Dynamic Chain Conformations in Dimyristoyl Glycerol–Dimyristoyl Phosphatidylcholine Mixtures. ^{2}H-NMR Studies

Karl Schorn and Derek Marsh
Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-37077 Göttingen, Germany

ABSTRACT The dynamic molecular lipid chain conformations in fully hydrated dimyristoyl phosphatidylcholine (DMPC)–dimyristoyl glycerol (DMG) mixtures have been investigated by ^{2}H-NMR spectroscopy of the individual lipid components, the *sn*-2 chains of which were perdeuterated or, in the case of DMG, specifically deuterated at the C-2 position. Mixtures of compositions corresponding to the three different regions of the binary phase diagram in which the fluid phase is lamellar (DMPC:DMG 70:30 mol/mol), inverted hexagonal (DMPC:DMG 45:55 and 40:60 mol/mol), or isotropic (DMPC:DMG 20:80 mol/mol) were investigated. The gel phase in all three regions of the phase diagram has a lamellar structure, with the lipid chains rotating about the molecular long axis but executing only limited angular excursions. In the fluid lamellar phase of the 70:30 mol/mol DMPC–DMG mixture the profile of segmental chain flexibility is similar to that in single-component phospholipid bilayers and is characterized by an order parameter plateau for both lipid components. The chain order of the DMPC component is greater than in bilayers of DMPC alone and is also greater than that of the DMG component. In the inverted hexagonal phase of the 45:55 mol/mol DMPC–DMG mixture the chain flexibility profile is characterized by more widely spaced segmental order parameters off the plateau region. The intrinsic degree of chain order in the inverted hexagonal phase is less than in the lamellar phase of the 70:30 mol/mol mixture, and the difference in chain order between the DMPC and DMG components is reduced relative to that in the lamellar phase. The unique conformational features at the C-2 position of the *sn*-2 chain that characterize bilayers of diacyl phospholipids are found also for the diacylglycerol molecules in the fluid lamellar phase and most probably also in the inverted hexagonal phase. The DMG molecules are therefore integrated in the membrane (or nonlamellar lipid phase) in a configuration that is similar to that of the phospholipids and different from the crystal structure of diacylglycerols.

INTRODUCTION

Diacylglycerols are the endogeneous activators of protein kinase C and have been implicated both in exocytotic membrane fusion and in potentiation of the action of certain lipolytic enzymes (for reviews see, e.g., Newton, 1993; Zidovetzki and Lester, 1992; Baker, 1988). In the membrane action of diacylglycerols, high local concentrations of the enzymatically generated species could well play a significant role. It is therefore of both interest and potential relevance to investigate the interactions of diacylglycerols in lipid membranes over a wide range of composition. Binary phase diagrams have been determined for hydrated phosphatidylcholine–diacylglycerol mixtures (Heimburg et al., 1992; López-García et al., 1994). A particular feature found is that the fluid phases of the mixtures at high diacylglycerol content are not of the lamellar bilayer type. The present study concentrates partly on the dynamic molecular conformations that underlie these differences. The ability of diacylglycerols to induce vesicle fusion (Siegel et al., 1989; Ortiz et al., 1992), in which the normal lamellar membrane topology is transiently disrupted, must certainly be related to their potential for forming nonlamellar lipid phases. The latter potential may also be implicated in their controlling effects on the conformational equilibrium of peripherally bound membrane proteins, as demonstrated for cytochrome *c* (Heimburg et al., 1991). Likewise, it is probable that the tendency to induce nonlamellar phases (rather than their actual formation) is responsible for enhanced activation of protein kinase C by diacylglycerols (Goldberg et al., 1994).

^{2}H-NMR was used previously to investigate the chain order in a variety of phosphatidylcholine–diacylglycerol mixtures (De Boeck and Zidovetzki, 1989, 1992; Goldberg et al., 1994). These studies were confined primarily to the lamellar phase and concentrated mostly on the labeled phosphatidylcholine component. Here, the full range of diacylglycerol contents is investigated by ^{2}H-NMR studies of both components, and emphasis is put on the dynamic conformation of the diacylglycerol. In the latter connection, the configuration of the *sn*-2 chain at the C-2 position is of considerable significance (cf. Hamilton et al., 1991; Smith et al., 1992). The studies are performed on dimyristoyl phosphatidylcholine–dimyristoyl glycerol (DMPC–DMG) mixtures with saturated chains that display well-defined lamellar and nonlamellar phases, as indicated by the phase

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Address reprint requests to Dr. Derek Marsh, MPI fuer biophysikalische Chemie, Abt. 010 Spektroskopie, Am Fassberg, D-37077 Gottingen-Nikolausberg, Germany. Tel.: 49-551-201-1285; Fax: 49-551-201-1501; E-mail: dmarsh@gwdg.de.

Abbreviations used: d_{27}-DMPC, 1-myristoyl-2-[$^2H_{27}$]myristoyl-*sn*-glycero-3-phosphocholine; d_2-DMPC, 1-myristoyl-2-[2-$^2H_2$]myristoyl-*sn*-glycero-3-phosphocholine; d_{27}-DMG, 1-myristoyl-2-[$^2H_{27}$]myristoyl-*sn*-glycerol; d_2-DMG, 1-myristoyl-2-[2-$^2H_2$]myristoyl-*sn*-glycerol; DMG, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; L_β, lamellar gel phase; L_α, fluid lamellar phase; H_{II}, inverted hexagonal phase.

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diagram established previously (Heimburg et al., 1992). The phase diagram is divided into three regions (I–III) that are defined by the formation of stoichiometric compounds in the gel phase with DMPC:DMG compositions of approximately 1:1 and 1:2 mol/mol. In regions I, II, and III the fluid phases are progressively of lamellar, inverted hexagonal, and isotropic structure, respectively. The present studies delineate the effects of composition on the chain order and conformation of both lipid components under the conditions obtained in these three critical regions of the phase diagram. The results should also be representative of many situations for other phospholipid–diacylglycerol mixtures, e.g., with different chain compositions. In many cases, however, the various characteristic features can be achieved at lower diacylglycerol concentrations, for instance with lipids that have unsaturated chains (cf. Das and Rand, 1986).

MATERIALS AND METHODS

Materials

Perdeuterated myristic acid and myristic acid specifically deuterated at the C-2 position were obtained from Larodan (Malmö, Sweden). Dimyristoyl phosphatidylcholine (DMPC) was from Fluka (Buchs, Switzerland), and 1-myristoyl lysophosphatidylcholine was from Sigma (St. Louis, MO). Phospholipase C from *Bacillus cereus* was obtained from Boehringer-Mannheim (Mannheim, Germany). We synthesized dimyristoyl phosphatidylcholine deuterated in the *sn*-2 chain (d_{27}-DMPC or d_2-DMPC) by condensing the corresponding deuterated myristic acid with 1-myristoyl lysophosphatidylcholine according to the methods of Mason et al. (1981). Dimyristoyl glycerol (DMG), either fully protonated or deuterated in the *sn*-2 chain (either d_{27}-DMG or d_2-DMG), was synthesized enzymatically from the corresponding phosphatidylcholine by the action of phospholipase C in ether/water (1:1, v/v) at 0°C. The DMG was extracted from the ether phase after the reaction had gone to completion, and purity was checked by thin-layer chromatography.

Sample preparation

In the region of 60–80 mg of deuterated DMPC or DMG was codissolved in dichloromethane with the desired amount of the undeuterated partner lipid to give ~100–~200 mg of the DMPC–DMG lipid mixture. Both lipid components dissolve well in dichloromethane, which is a suitable solvent for producing well-mixed DMPC–DMG preparations (Heimburg et al., 1992). The solvent was evaporated from the mixed lipid solution in a 10-mm-diameter NMR tube by a stream of nitrogen gas, and the sample was then kept under vacuum for at least 3 h. The dry lipid was hydrated with ~200 μL of deuterium-depleted water (Aldrich, Steinheim, Germany) by vortex mixing at a temperature above the chain-melting transition. The same sample as for ^{2}H-NMR could be used for ^{31}P-NMR without transfer of the hydrated lipid mixture.

NMR spectroscopy

Broad-line ^{2}H-NMR spectra were recorded on a Bruker MSL-300 spectrometer at a frequency of 46.1 MHz by use of the quadrupolar echo (90_x-τ-90_y) sequence. The length of the 90° pulse was 6 μs, the interpulse spacing was 30 μs, and the recycle delay was 1 s. The digitization rate was adjusted to give an effective sweep width of 200 kHz for samples in the gel phase and of 62.5 kHz for samples at temperatures in the fluid phase. Proton dipolar decoupled ^{31}P-NMR spectra were recorded at a frequency of 121.5 MHz with a 90° pulse of 11 μs and a recycle delay of 5 s. The decoupling power was gated on only during collection of the free induction decay. Temperature was regulated by a thermostatted nitrogen gas-flow system.

The quadrupole splitting of the perpendicular peaks in a Pake-doublet ^{2}H-NMR powder pattern is given by (Seelig, 1977)

$$\Delta\nu_\perp = \tfrac{3}{4}(e^2Qq/h)S_{CD}, \quad (1)$$

where (e^2Qq/h) is the quadrupole coupling constant and S_{CD} is the order parameter of the CD bond relative to the principal ordering axis (i.e., the bilayer normal). For the terminal CD_3 group, rapid rotation of the methyl group (even in solids) results in axial symmetry about the C—C linkage, the order parameter of which is related to S_{CD} by $S_{C—C} = 3S_{CD}$ [e.g., Seelig (1977)]. The quadrupole splittings for a crystalline phospholipid with perdeuterated chains were measured to be $\Delta\nu_\perp$ = 122 kHz and 34.5 kHz for the CD_2 and CD_3 segments, respectively (D. Marsh, unpublished). For rapid rotation about the long axis of an all-*trans* chain these values should be reduced by a factor of 2 (i.e., $|S_{CD}| = 0.5$) in both cases. The quadrupole splittings of the parallel shoulders in the Pake powder pattern have twice the above values.

Assuming axial symmetry, the order parameter of a chain segment, S_{mol}, is related to that of the CD bond by $S_{mol} = -2S_{CD}$ [e.g., Seelig (1977)]. The cross-sectional area per chain, A, can be related to the value of the chain-order parameter in the plateau region, $\bar{S}_{mol}$, by a lattice model for the chain isomerism (De Young and Dill, 1988):

$$A = 3A_0/(2\bar{S}_{mol} + 1), \quad (2)$$

where $A_0 \approx 20$ Å^2 is the cross-sectional area of an all-*trans* chain.

For a phospholipid with rapid rotation about the long axis, the chemical shift anisotropy ($\Delta\sigma = \sigma_{//} - \sigma_\perp$) in the ^{31}P-NMR spectrum is given by [cf. Seelig (1978)]

$$\Delta\sigma = \Delta\sigma_R \cdot S_{PO_4} \quad (3)$$

where $\Delta\sigma_R \approx -74$ ppm is the rotationally averaged value of the nonaxial chemical shift anisotropy (cf. Griffin et al., 1978) and S_{PO_4} is the order parameter of the principal axis for this rotational averaging relative to the bilayer normal. In inverted hexagonal (H_{II}) phases the value of S_{PO_4} is reversed in sign and reduced by a factor of 2, relative to the corresponding lamellar phase, by translational diffusion about the cylinder axis of the H_{II} tubes.

RESULTS

^{31}P-NMR spectra

Proton dipolar-decoupled ^{31}P-NMR spectra of hydrated DMPC–DMG mixtures with compositions of DMPC:DMG = 70:30, 40:60, and 20:80 mol/mol are given in Fig. 1. For the sample with low DMG content (DMPC:DMG, 70:30 mol/mol) the spectra of the DMPC component correspond to powder patterns from an axial system with negative chemical shift anisotropy that is characteristic of a lamellar phospholipid arrangement. This is the case over the entire temperature range 20-60°C. At the lower temperatures the linewidths are broader, corresponding to the lamellar gel phase (L_β). At higher temperatures the linewidths narrow (seen particularly in the high-frequency $\sigma_\perp$ peak) corresponding to a transition to the fluid lamellar (L_α) phase. For the sample with intermediate DMG content (DMPC:DMG, 40:60 mol/mol) the spectra at low temperatures are again characteristic of a L_β lamellar gel phase. At higher temperatures these convert to a powder pattern with smaller chemical shift anisotropy of opposite sign that is characteristic of a system with cylindrical symmetry. In the

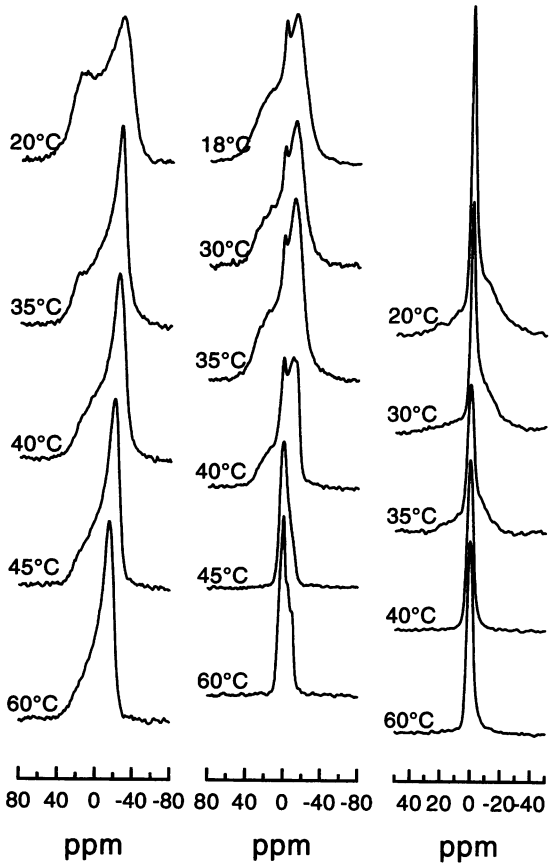

FIGURE 1 Temperature dependence of the proton-dipolar decoupled 121.5-MHz ^{31}P-NMR spectra of hydrated dimyristoyl phosphatidylcholine (DMPC)-dimyristoyl glycerol (DMG) dispersions with DMPC:DMG compositions of 70:30 mol/mol (*left*), 40:60 mol/mol (*center*), and 20:80 mol/mol (*right*). Chemical shifts (ppm) are referenced to external 85% phosphoric acid.

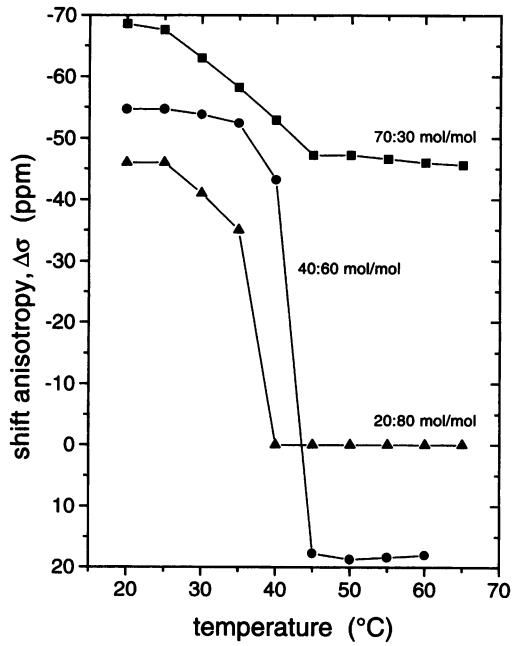

FIGURE 2 Temperature dependence of the effective chemical shift anisotropy, $\Delta\sigma$, in the ^{31}P-NMR spectra of hydrated DMPC–DMG dispersions with DMPC:DMG compositions of 70:30 mol/mol (■), 40:60 mol/mol (●), and 20:80 mol/mol (▲).

present case this corresponds to the inverted hexagonal (H_{II}) phase. For the sample with high DMG content (DMPC: DMG, 20:80 mol/mol) the spectra at lower temperature consist of a lamellar powder pattern with an isotropic component superimposed. With increasing temperature, the spectra convert to a solely isotropic component that is characteristic of an isotropic fluid or melt.

The temperature dependence of the effective chemical shift anisotropy, defined as the distance between the points of maximum slope in the flanks of the powder patterns, is given in Fig. 2 for hydrated DMPC–DMG mixtures of all three compositions. For the 70:30 mol/mol DMPC–DMG mixture the effective chemical shift anisotropy has a value of −68 ppm at 20°C that is representative of the lamellar gel phase. The shift anisotropy decreases over a broad temperature range of 25–45°C. This represents the region of gel–fluid phase separation for this particular mixture. At temperatures of 45°C and above, the chemical shift anisotropy of ~−(46–47) ppm is characteristic of a fluid lamellar phase. For the 40:60 mol/mol DMPC–DMG mixture the effective chemical shift anisotropy has a lower value of ~−55 ppm in the gel phase, which decreases to −43 ppm at 40°C. An abrupt change takes place at 45°C to the inverted hexagonal phase with chemical shift anisotropy of +18 ppm, which remains constant with increasing temperature in this single-phase region. For the 20:80 mol/mol DMPC–DMG mixture the effective chemical shift anisotropy of the lamellar powder pattern at low temperature in the gel phase is yet smaller (~−46 ppm) than for the 40:60 mol/mol mixture. This reduces to −35 ppm at 35°C, and from 40°C onward the system is solely isotropic, with no chemical shift anisotropy.

It will be noted that the effective chemical shift anisotropies, $|\Delta\sigma|$, in the gel phases of the samples that contain DMG are considerably lower than those typical of pure phospholipid bilayer gel phases. This increased motional freedom of the phospholipid headgroups arises because they are spaced apart by the intervening diacylglycerol molecules, which do not contribute to the bulk of the polar group regions of the lipid assemblies. Nevertheless, the ^{31}P linewidths from the DMPC–DMG mixtures remain characteristic of a phospholipid gel phase rather than of a fluid phase.

These ^{31}P-NMR results define the different natures of the fluid phase in the three major regions of the binary phase diagram and indicate the regions of lateral phase separation (cf. Heimburg et al., 1992) for the samples used in this study. The gel phase is principally lamellar in all three regions, and the progressive decrease in the effective chemical shift anisotropy with increasing DMG content indicates that the DMPC component is not totally phase separated in the gel phase; instead it is interacting with at least part of the DMG component.

^{2}H-NMR spectra of components with perdeuterated *sn*-2 chain

The broad-line ^{2}H-NMR spectra of hydrated 70:30 mol/mol DMPC–DMG mixtures in which either the DMPC component (d_{27}-DMPC) or the DMG component (d_{27}-DMG) is perdeuterated in the *sn*-2 chain are given in Fig. 3(*a*) and (*b*), respectively. For this composition the lipid is in a lamellar phase over the entire temperature range. The quadrupolar powder patterns for samples at the lower temperatures are broad and relatively structureless, as is characteristic of phospholipids in the gel phase (cf. Davis, 1979). The parallel shoulders in the spectra from the CD_2 segments extend over a range of $\Delta\nu_{//} \approx 118$ kHz, which is close to the value of 122 kHz expected for axial rotation of an all-*trans* chain. The perpendicular peaks are spread over a relatively broad range centered approximately on a splitting of $\Delta\nu_{\perp} \approx 55$ kHz, as seen from the sharpened peaks of the gel-phase component in the phase coexistence region at 35°C. The perpendicular peaks of the terminal CD_3 group have splittings of 12.6–13.2 kHz for the DMPC component and are substantially smaller for the DMG component. The spectra in the gel phase are therefore characteristic of axial chain rotation with slow limited *trans–gauche* isomerism. In the temperature region 30–35°C two separate spectral components are clearly distinguished that indicate the presence of coexisting gel and fluid lipid phases. The spectra from 45°C onward have smaller quadrupole splittings and are characteristic of the fluid lamellar phase. These spectra were collected at a slower digitization rate to yield better spectral resolution. The perpendicular peaks of the powder patterns from several of the chain CD_2 segments are resolved in the inner part of the spectra, within the major perpendicular peaks that are characteristic of the chain-order parameter plateau region. The overall splittings in the fluid phase are smaller for the DMG component than for the DMPC component. For example, the perpendicular quadrupole splittings for the plateau region are 19 and 24 kHz, and of the terminal methyl group are 1.5 and 2.5 kHz, for the DMG and the DMPC components, respectively, at 60°C. (It should be noted that the perpendicular peaks of the terminal methyl groups are off scale in Fig. 3 (*b*).)

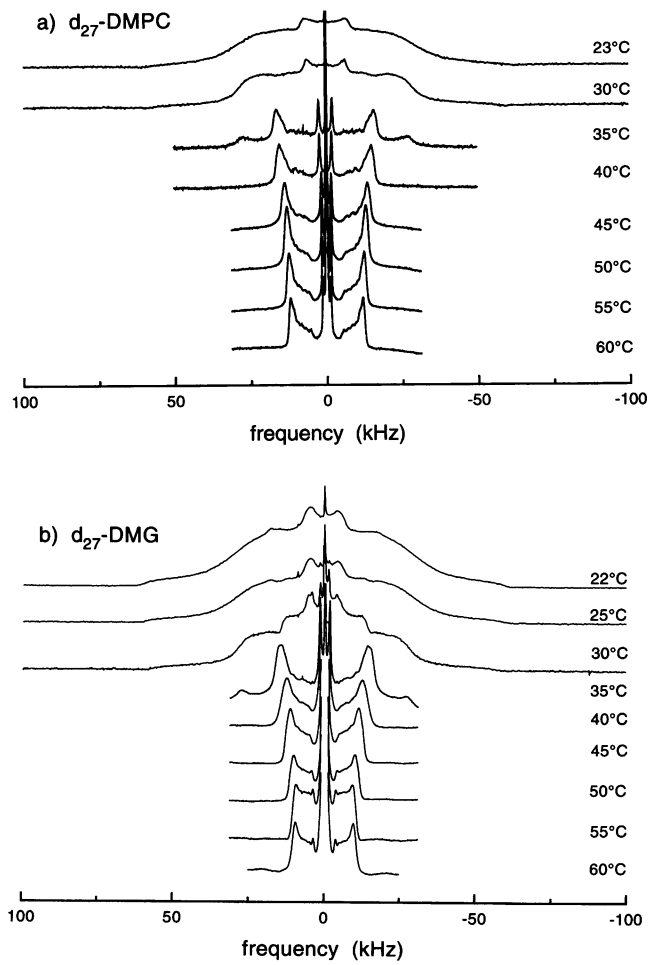

FIGURE 3 Temperature dependence of the 46.1-MHz ^{2}H-NMR spectra of (*a*) *sn*-2-chain perdeuterated dimyristoyl phosphatidylcholine and (*b*) *sn*-2-chain perdeuterated dimyristoyl glycerol in hydrated DMPC–DMG 70:30 mol/mol dispersions. The complementary lipid component in each mixture is not deuterated. For temperatures above 40°C the perpendicular peaks from the terminal CD_3 groups of the deuterated DMG are off scale in the central region of (*b*).

The temperature dependences of the resolved quadrupolar splittings, $\Delta\nu_{\perp}$, in the powder patterns of the d_{27}-DMPC and the d_{27}-DMG components (as determined from an expanded display) are given in Fig. 4 (*b*) and (*c*), respectively, for the 70:30 DMPC–DMG mixture. Tentative assignment of the quadrupole splittings to particular CD_2 segments is made from comparison with results from bilayers of d_{27}-DMPC alone (Fig. 4 (*a*)), which are included for comparison. The assignments for the latter were made on the basis of results with specifically deuterated samples (Oldfield et al., 1978). The temperature dependence and the relative ordering of the splittings are also aids to assignment, particularly with regard to the anomalous C-2 segment. In general, the quadrupole splittings for the DMG component are smaller than those for the DMPC component, at least for the plateau region and also for lower temperatures. The quadrupole splittings for the DMPC component in the mixture are, however, larger than those in bilayers of DMPC alone.

The ^{2}H-NMR spectra of d_{27}-DMPC and d_{27}-DMG in 45:55 mol/mol hydrated mixtures of DMPC with DMG are given in Fig. 5 (*a*) and (*b*), respectively. Mixtures of this composition are lamellar in the gel phase, but the fluid phase has an inverted hexagonal structure. The spectra of both components at low temperature in the gel phase are, on the whole, very similar to those of the 70:30 mol/mol DMPC–DMG mixture. At somewhat higher temperatures, in the phase coexistence region, the spectra for d_{27}-DMPC consist of two components. The fluid component in this region has a splitting of $\Delta\nu_{\perp} \approx 29$ kHz for the CD_2 segments, which is comparable with, although slightly less than, the corresponding values for the lamellar fluid component in the 70:30 mol/mol DMPC–DMG mixture at the

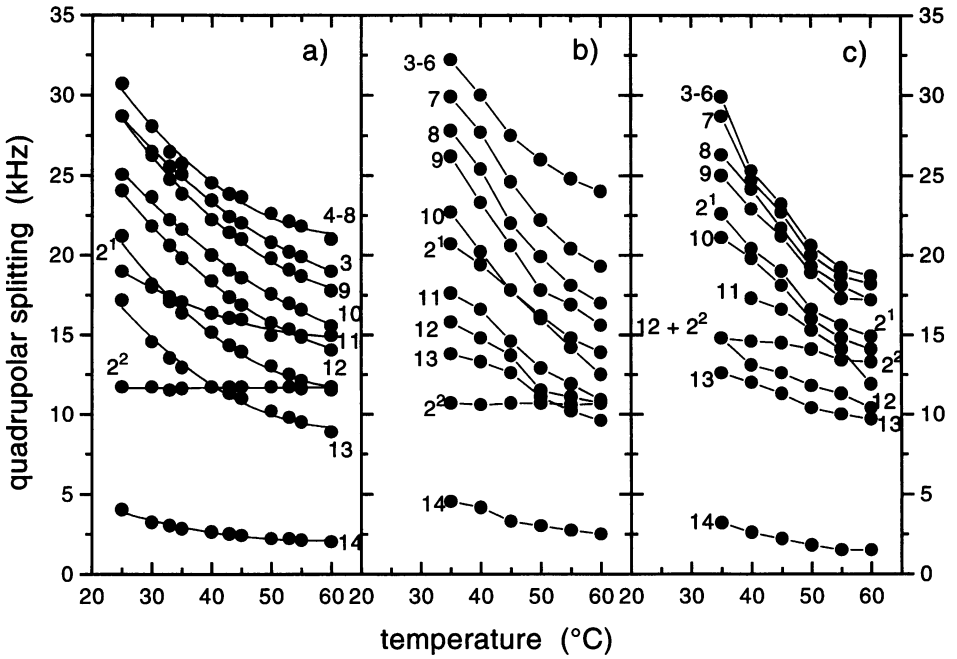

FIGURE 4 Temperature dependence of the quadrupole splittings, $\Delta\nu_\perp$, in the ^{2}H-NMR spectra of (*a*) hydrated d_{27}-DMPC dispersion [from Marsh et al., 1983], (*b*) d_{27}-DMPC in a hydrated DMPC–DMG 70:30 mol/mol dispersion, and (*c*) d_{27}-DMG in a hydrated DMPC–DMG 70:30 mol/mol dispersion. Assignments to individual CD_2 segments are given by the C-atom numbers next to the curves and are discussed in the text. The deuterons 2_1 and 2_2 at the C-2 position are inequivalent.

same temperatures (e.g., ≈32 kHz at 35°C). It therefore seems likely that the fluid phase has a lamellar structure in the phase coexistence region for the 45:55 mol/mol DMPC–DMG mixture. Indeed, the ^{31}P-NMR spectra in this temperature range (35–40°C) are characteristic of an almost wholly lamellar structure with very little H_{II}-phase component (Fig. 1). The ^{31}P-NMR spectra of this mixture at 40°C in Fig. 1 also show some evidence of a two-component nature that suggests the coexistence of lamellar gel and fluid phases. The resolution is far better, however, in the ^{2}H-NMR spectra of d_{27}-DMPC. In the fluid phase the quadrupole splittings in the ^{2}H-NMR spectra are greatly reduced, with maximum values of $\Delta\nu_\perp \approx 8$–11 kHz, compared with 20–30 kHz in the fluid lamellar phase of the 70:30 mol/mol DMPC–DMG mixture. The shape of the powder pattern is also very different from that for the fluid lamellar phase. The stepped triangular shape for the 45:55 mol/mol DMPC–DMG mixture arises from the closer spacing of the component quadrupole peaks and possibly also from a reduced length of the order parameter plateau region (cf. Sankaram and Marsh, 1989). Both this and the reduction in quadrupole splitting by more than a factor of 2 are characteristic features of the inverted hexagonal (H_{II}) phase (cf. Lafleur et al., 1990).

The temperature dependences of the resolved $\Delta\nu_\perp$ quadrupole splittings of both lipid components in the fluid phase of the 45:55 mol/mol DMPC–DMG mixtures are given in Fig. 6. Fewer resonances are resolved than in the case of the lamellar fluid phase of the 70:30 mol/mol DMPC–DMG mixtures because of the greater degree of overlap caused by the smaller splittings. Also, the splittings of the terminal CD_3 group are too small to be resolved from the overlapping isotropic peak. (The latter result presumably arises from small particles, but its contribution to the total integrated intensity is small, as is indicated by the ^{31}P-NMR spectra in Fig. 1 (*center*).) In general, the differences in quadrupole splittings between the DMPC and the DMG components in the mixture are considerably smaller than those for the fluid lamellar phase of the 70:30 mol/mol DMPC–DMG mixture, even when allowance is made for their intrinsically smaller absolute values. Moreover, the temperature dependence of the quadrupole splittings for both components is considerably smaller than in the fluid lamellar phase.

The ^{2}H-NMR spectra of d_{27}-DMPC and of d_{27}-DMG in a 40:60 mol/mol hydrated mixture of DMPC with DMG are rather similar to those of the corresponding deuterated components in the 45:55 mol/mol mixture (data not shown). Both of these mixtures should lie in the same central region of the binary phase diagram, for which the structure of the gel phase is lamellar and that of the fluid phase is of the inverted hexagonal type (cf. Heimburg et al., 1992). The one difference in the temperature dependence of the spectra is that, whereas the spectrum of the d_{27}-DMPC component at 50°C is of the inverse hexagonal type (as for the 45:55 mol/mol mixture), those at higher temperature are isotropic. This suggests that the composition of the 40:60 mol/mol mixture lies very close to the phase boundary with the third region of the phase diagram. In the fluid phase this phase boundary is presumably not vertical but skewed such that, at higher temperatures, the 40:60 mol/mol mixture crosses the boundary to the fluid isotropic phase that is characteristic of higher DMG contents.

The ^{2}H-NMR spectra of d_{27}-DMPC and of d_{27}-DMG in a 20:80 mol/mol hydrated DMPC–DMG mixture are given in Fig. 7 (*a*) and (*b*), respectively. Mixtures of this composition are lamellar in the gel phase but are isotropic in the fluid phase. In addition, the phase diagram indicates that excess

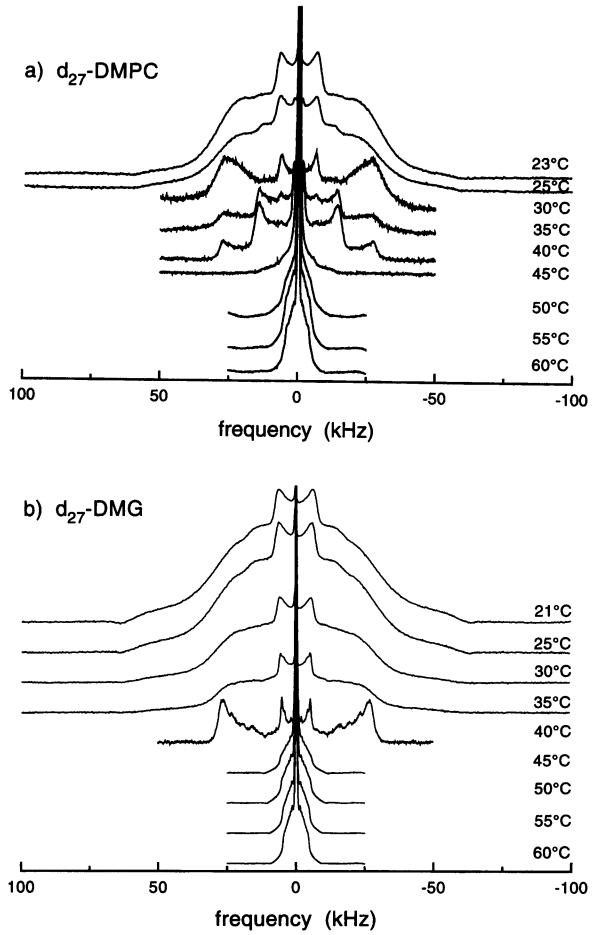

FIGURE 5 Temperature dependence of the 46.1-MHz ^{2}H-NMR spectra of (*a*) d_{27}-DMPC and (*b*) d_{27}-DMG in hydrated DMPC–DMG 45:55 mol/mol dispersions. The complementary lipid component in each mixture is not deuterated. For temperatures above 40°C the perpendicular peaks from the terminal CD_3 groups of the deuterated lipid are off scale in the central regions of both parts of the figure.

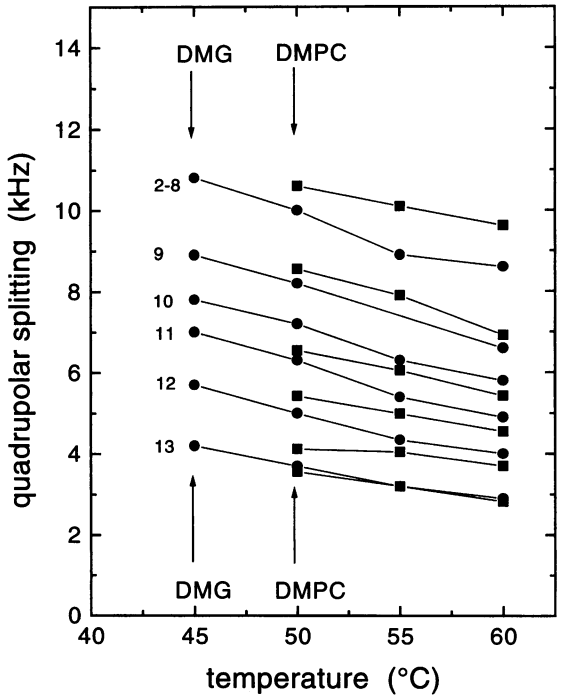

FIGURE 6 Temperature dependence of the quadrupole splittings, $\Delta\nu_\perp$, in the ^{2}H-NMR spectra of d_{27}-DMPC (■) and d_{27}-DMG (●) in hydrated DMPC–DMG 45:55 mol/mol dispersions. Tentative assignments to individual CD_2 segments are given by the C-atom numbers adjacent to the curves.

crystalline DMG (over that which forms the DMPC–DMG 1:2 mol/mol compound) should be present in the low-temperature phase (cf. Heimburg et al., 1992). The spectra of both components at low temperature in the gel phase are very similar to those discussed previously for the lamellar gel phases in the two other regions of the phase diagram. The one exception is that an additional component is seen in the low-temperature spectra of the d_{27}-DMG component that has a quadrupole splitting of $\Delta\nu_\perp$ = 34–35 kHz. This value is characteristic of a terminal CD_3 group in a solid phase for which the only motion is rapid rotation of the CD_3 group itself, i.e., in the absence of rotation about the long axis of the chain. This additional component therefore represents excess crystalline DMG in the low-temperature phase. The remainder of this powder pattern is not seen because it is too broad to be observed with the concentration and data collection conditions used. In the fluid phase only an isotropic spectrum is seen for both lipid components of the mixture, as is expected.

^{2}H-NMR of DMG deuterated at the C-2 position

The quadrupole splittings for the C-2 segment in the *sn*-2 chain of DMG have tentatively been identified in the spectra from the perdeuterated chains. To define better this conformationally crucial part of the molecule, experiments have also been performed with the specifically deuterated derivative. The ^{2}H-NMR spectra of d_2-DMG in a 70:30 mol/mol hydrated DMPC–DMG mixture are given in Fig. 8. As mentioned above, these samples are in the lamellar phase at all temperatures studied. The spectra at lower temperature in the gel phase are broad and structureless, and it is difficult to deduce any specific orientational information from these spectra. The spectra in the fluid phase consist principally of three Pake doublets. The two major inner doublets are attributed to the two (inequivalent) deuterons at the C-2 position. The third doublet of larger splitting is attributed to partial deuteration of the neighboring C-3 segment because its quadrupole splittings correspond closely to those of the outer plateau region in the spectra of perdeuterated d_{27}-DMG in an equivalent sample that were given in Fig. 3 (*b*). (High-resolution proton NMR reveals that the d_2-DMG sample is devoid of the 1, 3-isomer.) From the size of the splitting of the innermost doublet and its rather small temperature dependence ($\Delta\nu_\perp$ = 15.8 kHz at 40°C and 13.6 kHz at 60°C), this doublet is assigned to the C-2_2 deuteron (cf. Fig. 4). The splittings of the intermediate doublet, then assigned to the C-2_1 deuteron, are consistent with the as

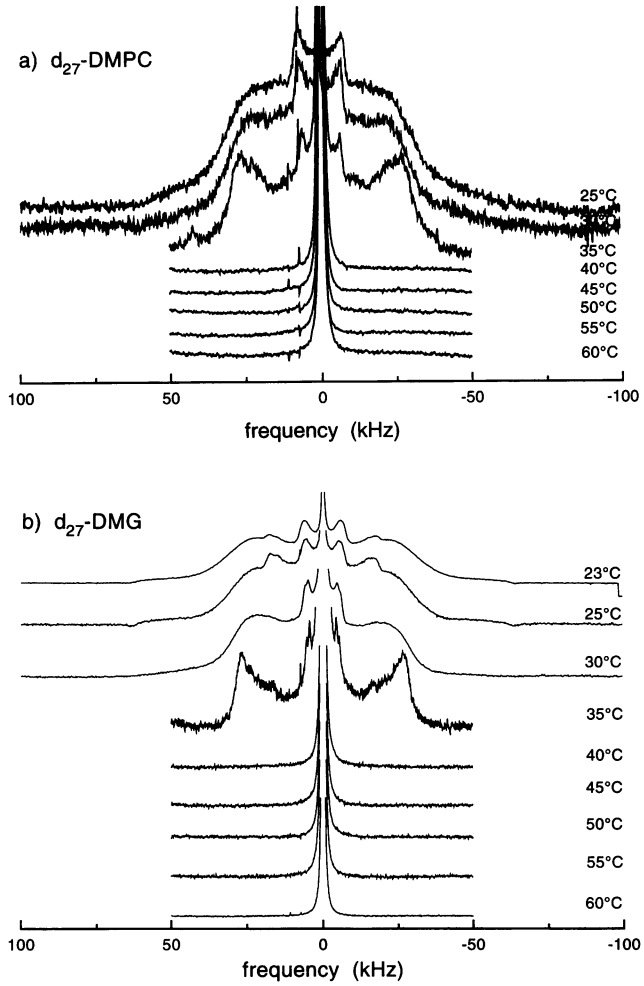

FIGURE 7 Temperature dependence of the 46.1-MHz ^{2}H-NMR spectra of (*a*) d_{27}-DMPC and (*b*) d_{27}-DMG in hydrated DMPC–DMG 20:80 mol/mol dispersions. The complementary lipid component in each mixture is not deuterated.

signment given for this position in the spectra of the corresponding sample with perdeuterated chains in Fig. 4 (*c*) ($\Delta\nu_\perp$ = 21.5 kHz at 40°C and 15.9 kHz at 60°C).

The ^{2}H-NMR spectra of d_2-DMG in a 45:55 mol/mol hydrated DMPC–DMG mixture are given in Fig. 9. For this composition the samples are lamellar in the gel phase and have an inverted hexagonal structure in the fluid phase. At low temperature in the gel phase the spectra are broad and structureless. In the fluid phase at higher temperature the spectra apparently consist of essentially a single Pake doublet. However, there is a broadening in the outer flanks of the perpendicular peaks that is seen most clearly as small shoulders in the 45°C spectrum. This suggests that the spectra may consist of two unresolved doublets. The quadrupole splitting of the perpendicular peaks is $\Delta\nu_\perp$ = 7.5 kHz at 45°C and 6.4 kHz at 60°C. The limits of the unresolved shoulders associated with these peaks correspond to quadrupole splittings of ~9 and ~7 kHz at 45°C and 60°C, respectively. Scaling the observed splittings for the C-2

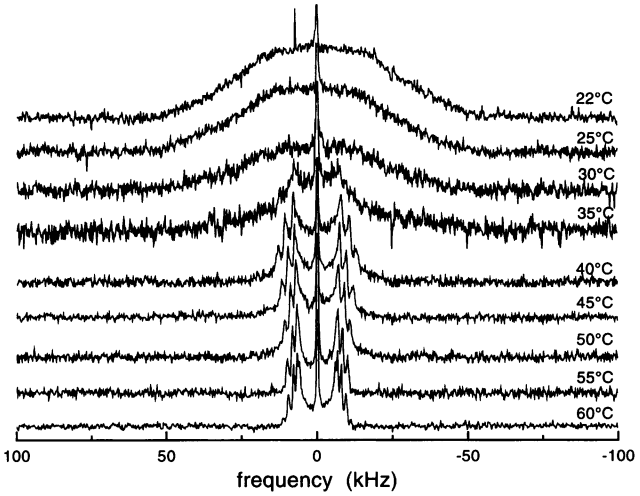

FIGURE 8 Temperature dependence of the 46.1-MHz ^{2}H-NMR spectra of d_2-DMG (deuterated on C-2 of the *sn*-2 chain) in a hydrated DMPC–DMG 70:30 mol/mol dispersion. The DMPC component is not deuterated.

position of d_2-DMG in the lamellar phase (Fig. 8) by the ratio of the quadrupole splittings of the plateau region in the H_{II} and lamellar phases (Fig. 4) yields values that are very close to those quoted above for the perpendicular peaks and their shoulders in Fig. 9. The plateau region is that which is appropriate when one is considering the C-2 position and yields a scaling factor of 0.46–0.47 for the H_{II} phase relative to the L_α phase. This therefore suggests that the two deuterons at the C-2 position of d_2-DMG are also inequivalent in the H_{II} phase of the 45:55 mol/mol DMPC–DMG mixture and predicts that the difference in their quadrupole splittings would be too small to be resolved completely in the spectra of Fig. 9. (A similar scaling also predicts that the splitting of the third doublet in Fig. 8 would also remain unresolved further in the outer flanks of the perpendicular peaks in Fig. 9.)

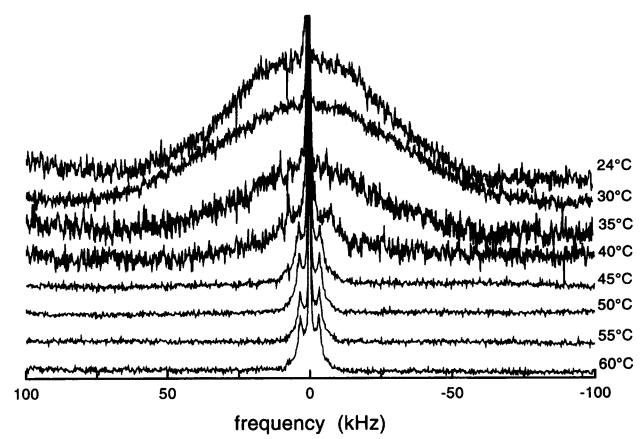

FIGURE 9 Temperature dependence of the 46.1-MHz ^{2}H-NMR spectra of d_2-DMG in a hydrated DMPC–DMG 45:55 mol/mol dispersion. The DMPC component is not deuterated.

The ^{2}H-NMR spectra of d_2-DMG in a 20:80 mol/mol hydrated DMPC–DMG mixture were also recorded. In the gel phase at low temperature the spectra consist of a very broad essentially structureless line. In the fluid phase at higher temperature the spectra are composed of a single isotropic line (data not shown). For this composition it is unfortunately not possible to obtain information on the orientation of the C-2 segment of DMG from the ^{2}H-NMR spectra.

DISCUSSION

At the molecular level the specific functional membrane properties of diacylglycerols undoubtedly are related both to their chain configuration and to their effects on the segmental order of the phospholipid chains. In the present study we have investigated the phase behavior of DMPC–DMG mixtures and the dynamic chain conformations of both lipid components in the fluid phases of mixtures that correspond to the three different regions of the binary phase diagram.

Broadly speaking, the ^{2}H-NMR spectra of the perdeuterated components support the assignments of the phase behavior given in the previously established phase diagram for DMPC–DMG mixtures (Heimburg et al., 1992; Schorn and Marsh, 1996a). Apart from the appearance of two-component ^{2}H-NMR spectra in the regions of gel–fluid phase coexistence that was observed previously in the spectra from mixtures of long-chain diacylglycerols with dipalmitoylphosphatidylcholine (De Boeck and Zidovetzki, 1992), the following features that emerge from the present study are of particular note. First is the demonstration of crystalline DMG coexisting with the lamellar gel phase at high DMG contents in the third region of the phase diagram. The second is the conclusion that the fluid phase in the coexistence region for intermediate compositions has a lamellar structure, whereas at higher temperatures in the single-phase region it is of the inverted hexagonal type. The third is the finding that the fluid–fluid phase boundary with the region of high DMG content is crossed at higher temperature by the DMPC–DMG mixture of 40:60 mol/mol composition. The last-named feature indicates that the boundary between the fluid phases of the second and third regions of the phase diagram is not perpendicular but is inclined to the composition axis.

The ^{2}H- and ^{31}P-NMR spectra indicate that the lipid molecules are rotating rapidly, relative to a frequency scale of 250 kHz, in the lamellar gel phases for all samples studied (Figs. 1, 3, 5, and 7). The single exception is the crystalline component of DMG that is present in the third region of the phase diagram (Fig. 7). The quadrupole splittings, $\Delta\nu_\perp \approx 55$ and 11 kHz at 35°C for the CD_2 and CD_3 segments, respectively, are smaller than those expected simply for an all-*trans* chain rotating rapidly about its long axis (61 and 17.2 kHz, respectively; cf. Eq. 1). This indicates that additional rapid chain motions of limited amplitude are present in the gel phase. The considerable narrowing of the spectra indicates that the rate of long-axis rotation and these additional motions increases with increasing temperature in the gel phase (cf. Schorn and Marsh, 1996b). The additional motions most probably correspond to limited *trans–gauche* isomerism and limited angular excursions of the chain axis (cf. Moser et al., 1989).

The ^{2}H NMR spectra of the 70:30 mol/mol DMPC–DMG mixture in the fluid phase (Figs. 3 and 4) indicate that the typical flexibility profile, with a plateau in chain segmental order in the region closer to the polar headgroups, is retained for the phospholipid bilayer that contains 30 mol% DMG. This holds for the DMG component as well as for the DMPC component and indicates further that the chains of the diacylglycerol molecules are integrated into the hydrophobic region of the bilayer with an orientation similar to that of the host phospholipid chains. Predictably, the latter condition is found to hold also at much lower DMG contents (Sanders, 1994). The degree of chain order of the DMPC component is, however, increased substantially relative to that in DMPC bilayers that do not contain DMG. The latter result is in agreement with electron spin resonance data from spin-labeled lipid components in the same system (Schorn and Marsh, 1996a). It has also been found to be the case from ^{2}H-NMR studies of phospholipid–diacylglycerol mixtures of other chain compositions (De Boeck and Zidovetzki, 1989, 1992). This effect corresponds to an increase in the chain-packing density of the DMPC component on addition of DMG. From the chain-order parameter plateau values (cf. Eq. 2) it can be calculated that the areas per DMPC molecule, at 35°C and 60°C, are 65 and 71 Å^2, respectively, in the absence of DMG and are decreased to 59 and 67 Å^2, respectively, in the presence of 30 mol% DMG (cf. also De Boeck and Zidovetzki, 1989).

The quadrupole splittings in the ^{2}H-NMR spectra of the d_{27}-DMG component of the 70:30 mol/mol DMPC–DMG mixture are considerably smaller than those of the d_{27}-DMPC component (Fig. 4). From electron spin resonance experiments on the spin-labeled lipid components it was concluded recently that the diacylglycerol molecules are situated approximately two CH_2 segments deeper into the hydrophobic region of the fluid bilayer, in 70:30 mol/mol DMPC–DMG mixtures, than is the phosphatidylcholine component (Schorn and Marsh, 1996a). The reduction in chain order of the DMG component relative to the DMPC component found in the present ^{2}H-NMR studies is at least qualitatively consistent with this interpretation. Effective areas per DMG molecule, deduced from Eq. 2, are 60 and 75 Å^2 at 35°C and 60°C, respectively. Comparison with the corresponding values for DMPC must take account of the difference in vertical location of the two components. However, the considerably steeper temperature dependence in the plateau region for the DMG component indicates an enhanced chain rotational isomerism and the corresponding propensity of the deeper-situated DMG component to induce H_{II} phases at higher mole fractions.

The ^{2}H-NMR spectra of DMG deuterated at the conformationally unique C-2 position (Fig. 8) are complicated by

partial labeling at the β position to the carbonyl, which is known also to be partially activated. (Additionally, limited chain migration cannot be excluded entirely.) Nevertheless, the spectra clearly demonstrate that the orientational inequivalence of the two deuterons at the C-2 position of the *sn*-2 chain, which is a characteristic feature of diacyl phospholipids (Seelig and Seelig, 1975; Seelig and Browning, 1978), is preserved in the DMG component. Hence, the conformation and the orientation of the glycerol backbone that are found in phospholipids and result in a bent configuration of the *sn*-2 chain at the C-2 position are also features of the DMG molecules in the fluid lamellar phase of the 70:30 mol/mol DMPC–DMG mixture. The integration of the DMG molecules in the fluid bilayer therefore is achieved with a configuration about the glycerol backbone similar to that of the host phospholipid molecules. A similar conclusion has been reached on the basis of ^{13}C-carbonyl NMR for dipalmitoyl glycerol at 10 mol % in egg phosphatidylcholine bilayers (Smith et al., 1992). This molecular conformation of the diacylglycerol in fluid phospholipid bilayers differs, however, from that found in the crystal structure (Pascher et al., 1981; Dorset and Pangborn, 1988).

The ^{2}H-NMR spectra of the 45:55 mol/mol DMPC–DMG mixture in the fluid phase consist of powder patterns that are characteristic of the spread of chain segmental order parameters in the H_{II} phase for both the d_{27}-DMPC and the d_{27}-DMG components (Fig. 5). Comparison of Figs. 4 and 6 reveals that the segmental order parameters are more evenly spaced for the lipid chains in the H_{II} phase than they are for the chains in the lamellar L_α phase, at least for positions closer to the terminal methyl group. This reflects the difference in geometry between the two phases, which allows more freedom at the chain ends in the hydrophobic region of the inverted hexagonal structure. The quadrupole splittings in the H_{II} phase are also found to be less than half of those in the lamellar L_α phase, which again reflects a difference in the intrinsic chain order between the two phases. The effective areas per lipid molecule, deduced from the chain-order parameters in the plateau region (Eq. 2), are in the region of 71–77 Å^2, depending on lipid and temperature but with considerably smaller variation than in the L_α phase. These values represent the chain areas in the hydrophobic interior of the H_{II} phase, i.e., in the region of the order parameter plateau. The geometry of the H_{II} phase dictates that they should be larger than in the L_α phase if the areas per headgroup are comparable in the two phases. Additionally, the difference in chain order between the DMG and DMPC components is smaller in the H_{II} phase than it is in the lamellar phase. The DMG component, however, does still have somewhat smaller segmental chain-order parameters than the DMPC component. This is in agreement with electron spin resonance studies on the spin-labeled lipid components, from which it was suggested that the diacylglycerol component is situated less deeply (by approximately one CH_2 group) in the hydrophobic region of the H_{II} phase than it is in the fluid lamellar phase (Schorn and Marsh, 1996a).

The ^{2}H-NMR spectra of the DMG component deuterated at the C-2 position in the H_{II} phase of the 45:55 mol/mol DMPC–DMG mixture consist of two unresolved doublets (Fig. 9). Extrapolation from the spectra in the fluid lamellar phase of the 70:30 mol/mol DMPC–DMG mixture suggests that the configuration of the glycerol backbone with the *sn*-2 chain bent at the C-2 position is retained by the DMG molecules in the H_{II} phase. The lack of resolution prevents a completely definite statement, but the data are consistent with this interpretation.

The ^{2}H-NMR spectra for both lipid components in the 20:80 mol/mol DMPC–DMG mixtures are isotropic in the fluid phase. The lipid molecules, or their aggregates, are therefore rotating isotropically at a frequency that is much greater than 50 kHz. The DMG component consists of an isotropic melt. Results from conventional spin-label electron spin resonance spectroscopy, which is sensitive only to much faster motions, indicate, however, that the phosphatidylcholine component forms inverted micelles (Schorn and Marsh, 1996a). The NMR results show that these micelles must rotate isotropically on the ^{2}H-NMR time scale, within the liquid-DMG component.

The present studies have delineated several significant features of the dynamic molecular configurations that are involved in diacylglycerol–phospholipid interactions under different conditions. These studies have been confined to lipids with homogeneous saturated chain compositions. However, by exploring all the different regions of the binary phase diagram one can extrapolate the results to many of the relevant situations that involve lipids of different chain compositions and phospholipids of different headgroup types. It is well known, for instance, that phosphatidylethanolamines and certain membrane phospholipids with unsaturated chains have a stronger propensity to form inverted hexagonal phases than do phosphatidylcholines with saturated chains. Also, diacylglycerols may vary in their efficiency both to activate protein kinase C and also to induce H_{II}-lipid phases (see, e.g., Zidovetzki and Lester, 1992). Therefore, some of the effects reported here at high concentrations of diacylglycerol may be achieved more readily at lower concentrations with the specific lipids of the biological milieu.

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