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^{2}H- AND ^{31}P-NMR STUDIES OF BILAYERS COMPOSED OF 1-ACYLLYSOPHOSPHATIDYLCHOLINE AND FATTY ACIDS

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1-Palmitoyllysophosphatidylcholine has been mixed in equimolar amounts with specifically deuterated palmitic acid and the structural properties of the lipid/water phase have been studied by ^{2}H- and ^{31}P-nuclear magnetic resonance. The order profile of the free palmitic acid is very similar to that of the parent compound 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine at temperatures above the gel-to-liquid crystal phase transition. The bending of the *sn*-2 chain which is typical for diacyl lipids is not observed for the free palmitic acid. The mixture of lysolipid and palmitic acid exhibits well-defined quadrupole splittings even at temperatures below the gel-to-liquid crystal phase transition. Hence it is possible for the first time to establish an order profile in the gel-state of the lipid bilayer phase. Between carbon atoms 5 to 12 the palmitic acid chain is found to assume the extended all-*trans* conformation with a very small contribution from *gauche* defects. Towards the methyl terminal a distinct increase in the *gauche* probability can be noted. The motion of the phosphocholine headgroup was also studied by ^{2}H- and ^{31}P-NMR using selectively deuterated 1-palmitoyllysophosphatidylcholine. The headgroup has a considerably larger motional freedom in the mixture of lysolipid and palmitic acid than in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. In addition, the average headgroup conformations are also different in the two systems.

Introduction

The hydrolysis of phospholipids by phospholipase A_2 leads to the production of equimolar amounts of lysophospholipid and free fatty acid [1]. In spite of the fact that the individual compounds act as detergents, the equimolar mixture of the two retains the bilayer structure as has been demonstrated for pure two-component mixtures [2,3]. This finding sheds light on some unexpected results obtained with erythrocyte membranes [4,5]. Erythrocyte membranes which are subjected to phospholipase A_2 treatment do not disintegrate but preserve their bilayer structure, most probably via the formation of a functional dimer of lysophospholipid and free fatty acid. The primary evidence for the bilayer structure of the hydrolysed lipids has come from ^{31}P-NMR. The ^{31}P-NMR spectra of phospholipase-treated erythrocytes as well as those of equimolar mixtures of lysophospholipids and fatty acids exhibit a negative chemical shielding anisotropy which is usually considered to be a sufficiently strong evidence for the bilayer organisation of the lipids involved. A large variety of studies on model membranes and biological membranes where the bilayer structure had been established unambiguously by X-ray or neutron diffraction has indeed demonstrated that lipid bilayers (in the liquid crystalline state) are characterized by a negative chemical shielding anisotropy, $\Delta\sigma$, of about -40 ppm to -50 ppm [6–8]. In contrast, phospholipids organized in an

inverted hexagonal phase (H_{II} phase) give rise to a positive chemical shielding anisotropy of about 20 ppm [9,10]. The change in sign and the reduction in magnitude by a factor of two are caused by the additional motion of the phospholipids around the cylinder axis and does not imply a change in the headgroup structure [8,9].

The justification of this empirical interpretation of ^{31}P-NMR spectra must be scrutinized carefully in the case of lysophospholipids since the experimentally observed chemical shielding anisotropy of 1-acyllysophospholipids is only -15 ppm to -20 ppm as compared to $\Delta\sigma \approx 50$ ppm for diacylphospholipids [2]. This difference is indicative of a structural reorganisation of the phospholipids which could just be a conformational change of the phosphocholine headgroup but could also involve an additional change in the phase structure. It follows from theoretical considerations that no decision between these two alternatives can be made on the basis of ^{31}P-NMR spectra alone [11]. In the present study the structural properties of mixtures of 1-palmitoyllysophosphatidylcholine and palmitic acid were therefore studied with ^{2}H-NMR since this method allows a wider range of molecule segments to be investigated. Moreover, comparative ^{2}H-NMR data are already available for the bilayer-forming parent compound 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [12]. The purpose of the present work is then to provide a detailed comparison between the two systems at the level of both the hydrocarbon chains and the phosphocholine headgroup.

Materials and Methods

Selectively deuterated palmitic acids and chain deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholines (16 : 0/16 : 0-PC) were from the same batch as in Ref. 12. In the free palmitic acid the deuterium label was attached at carbon atoms 2, 3, 4, 5, 9, 12, and 15.

16 : 0/16 : 0-PC's deuterated at the methylene segments of the phosphocholine segments and at the *sn*-3 segment of the glycerol backbone were prepared as described in Ref. 6.

1-Acyllysophosphatidylcholines were prepared by the action of phospholipase A_2 (*Crotalus atrox* venom) on 16 : 0/16 : 0-PC in moist diethyl ether [13].

The codispersions were prepared by dissolving equimolar amounts (approx. 0.1 mmol) of 1-palmitoyllysophosphatidylcholine and palmitic acid in chloroform/methanol (2 : 1, v/v). The solvent was evaporated under vacuum and the dry film was dispersed in 500 mg buffer containing 150 mM KCl, 10 mM Tris, and 0.2 mM EDTA at pH 7.5. The buffer was made up with deuterium depleted water.

Nuclear magnetic resonance spectra were recorded at 46 MHz for deuterium and 121 MHz for phosphorus with a Bruker CXP-300 spectrometer. The quadrupole echo mode was used for the ^{2}H-NMR spectra. The ^{31}P-NMR spectra were proton-decoupled with a high-power decoupler. The experimental conditions are the same as described elsewhere [14].

Results

Hydrocarbon region

The lipid conformation in multilamellar dispersions of lipid bilayers can be determined by ^{2}H-NMR (for reviews, see Refs. 15–17). In particular, the order profile of bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine above the gel-to-liquid crystal phase transition is well-established [12]. ^{2}H-NMR has also demonstrated that the *sn*-1- and *sn*-2-fattyl acyl chains are conformationally inequivalent near the glycerol backbone [18,19]. Related ^{2}H-NMR studies have been performed at temperatures below the phase transition [20,21]. Under these conditions the lineshape of the ^{2}H-NMR spectra is rather broad and and is determined, in part, by slow motions. Model-dependent spectral simulations are required in order to arrive at molecular interpretations.

A comparison of ^{2}H-NMR spectra of an aqueous dispersion of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine with those of an equimolar mixture of 1-palmitoyllysophosphatidylcholine and palmitic acid is shown in Fig. 1. The diacylphospholipid was deuterated at carbon atom 9 of both fatty acyl chains, whereas the mixture was deuterated at the C-9 position of the free palmitic acid only.

Let us first consider the liquid-crystalline

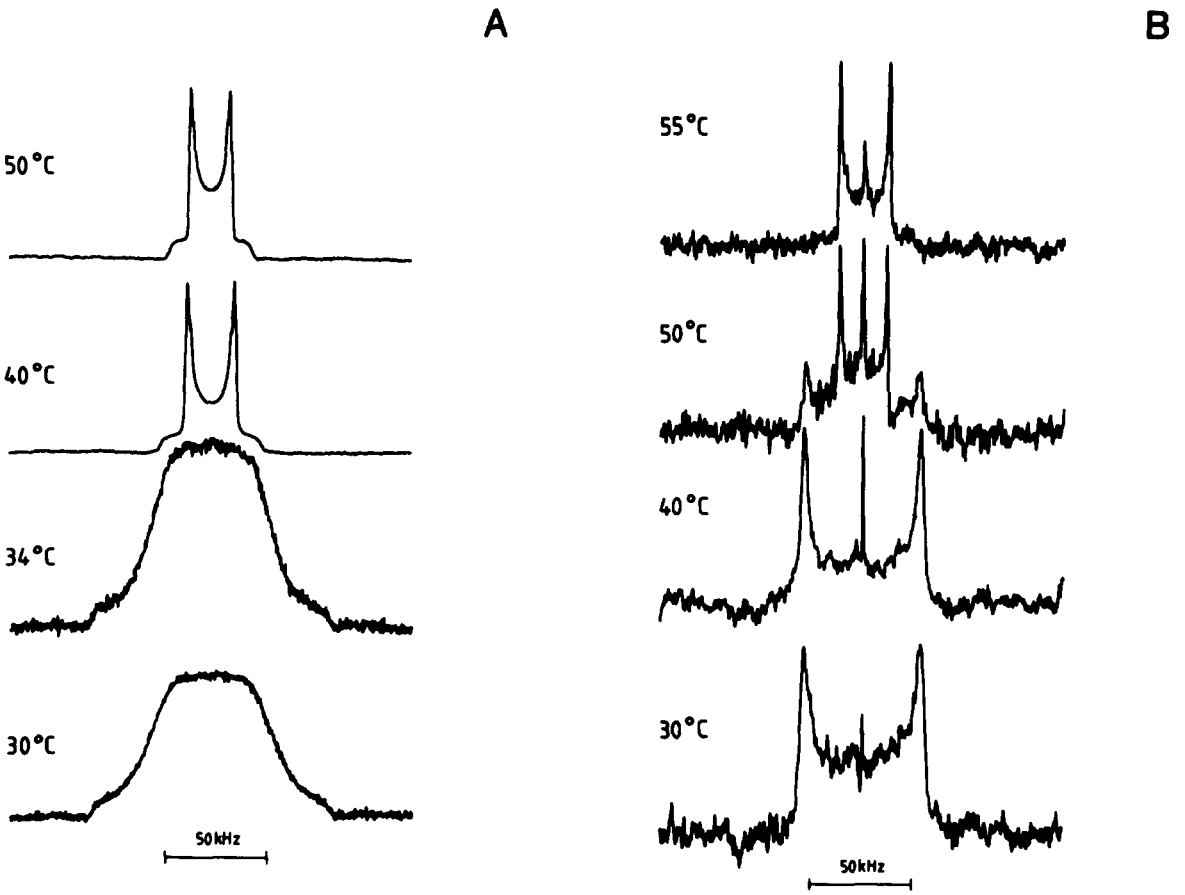


Fig. 1. (A) ^{2}H-NMR spectra at (46.1 MHz) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in water above and below the phase transition. The lipid is deuterated at the C-9 position of both hydrocarbon chains. (B) ^{2}H-NMR spectra of an equimolar mixture of 1-palmitoyl-lysophosphatidylcholine and palmitic acid. The C-9 position of the free palmitic acid is deuterium-labelled.

phases. Above 50°C both systems exhibit very similar ^{2}H-NMR spectra, characteristic of liquid-crystalline microdomains with uniaxial symmetry. From the quadrupole splitting $\Delta\nu_Q$ (defined as the separation of the most intense peaks in the spectrum) it is possible to derive the absolute value of the deuterium order parameter S_{C^2H} according to

$$|s_{C^2H}| = (4/3)(e^2qQ/h)^{-1}\Delta\nu_Q \quad (1)$$

with $(e^2qQ/h) = 170$ kHz. S_{C^2H} is a quantitative measure for the angular fluctuations of the C^2H bond vector and can be related to the segmental fluctuations of the long molecular axis S_{mol} according to [12]:

$$S_{mol} = 2|S_{C^2H}| \quad (2)$$

S_{mol} renders itself more easily to a molecular interpretation: for stiff and extended all-*trans* hydrocarbon chains S_{mol} equals unity for all segments. While Eqn. 1 is strictly valid for systems with uniaxial motional symmetry the transformation from $|S_{C^2H}|$ to S_{mol} is based on specific assumptions which are fulfilled to a good approximation for the inner region of the hydrocarbon layer but break down close to the water interface (cf. below). The variation of the order parameters $|S_{C^2H}|$ and S_{mol} as a function of labelled carbon atom above the phase transition is summarized in Fig. 2A. The order profiles of the two systems are found to be virtually superimposable from C-5 to C-15 and exhibit only small quantitative differences close to the lipid/water interface, i.e. from C-3 to C-5.

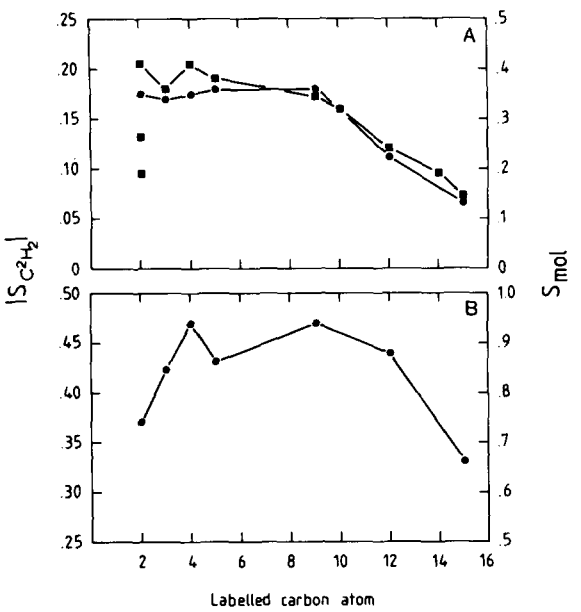


Fig. 2. Comparison of order profiles. (A) Order profile above the phase transition (at 50°C). ■———■, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ●——— ●, 1-palmitoyl-lysophosphatidylcholine + palmitic acid. (B) Order profile of 1-palmitoyl-lysophosphatidylcholine below gel-to-liquid crystal phase transition (at 30°C).

Hence it may be concluded that the hydrolysis of the ester bond at the *sn*-2 position does not entail a large increase in motional freedom for the liberated palmitic acid.

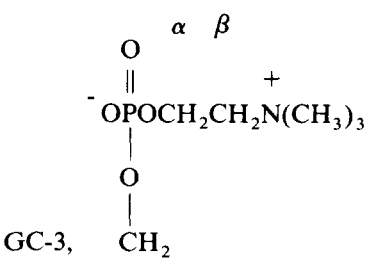
A different situation is encountered for the C-2 position. The ^{2}H-NMR spectrum of 1,2-di-[2-$^2H_2$]palmitoyl-*sn*-glycero-3-phosphocholine consists of three quadrupole splittings with separations of about 27.2, 19.2 and 12 kHz at 50°C [12]. The large splitting arises from the two deuterons of the *sn*-1 chain while the two smaller splittings must be assigned to the two deuterons of the *sn*-2 chain [18,22]. The reason for the smaller splittings is a bend in the *sn*-2 chain. The *sn*-2 chain starts out parallel to the bilayer surface and bends parallel to the *sn*-1 hydrocarbon chain only after the C-2 segment. This unique structural feature of the *sn*-2 chain is seen most clearly in the two single-crystal structures of phospholipids which are available to date [23,24]. The *sn*-1 chain, on the other hand, is perpendicular to the bilayer surface at all carbon segments. The quadrupole splitting observed for the C-2 position of free palmitic acid in mixture with 1-palmitoyllysophosphatidylcholine clearly corresponds to that of the *sn*-1 chain of the diacyl lipid. This leads to the conclusion that the splitting of the ester linkage removes the bend at the C-2 position leading to a more extended conformation of the released palmitic acid.

We may now proceed to a discussion of ^{2}H-NMR spectra at and below the phase transition. Aqueous dispersions of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine are characterised by a sharp gel-to-liquid crystal phase transition with a transition temperature of $t_c = 41$°C [25] which is also reflected in the ^{2}H-NMR spectra (Fig. 1). Above the phase transition the spectra exhibit the typical line-shape of non-oriented liquid crystals with well-defined quadrupole splittings as discussed above. Below the phase transition the spectra are considerably broadened and the evaluation of the residual quadrupole splitting requires a computer simulation of the spectrum. This situation is quite different for the mixture of 1-palmitoyl-lysophosphatidylcholine and palmitic acid. Again the system is characterized by a gel-to-liquid crystal transition, this time centered around 45°C, but differential scanning calorimetry [2] as well as ^{2}H-NMR reveal that the transition is much broader compared to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. The ^{2}H-NMR spectra obtained in the transition region are characterized by a superposition of two well-defined quadrupole splittings (Fig. 1B). With decreasing temperature the intensity of the outer splitting grows at the expense of the inner splitting until at about 40°C only the outer splitting is retained. The ^{2}H-NMR spectra thus demonstrate the coexistence of a gel phase and a liquid-crystalline phase over a temperature interval of at least 10 K. The second point of interest is the appearance of the ^{2}H-NMR spectra in the gel-phase. The intrinsic linewidth is much reduced compared to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and the residual quadrupole splitting can be measured in the conventional manner since the spectra conform to the theoretical lineshape expected for systems with uniaxial motional symmetry. It may be noted in Fig. 1 that the quadrupole splittings below the phase transition are about a factor of two larger than above the phase transition indicating a considerable stiffening of the

hydrocarbon chains. Due to the well-defined lineshapes it is possible to derive an order profile also for the gel state (Fig. 2B). Inspection of Fig. 2B reveals that the chain order parameter S_{mol} reaches almost its maximum value of $S_{mol} = 1.0$ in the region between C-5 to C-13. The drop of S_{mol} towards the terminal methyl group parallels that observed for the liquid-crystalline state (Fig. 2A) and may be explained by an increased flexibility of the very last chain segments. The same explanation could apply to the first two segments, however an alternative possibility appears to be more probable: the denser packing in the gel-state could force the palmitic acid into a bent conformation such as to preform the structure observed in single crystals of diacylphospholipids. As has been mentioned above and has been quantitated previously [26] such a bend (which is still in dynamic equilibrium with the extended chain conformation) would considerably reduce the residual quadrupole splittings. Thus the decrease in the order profile between positions C-4 to C-2 could be the result of a geometric constraint rather than of an increase in segmental freedom.

Polar headgroup

In order to simplify the discussion, the following nomenclature for the deuterated headgroup segments is introduced:



In the experiments described below the deuterium label is always attached at 1-palmitoyllysophosphatidylcholine and non-deuterated palmitic acid is added as the second component. Typical ^2H- and ^{31}P-NMR spectra of the headgroup segments are reproduced in Fig. 3. All measurements were made at 55°C and hence refer to the liquid-crystalline state of the mixture. Related studies on the parent compound, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, have been published previously [6,27] and a numerical com-

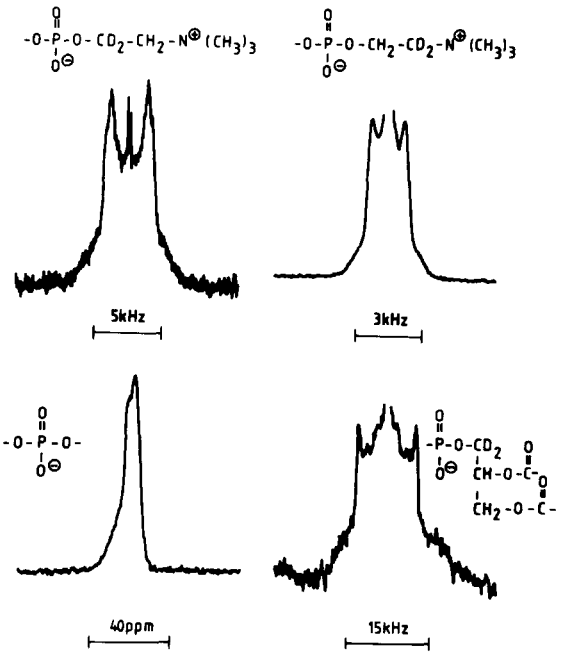


Fig. 3. ^2H- and ^{31}P-NMR spectra of the headgroup segments of 1-palmitoyllysophosphatidylcholine in equimolar mixture with palmitic acid at 55°C. D, ^2H.

TABLE I

QUADRUPOLE SPLITTINGS $\Delta\nu_Q$ (kHz) AND CHEMICAL SHIELDING ANISOTROPIES $\Delta\sigma$(ppm) OF VARIOUS HEADGROUP SEGMENTS

	PO_4 (ppm)	GC-3 (kHz)	α-C^2H_2 [b] (kHz)	β-C^2H_2 [b] (kHz)
1-Palmitoyllysophosphatidylcholine/palmitic acid [a]				
55	−19	11.2	3.6; 2.8 [c]	1.6
50	−22	11.3	3.0	2.4
40	−30	broad	broad	broad
30	−35		broad	broad
1,2-Dipalmitoyl-*sn*-3-phosphocholine [d]				
55	−45.5	26.2; 28.7 [e]	5.9	4.6
50	−47.0	26.8; 29.8	5.9	5.0
40	−53.0	broad	~ 6.5	~ 7.2
30			broad	broad

[a] 1 : 1 molar ratio.

[b] The headgroup splittings are very sensitive to even small changes in the phase composition.

[c] Two quadrupole splittings are observed which must be assigned to the individual deuterons of the α-C^2H_2 group (cf. also Ref. 27).

[d] Data interpolated from Refs. 6 and 27.

[e] Two quadrupole splittings which must be assigned to the individual deuterons (Haberkorn, R.A. et al., unpublished data).

parison of the two systems is shown in Table I. The unique feature of the mixture are the rather small residual anisotropies observed at all headgroup segments. Compared to the diacylphospholipid all quadrupole splittings as well as the chemical shielding anisotropy are reduced by a factor of 2.8 to 1.8 with the extent of reduction varying somewhat from segment to segment. In contrast to the ^{2}H-NMR spectra of the hydrocarbon region the headgroup spectra below the phase transition temperature do not exhibit well-defined quadrupole splittings but give rise to rather broad lineshapes.

Discussion

Hydrocarbon chain flexibility

The ^{2}H-NMR spectra of the hydrocarbon region provide evidence for a close similarity between bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and mixtures of 1-palmitoyllysophosphatidylcholine and palmitic acid, at least as far as the liquid crystalline state is concerned. From the almost quantitative agreement of the $|S_{C^2H}|$-order profiles of the two systems the following two conclusions can be drawn: (1) Since 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine is known to adopt the bilayer configuration the same appears to hold true for the mixture of lyso-lipid and fatty acid. A hexagonal phase can be excluded in all likelihood because additional motion around the cylinder axis reduces all quadrupole splittings by a factor of two. Hence, in order to produce the same experimental quadrupole splittings as the bilayer the hexagonal phase must possess much stiffer hydrocarbon chains. However, considering the unfavorable geometry of a hexagonal phase the opposite behavior is expected in order to avoid packing problems. Indeed, ^{2}H-NMR studies on 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine have demonstrated that the hydrocarbon chains are more disordered in the hexagonal phase (H_{II}) than in the bilayer [29]. (2) The free palmitic acid in the mixture assumes approximately the same average location in the bilayer as the covalently linked palmitic acid in the parent compound. If there was a shift in the segment positions this would be reflected in a corresponding shift of the order profile along the abscissa which is however not observed. This conclusion is at variance with an earlier interpretation based on measurements of the transition temperature T_c [3]. Since the transition temperature reflects an overall thermodynamic behavior, the NMR approach is probably better suited to reveal the details of the molecular structure.

The other interesting feature about the hydrocarbon region are the well-defined lineshapes in the so-called gel state of the mixture. The spectra correspond to fast-limit axially symmetric powder patterns [15] which indicates that the diffusion rates in the gel state are still fast on the ^{2}H-NMR time scale. This is in contrast to the behavior of the same fatty acid in bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [30] or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [31] where the motional rates in the gel-state are no longer in the fast limit. While the latter spectra require model-dependent computer simulations, the ^{2}H-NMR spectra of the mixture can easily be interpreted in terms of S_{C^2H} order parameters. Fig. 2B therefore represents the first experimentally observed order profile of a bilayer in the gel phase.

If it is assumed that in the gel state the palmitic acid chains are in the all-*trans* conformation and are rotating rapidly around their long molecular axis then the quadrupole splitting of the powder-pattern should be $\Delta\nu_Q \approx 63.75$ kHz, corresponding to $|S_{C^2H}| = 0.5$ and $S_{mol} = 1.0$. This limiting value is, in fact, approached experimentally in the middle part of the hydrocarbon layer. For example, the quadrupole splitting at the C-9 position measures 59.9 kHz ($S_{C^2H} = -0.47$; $S_{mol} = 0.94$) at 30°C. However, long axis diffusion alone is not sufficient to explain the decrease of the order profile towards the methyl terminal. Additional motional averaging can be achieved by invoking *trans-gauche* isomerisations around individual carbon-carbon bonds. In terms of this model the middle part of the chain shows the lowest *gauche* probability P_g which may be estimated according to Ref. 12

$$P_g = (1 - S_{mol})/1.125$$

For the C-9 position at 30°C this relation yields $P_g^{(9)} \approx 0.05$. The *gauche* probability increases towards the methyl terminal and the same reasoning

applied to the C-12 position ($S_{mol} \approx 0.88$) leads to $PO_g^{(12)} = 0.11$. These numbers are only crude approximations but are in agreement with related estimates on bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine based on computer simulations of gel-state spectra [31].

Phosphocholine headgroup

The average orientation of the phosphocholine head group is parallel to the bilayer surface, at least in bilayers of 1,2-dimyristoyl- and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [24,32,33]. In addition it is known from NMR and dielectric studies that the phosphocholine dipoles rotate rapidly in the plane of the membrane with a diffusion constant of about $2 \cdot 10^8\ s^{-1}$ corresponding to a NMR correlation time τ_c of approx. 1 ns for the phosphate group [34–36]. ^{2}H-NMR investigations have further demonstrated that the phosphocholine group is not locked into a rigid conformation but undergoes conformational transitions [36,37]. In this respect it is interesting to note that the correlation times for the reorientation of the C^2H bond vectors of the choline α- and β-segments as determined from ^{2}H-spin lattice relaxation times are about one order of magnitude shorter than that of the whole phosphocholine dipole [35].

Compared to this rather detailed picture for the phosphocholine group in diacyl lipids the data presented here allow only much more limited conclusions. The new feature of the mixture of lysophospholipid and free fatty acid is the reduction of all head group anisotropies, including that of the backbone GC-3 segment. The molecular interpretation of these changes leads to two conclusions. (1) The phosphocholine headgroup in lysophosphatidylcholine is less restricted in its movement and samples a larger conformational space. This explains the large reductions in the quadrupole splittings and the chemical shielding anisotropy by more than a factor of two. (2) Since the reduction factor is dependent on the segment position, this is also indicative of a conformational change of the phosphocholine headgroup. Without a conformational change segments should experience exactly the same relative reduction. The nature of the conformational change cannot be established with the presently available data.

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