed to reflect the tendency of POPE to adopt the H_{II} phase. The cone shape leads to the existence of additional lateral pressure that increases the order of the acyl chains. This may be also described by the curvature arguments, as a large difference existing between the actual radius of curvature ($R \simeq \infty$ for a bilayer) and the intrinsic spontaneous radius of curvature. The tension created by this difference is a potential explanation for the increased order.

Similar results are obtained on lipid mixtures. The introduction of a cone-shaped lipid in a bilayer increases the order of the acyl chains. This has been shown by the addition of POPE in a POPC matrix (Cullis *et al.* 1986); the order has been measured using POPC labelled on the 11th position of the oleoyl chains, next to their double bond (Fig. 7). A correlation between the proportion of POPE (the cone-shaped lipid) in the bilayer and the quadrupolar splitting measured by POPC has been found. This indicates that when constrained in the bilayer structure, the tendency of POPE for the H_{II} phase influences the hydrocarbon order.

These results indicate that the size of the polar head group, which is a determinant for polymorphic behavior, also affects the order parameters along the acyl chains. Other factors that modulate polymorphism, such as temperature or the presence of unsaturation in the acyl chains, have been also shown to affect the lipid chain order parameters (Seelig and Seelig 1977, 1980). These results encourage investigations of the relationship between the lipid polymorphic preferences and the orientational order of the acyl chains.

Conclusion

Order parameters (their values and their distribution along the chain) and lipid polymorphism appear to be intimately related. It is proposed that the forces which modulate polymorphism are also crucial in the regulation of the chain order. The information obtained by ^{2}H-NMR concerning this order may be significant for the understanding of lipid polymorphism, particularly if it allows us to characterize

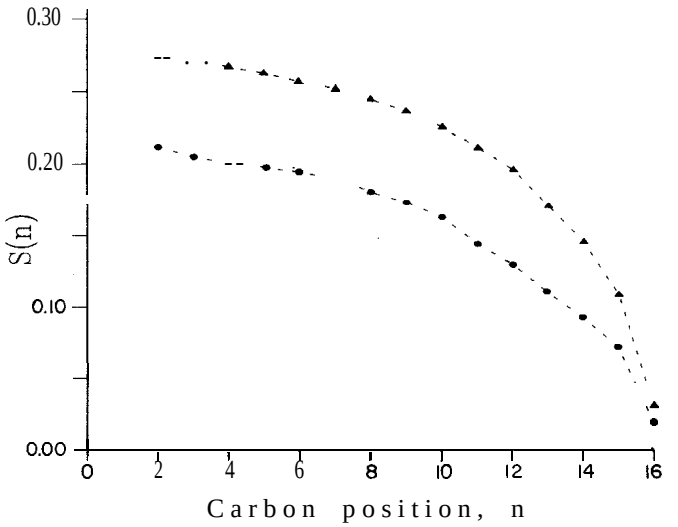

FIG. 6. Orientational order profile of POPC (●) and POPE (A) at 30°C.

the polymorphic tendency by a measurable and well-defined parameter such as the orientational order. The dePakeing method for the determination of the order profile using a perdeuteriated saturated chain is important in this regard as a straightforward way to obtain information on crucial features of the lipid structure.

In this investigation of polymorphism, ^{2}H-NMR spectroscopy is not used to characterize the local structure of the acyl chains, but to provide a description of the reaction of the lipid ensemble in the membrane to different forces. Order parameters should be considered in the same spirit as thermodynamic measurements, since the value measured is actually a mean value of a large number of lipids. Statistical mechanical treatments have proposed a correlation between the order of the acyl chains and the bilayer thickness (Seelig and Seelig 1974; Mouritsen and Bloom 1984; DeYoung and Dill 1988). Investigations are in progress to establish other possible relationships between the order and different parameters such as R_0. In contrast to many other techniques, ^{2}H-NMR does not give a single parameter, but provides a description of the profile of the order across the bilayer.

The regulation of the lipid composition of biological membranes is likely necessary to optimize certain characteristics of the lipid matrix. Various parameters have been proposed: bilayer and nonbilayer forming lipid balance (Wieslander *et al.* 1980), lipid fluidity (Chapman 1980), bilayer thickness (Bloom and Mouritsen 1988), or the spontaneous radius of curvature (Gruner 1985). The regulation of the order of the hydrophobic core of the bilayer by lipids with different shapes or different R_0 might also be a significant factor in the control of lipid distribution in biological membranes.

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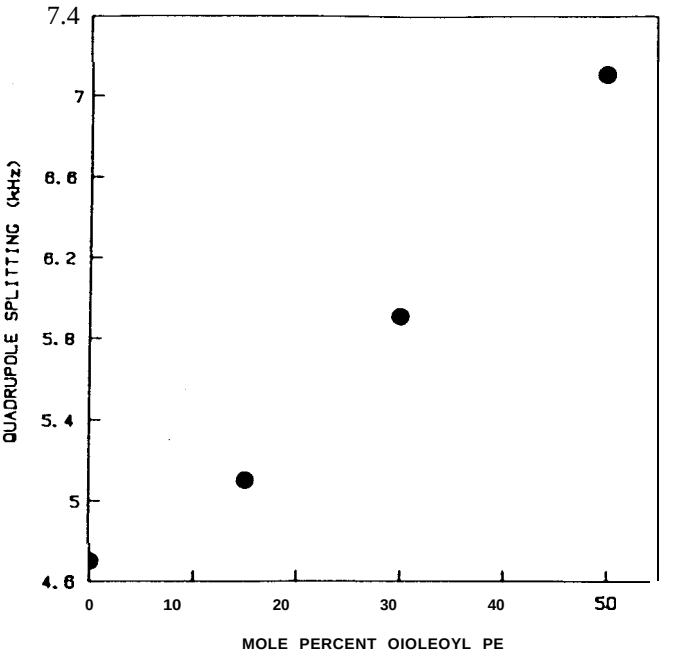

FIG. 7. Effect of the addition of a cone-shaped lipid (DOPE) in a bilayer matrix (DOPC) on the order parameter probed at the 11th position of the oleoyl chains of DOPC. (From Cullis *et al.* 1986, with permission.)

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