A MODEL OF ORIENTATIONAL ORDERING IN PHOSPHATIDYLCHOLINE BILAYERS BASED ON CONFORMATIONAL ANALYSIS OF THE GLYCEROL BACKBONE REGION

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ABSTRACT: Molecular and conformational ordering in aqueous multilamellar suspensions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) have been examined by deuterium nuclear magnetic resonance (^{2}H NMR) in the liquid crystalline (L_α) phase. Motionally averaged quadrupolar splittings $\bar{\nu}_Q$ from six sites in the vicinity of the glycerol backbone have been analyzed by a molecular frame and order matrix approach in which the usual assumption of a freely-rotating molecule is not invoked. By assuming a relatively rigid glycerol backbone region, the six $\bar{\nu}_Q$ values are found to be consistent with a conformation of the glycerol backbone that is almost identical to that of one of the two structures in crystalline DMPC dihydrate (Pearson, R. H., and I. Pascher, 1979, *Nature (Lond.) 281*: 499–501). The orientation of the most-ordered axis of the DMPC molecule is found to be tilted at an angle of 27 ± 2° with respect to the long axis of the *sn*-1 chain in its extended all *trans* conformation. The ordering of the most ordered molecular axis with respect to the bilayer normal is expressed by an order parameter of $S_{zz} \simeq 0.6 \pm 0.1$, consistent with values in analogous thermotropic liquid crystals.

INTRODUCTION

The conformation and dynamic properties of phospholipids have been intensely studied by a wide variety of physical techniques. High-resolution nuclear magnetic resonance (NMR) techniques have been the most widely used to examine rapid isotropic motion of phospholipids dissolved in organic solvents (1–6), while x-ray diffraction techniques have proved the most informative in the solid state (7–12). It is the structural behavior of phospholipids aggregated in the presence of water into ordered (usually lamellar) bilayer structures, however, that is of greatest interest to biologists. The bilayer structure has been shown to be an integral part of biological membranes and a knowledge of the structural behavior of component lipids is essential for an understanding of membrane assembly, architecture, and properties.

In the past decade, ^{2}H NMR has been extensively used to describe the conformation, order and dynamics of phospholipids in ordered bilayer structures (13–16). These studies have focused principally on the liquid crystalline phase where the correlation times for orientational motions of the molecule and its segments are short on the time scale of the NMR measurement ($\tau < 10^{-5}$s). Of the several parameters measured by the ^{2}H NMR technique, perhaps the most informative in the fast-motion regime is the time-averaged quadrupolar coupling constant ($\bar{\nu}_Q^i$) given by

$$\bar{\nu}_Q^i = \nu_Q \langle 3/2 \cos^2\sigma_i - 1/2 \rangle, \quad (1)$$

where ν_Q is the solid-state quadrupolar coupling constant associated with the C—^{2}H$_i$ bond. The term $\langle 3/2 \cos^2\sigma_i - 1/2 \rangle$, or the order parameter S, is a time average of the quantity in brackets, taken over all the angles (σ_i) that the C—^{2}H$_i$ bond direction assumes with respect to the time averaged principal axis of the electric-field-gradient tensor, during the time course of the NMR measurement.

A principal goal in the study of membrane structure by ^{2}H NMR, is the interpretation of S in terms of a molecular model of the bilayer that accounts for the relative importance of the various motions that time average the quadrupole interaction for a particular C—^{2}H$_i$ segment. It is widely believed that translational diffusion and vesicle tumbling in multilamellar preparations of phospholipids, are not significant in this time-averaging process. It has been difficult, however, to quantify the relative contributions to the time averaging of ν_Q, of segmental motions produced by rotational isomerization about C—C single bonds, and of rigid-body reorientations produced by motion of the molecule as a whole. The close similarity in $\bar{\nu}_Q$ values for equivalent positions at identical reduced

temperatures in the various phospholipid classes (14) infers that rigid-body reorientations may be significant, and that orientational ordering is not just a molecular property but a property of the phase itself.

The first approach used in the interpretation of $\bar{\nu}_Q^i$ values from phospholipid bilayer membranes assumed that rigid-body reorientations were insignificant and that most time averaging of ν_Q arose from rotational isomerization about C—C bonds in the hydrocarbon chains (17). It was also assumed by these authors that orientational ordering about the director axis in the bilayer is axially symmetric. The first of these assumptions was questioned by Petersen and Chan (18) who assigned an instantaneous chain axis in the molecule and expressed the order parameter, S, as the product of S_α, a chain order parameter and S_γ, an intramolecular order parameter. Oldfield et al. (19) using this treatment and specifically ^{2}H-labeled cholesterol as a probe, estimated a value of S_α of 0.78 for pure (infinite cholesterol dilution) 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers at 23°C. Their results supported the hypothesis that rigid-body reorientations are significant in time averaging the quadrupolar interaction in the ^{2}H NMR spectra of phospholipid bilayers.

Here we have applied an even more general treatment, which does not assume that ordering is necessarily symmetric about the director axis, for the analysis of the order parameters measured in specifically ^{2}H-labeled phosphatidylcholines. This approach has the potential of capturing all the information possible from the motionally averaged quadrupole interaction in the fast motion regime (20). In this treatment, the instantaneous chain axis of Petersen and Chan (18) is replaced by a molecular frame fixed to some region of the phosphatidylcholine molecule. The method, which has already been successfully used in the measurement of molecular ordering in thermotropic liquid crystals (20–22) utilizes ^{2}H NMR date from several nonequivalent sites in the lipid molecule. As noted by Burnell and De Lange (23), this molecular frame and order matrix approach is only valid for a group of sites located in a rigid segment of the molecule. Although there is no independent evidence that such a region exists in phosphatidylcholine we have assumed, for reasons outlined in the Discussion, that the glycerol backbone is essentially rigid. This moiety in phosphatidylcholine is widely regarded as being much less flexible than either the polar headgroup or hydrocarbon chains (24). The validity of our assumption will be tested by how successfully calculated molecular order parameters are in agreement with corresponding values in analogous thermotropic liquid crystals, and by the consistency of calculated ^{13}C NMR chemical shift anisotropies with experimental measurements (25). Thus, the assumptions made in earlier treatments have been replaced in our analysis by the assumption of glycerol backbone rigidity. ^{2}H NMR data from six positions in the vicinity of the glycerol backbone of DMPC have been obtained as a function of temperature in the L_α phase. Our results are consistent with data reported for these same sites in several related phospholipids (26). Analysis of the results gives a model of ordering in the bilayer that is fundamentally different from the model described by Gally et al. (26), on the basis of their analysis of ^{2}H NMR data from the same region in several phospholipids. In our model we do not invoke rapid interconversion of two conformers by rotation about the C_1—C_2 bond of the glycerol backbone, in order to explain the nonequivalence of $\bar{\nu}_Q$ for the 1R and 1S glycerol segments. Unlike their model, our analysis satisfactorily explains the observed nonequivalence of $\bar{\nu}_Q$ for the 3R and 3S glycerol segments.

THEORY

The theoretical approach to understanding the values of the ^{2}H NMR spectral splittings closely follows what we have used in some deuterated thermotropic and lyotropic liquid crystals (20, 21, 27).

The ^{2}H quadrupolar interaction in the L_α phase is observed as a splitting ($\Delta\nu_i$) between the 90° singularities of the spectral patterns which can be related to the time-averaged quadrupole splitting $\bar{\nu}_Q^i$, by the expression $\bar{\nu}_Q^i = 4/3\ \Delta\nu_i$.

Since the quadrupole splitting $\bar{\nu}_Q^i$ depends on the time average $\langle 3/2 \cos {}^2\sigma_i - 1/2\rangle$ in Eq. 1, it is useful to express this average in terms of the orientational motions of the molecule and its segments. This can be done by comparing the averaging at one site in the molecule with that at another. To accomplish this task it is common procedure to transform the expression in the time-average brackets to a molecular frame x, y, and z (27). Under this transformation:

$$\langle 3/2 \cos {}^2\sigma_i - 1/2\rangle = \sum_{p,q} \cos \alpha_p^i \cos \alpha_q^i \langle 3/2 \cos \theta_p \cos \theta_q - 1/2\delta_{pq}\rangle, \quad (2)$$

where the $\cos \alpha_p^i$ ($p, q = x, y, z$) are the direction cosines of the ith C-^{2}H bond in the molecular frame, and $\cos \theta_p$ are the direction cosines of the time averaged principal axis of the quadrupole interaction (V_{zz}) relative to the molecular frame (Fig. 1 *a*). Variations in $\cos \theta_p$ reflect fluctuations in the orientation of the molecular frame. In the work presented here we examine the order of the glycerol moeity of the molecule that we consider to be relatively rigid such that values of $\cos \alpha_p$ may be considered as approximately constant. Fixing the molecular frame to this relatively rigid region of the molecule, Eq. 2 can be expressed as (27)

$$\langle 3/2 \cos {}^2\sigma_i - 1/2\rangle = a_{zz}^i S_{zz} + 1/3(a_{xx}^i - a_{yy}^i)(S_{xx} - S_{yy}) + 4/3\ a_{xy}^i S_{xy} + 4/3\ a_{xz}^i S_{xz} + 4/3\ a_{yz}^i S_{yz}. \quad (3)$$

The quantities $a_{pq}^i = (3 \cos \alpha_p^i \cos \alpha_q^i - \delta_{pq})/2$ depend on the conformation of the molecule. It should be noted that the conformational parameters a_{pq}^i are not time averages, unlike the molecular order parameters S_{pq}, which have identical values at all sites in the rigid segment. For a suitable choice of the molecular frame, the order matrix can be diagonalized (20). The z' axis of this principal molecular axes x', y', and z' frame according to the convention $S_{x'x'} \leq S_{y'y'} < S_{z'z'}$, is the most-ordered axis of the molecule.

In applying Eq. 3 to the C-1 and 3 positions of the glycerol moeity it is convenient first of all to fix the molecular frame to the C-2 position of the *sn*-1 chain. Our results (vide infra) and those of Gally et al. (26) show that the spectral splitting for the two deuterium spins at this site in DMPC, are equivalent at all temperatures in the L_α phase, indicating that their quadrupole interactions are equivalent. This feature allows us to establish one of the principal molecular axes (which we choose as the x' axis) to be normal to the plane that bisects these two deuterium sites (Fig. 1 *b*). The y and z axes lie in this plane (Fig. 1 *b*). Reorientation of the molecular

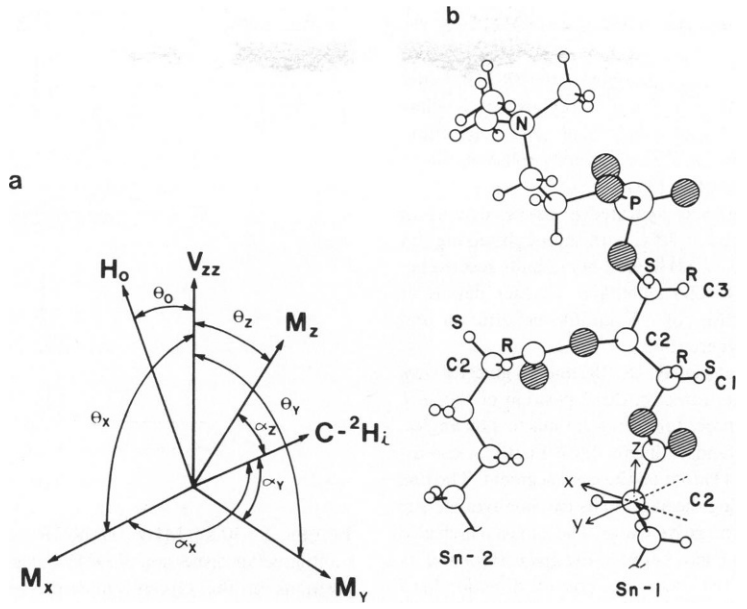

FIGURE 1 (*a*) Angle designations used in text. H_o is the direction of the applied magnetic field. V_{zz}, the principal component of the electric field gradient tensor has been shown to be parallel to the bilayer normal at several sites in the molecule. The instantaneous molecular orientation is indicated by the molecular frame M_x, M_y, M_z, which is attached to some rigid segment of the molecule. The direction of the carbon-deuterium bond is indicated by C—^2H_i. (*b*) Position of arbitrarily chosen molecular frame (M_x, M_y, M_z) in DMPC molecule. Hatched circles represent oxygen atoms while large clear circles are carbons. Only the upper portions of the *sn*-1 and *sn*-2 chains are indicated.

frame about x' maintains the equivalence of the deuterium quadrupole interaction of this segment. With this choice of frame there remain only three terms in the Meier-Saupe order matrix that are important in the spectral splitting. Applying Eq. 3 to Eq. 1 these are

$$\bar{\nu}_Q^i = \nu_Q \{a_{zz} S_{zz}^i + 1/3(a_{x'x'}^i - a_{yy}^i)(S_{x'x'} - S_{yy}) + 4/3 a_{yz}^i S_{yz}\}. \quad (4)$$

In the application of Eq. 4 one has the freedom to arbitrarily choose the orientation of z (and consequently y) relative to the C-2 segments. Since the long axis of the molecule is parallel to the direction corresponding to the all-*trans* or extended conformation, we choose the z axis to lie in that direction, in which case, the y axis bisects the tetrahedral ^{2}H—C—^{2}H bond of the C-2 position of the *sn*-1 chain. In this case the angles α_p for $p = (x', y, z)$ are (35.26°, 54.74°, 90°) and (125.26°, 54.74°, 90°) for each deuterated site of this segment that yields equivalent values of a_{pq}. In the application of Eq. 4, a value of ν_Q of 170 kHz has been used, which is the experimentally determined value of the deuterium quadrupole coupling constant in a range of aliphatic compounds (28, 29).

The next step in our analysis is to express the orientation of each carbon-deuterium bond in the glycerol region, in this chosen arbitrary frame. For a given conformation of this rigid segment, values of torsion angles about the C—C, and C—O single bonds, as well as C—C—C, C—O—C, and C—C—O bond angles are required for these coordinate transformations. Bond angle values used were those reported by Sundaralingam (30), whereas various torsion angle values were tested. Further details of these coordinate transformations are described in the Materials and Methods section.

From these torsion and bond angles for a chosen conformation, all of the values of a_{pq}^i can be calculated for each of the sites on the glycerol backbone. For reasons outlined in the Results our analysis used data from only the 1R, 1S, 3R, and 3S sites. For each experimental quadrupole splitting there will be a corresponding Eq. 4. Since there are three unknown molecular order parameters, S_{zz}, $S_{x'x'} - S_{yy}$ and S_{yz}, only three nonequivalent quadrupole splittings are required to determine their values. What is being sought in this analysis is a conformation of the rigid segment that gives the same three S_{pq} values for any three of the six possible Eqs. 4 that may be written. Thus, we wish to determine a conformation that gives a self-consistent set of quadrupole splittings from all six sites in the glycerol region. In applying Eq. 4 to these measured values of $\bar{\nu}_Q^i$ it must be remembered that only the absolute values of $\bar{\nu}_Q^i$, are measured. The correct signs to use can only be determined by retaining only those results in which $-0.5 \le S_{zz} \le 1$, $-1 \le S_{yz} \le 1$, and $(S_{x'x'} - S_{yy}) \le (1 - S_{zz})$.

A finite value of S_{yz} indicates that the z axis (extended axis of the chain) is tilted at an angle γ relative to the most-ordered axis of the molecule (this is the axis that diagonalizes the Meier-Saupe order matrix). Diagonalization of the Meier-Saupe order matrix yields (21):

$$\gamma = 1/2 \tan^{-1}\{4/3\, S_{yz}[S_{zz} + (S_{x'x'} - S_{yy})/3]\}.$$

The value of $S_{z'z'}$ for the most-ordered axis and the anisotropy of the order $(S_{x'x'} - S_{y'y'})$ are given by

$$S_{z'z'} = S_{zz}(3/2 \cos^2\gamma - 1/2) - 1/2(S_{x'x'} - S_{yy}) \sin^2\gamma + S_{yz} \sin 2\gamma$$

$$(S_{x'x'} - S_{y'y'}) = S_{zz}(\cos^2\gamma - 1) + 1/3(S_{x'x'} - S_{yy})(1 + \cos^2\gamma) + 2/3\, S_{yz} \sin 2\gamma.$$

$S_{z'z'}$ and $S_{x'x'} - S_{y'y'}$ express the ordering of the most-ordered molecular axis, with respect to the director axis, which has been determined for several sites in the hydrocarbon chain to be parallel to the bilayer normal (17). In our treatment, we assume that this is also the case for the sites on the glycerol backbone in the L_α phase.

MATERIALS AND METHODS

DMPC selectively ^{2}H-labeled in the glycerol moeity was synthesized by the following procedure. *rac*-Isopropylidene [1,3-$^2H_4$] glycerol was synthesized from diethyl acetoxymalonate as described by Wohlgemuth et al. (31). Selectively deuterated 1,2-dimyristoyl-*rac*-glycerol was prepared in several steps starting with *rac*-isopropylidene [1,3-$^2H_4$] glycerol (32).

1,2-Dimyristoyl-*rac*-glycerol was converted to ^{2}H-labeled DMPC by the procedure of Eibl (33).

DMPC, selectively ^{2}H-labeled at the 2 position of the *sn*-1 and *sn*-2 chains was synthesized by acylation of L-α-glycerophosphorylcholine cadmium chloride with [2,2-^{2}H] tetradecanoic acid (34). Deuterium exchange at the α-position of tetradecanoic acid was accomplished using a modification of the method of Aasen et al. (35).

^{2}H-labeled DMPC (75–100 mg) was hydrated in excess deuterium depleted water (150 μL) by warming at 30°C with thorough mixing. ^{2}H NMR spectra were recorded at 30.87 MHz on a home-built spectrometer, using a modified quadrupolar echo technique. Further details of instrumentation, sample temperature control, sample preparation and sample analysis are described elsewhere (36).

The orientation of C—^{2}H bonds at the 1R, 1S, 3R, and 3S glycerol sites in the arbitrarily chosen molecular frame on the 2 position of the *sn*-1 chain, were found by the following procedure using various torsion angles, and the bond angles reported by Sundaralingam (30). Firstly, a coordinate system is fixed to a given C or O atom on the rigid segment. The line which bisects the two bonds attaching the atom to its two nonhydrogen or nondeuterium neighbors forms the negative y axis. The z axis, which also lies in the plane formed by the two bonds between the given atom and its nearest neighbors, is orthogonal to the y axis. The positive direction for z is chosen so that the angle between it and the bond to the nearest neighbor closest to the 2 position of the *sn*-1 chain, is greater than 90°. The x axis, is normal to this plane and chosen to form a right-handed coordinate system. This coordinate system is now transformed by three successive rotations into the equivalent coordinate system attached to the adjacent C or O atom. The first rotation, of an angle of ½ (180°-bond angle) about the x axis in a counterclockwise direction, aligns the z axis along the bond joining the given atom to the nearest neighbor. The second rotation is counterclockwise about this new z axis by an angle given by the torsion angle. The third rotation is a counterclockwise rotation about the new x axis by an angle of ½(180°-bond angle). Hence, by applying three successive rotations, the directions in one coordinate system of a C—^{2}H bond on one atom, can be found in terms of another equivalent coordinate system attached to the nearest neighbor.

Finally, this process of nearest-neighbor transformation can be repeated atom by atom along the rigid segment until the C-2 position of the *sn*-1 chain is reached. This process was done for dueterons on the 1R, 1S, 3R, and 3S positions of the glycerol to give the orientation of each of these C—^{2}H bonds in the arbitrarily chosen frame on C-2 of the *sn*-1 chain. From the orientation of each of the above C—^{2}H bonds, a conformation parameter d^i_{pq} was calculated.

RESULTS

Representative 30.87 MHz ^{2}H NMR spectra of DMPC deuterated at the 1R, 1S, 3R, and 3S positions of the glycerol moeity are shown in Fig. 2. In making assignments in these spectra it was assumed that the quadrupole splittings in DMPC were very similar to values reported for the same positions in dipalmitoylphosphatidylcholine (37) and other phospholipids (26, 31, 38). Stereospecific monodeuteration of several of these phospholipids showed that the two splittings from the 1-glycerol segment, and the two splittings from the 3-glycerol segment reflect motional inequivalence of the individual deuterons (26).

The temperature dependence of the quadrupole splittings in the 30.87 MHz ^{2}H NMR spectrum of DMPC, ^{2}H-labeled in the 1R, 1S, 3R, and 3S positions of the glycerol moeity, is shown in Fig. 3. ^{2}H NMR data from the 2 position of the glycerol backbone of DMPC was not utilized in the spectral analysis. Because of the close similarity of $\Delta\nu$ for the 2 and 3R positions, their measurement would have required separate experiments, thus increasing the possibility of introducing errors into the order matrix treatment.

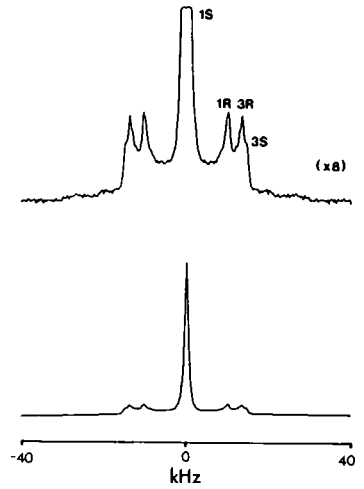

FIGURE 2 30.87 MHz ^{2}H NMR spectrum at 28°C of an aqueous multilamellar dispersion of DMPC deuterated in the 1R, 1S, 3R, and 3S positions of the glycerol moeity. Assignments are indicated on the expanded spectrum.

^{2}H NMR spectra of DMPC, deuterated at the 2 positions of the *sn*-1 and *sn*-2 chains were also recorded over the temperature range, 23 to 43°C. Our data were found to be consistent with published results for DMPC and other phospholipids, ^{2}H-labeled in the 2 positions of their hydrocarbon chains (39–45). In order to compare these $\Delta\nu$

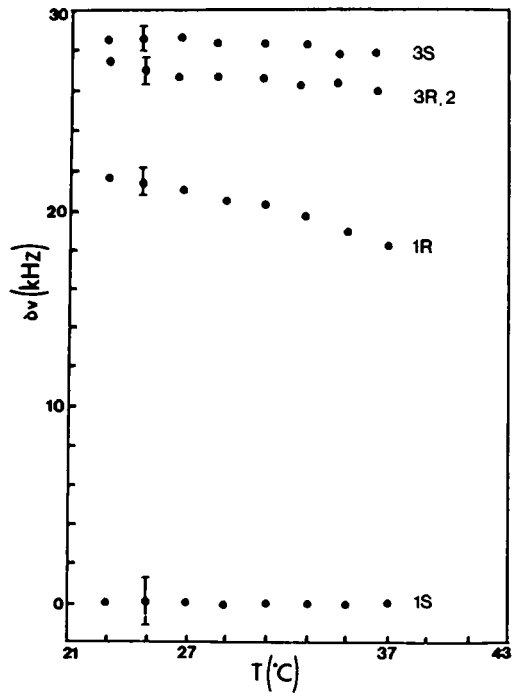

FIGURE 3 Temperature dependence of quadrupole splittings in the ^{2}H NMR spectrum of DMPC, which is ^{2}H-labeled in the 1R, 1S, 3R, 3S positions (●).

values directly with data from the glycerol backbone, we recorded a ^2H NMR spectrum of a mixture of glycerol-, and acyl chain-, ^2H-labeled DMPC.

With experimental quadrupole splittings from six sites in the glycerol region, it is possible to determine a^i_{pq} values using various trial conformations of this region. These conformational parameters together with Eq. 4 were used to solve for S_{pq}. Our criteria for a suitable a^i_{pq} is that they give the same S_{pq} at most sites in the glycerol, and that the calculated S_{pq} satisfy the conditions listed above in the Theory section. In addition, it is reasonable to expect that $S_{x'x'} - S_{y'y'}$ (in the diagonalized frame) should be small because of the approximately cylindrical shape of the DMPC molecule. Also, γ, the angle through which the arbitrary frame is tilted to diagonalize the molecular order matrix should be small, since the most-ordered molecular axis in the molecule would not be too different from the direction corresponding to the all-*trans* conformation of the *sn*-1 chain.

Certain conformations were eliminated immediately on the basis of simple symmetry considerations. The single-crystal data of Pearson and Pascher (10) show that the DMPC dihydrate molecule exists in the solid state in two conformations. These differ in that the glycerol C(1)—C(2) bond may occur in either the *trans* (t) or the *gauche* (g) conformation. In the *trans* state, both deuterons make the same angle with the z axis in our arbitrary frame. Since the two deuterons on the 2 position of the *sn*-1 chain are equivalent in the ^2H NMR spectrum and they also make the same angle with respect to the z axis, the 1R and 1S deuterons on the glycerol should be equivalent in this conformation. This is not found to be the case, experimentally.

Of the many conformations tested we find that the only one to meet the above criteria is a conformation very similar to the DMPC-B structure in the solid state (10). Torsion angles for this conformation are described in the literature (10, 24) and the calculated orientations of the various C—^2H bonds of the glycerol region in the arbitrary molecular frame are given in Table I. The a^i_{pq} determined from these angles in Table I, have been used in Eq. 4 together with the experimentally measured quadrupole splittings, to solve for S_{pq}. Table II shows the $S_{x'x'}$, $S_{x'x'} - S_{y'y'}$, and γ values calculated at 23°C for different sets of three sites in the glycerol region.

TABLE I
CALCULATED C—^2H BOND ORIENTATIONS IN THE CHOSEN MOLECULAR FRAME* FOR THE DMPC B CONFORMATION (10)

C—^2H bond position	$\Delta\nu/129$	α_x	α_y	α_z
1R	0.1669	151	61	56
1S	0.0	103	167	101
3R	0.2108	120	116	89
3S	0.2269	126	46	60
2S (1-chain)	0.2400	35.26	54.74	90
2R (1-chain)	0.2400	144.74	54.74	90

*Molecular frame chosen such that 2R, S deuterons were in xy plane bisecting the y axis.

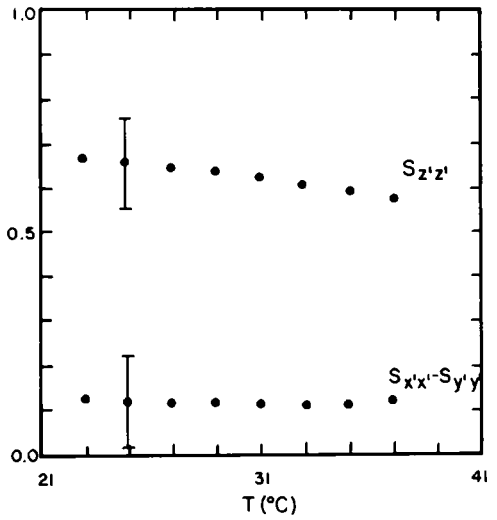

FIGURE 4 Temperature dependence of the molecular order parameters $S_{z'z'}$ and $S_{x'x'} - S_{y'y'}$.

Since the quadrupole splittings vary with temperature (Fig. 3) we have determined, using Eq. 4, the temperature dependence of $S_{z'z'}$, $S_{x'x'} - S_{y'y'}$, and γ. Fig. 4 shows that $S_{z'z'}$ decreases with increasing temperature, while $S_{x'x'} - S_{y'y'}$ is temperature independent. Furthermore, the angle γ was found to be 26 ± 3°C and also temperature invariant.

To test the validity of this analysis we have checked $S_{z'z'}$ and γ values in Table II by the following procedure. Since $S_{x'x'} - S_{y'y'}$ values are small, the assumption was made, as others have done, that ordering is symmetric about the director axis, i.e.,

$$S_{x'x'} - S_{y'y'} = 0.$$

If this is so, then the time-averaged quadrupole coupling constant ($\bar{\nu}^i_Q$) is given by

$$\bar{\nu}^i_Q = \nu_Q (a^i_{z'z} S_{z'z}), \tag{5}$$

TABLE II
CALCULATED MOLECULAR ORDER PARAMETERS $S_{p'q'}$ IN THE DIAGONALIZED FRAME FOR DIFFERENT SETS OF SITES IN THE GLYCEROL REGION*‡

Sites	$S_{z'z'}$	$S_{x'x'} - S_{y'y'}$	γ
1R, 1S, 2RS§	0.6 ± 0.1	0.1 ± 0.1	27 ± 2°
3R, 3S, 2RS§	0.6 ± 0.1	0.1 ± 0.1	26 ± 2°
3R, 1R, 2RS¶	0.6 ± 0.1	0.1 ± 0.1	26 ± 2°
1S, 3S, 2RS§	0.6 ± 0.1	0.1 ± 0.1	27 ± 2°
1R, 3S, 2RS§	0.6 ± 0.1	0.1 ± 0.1	26 ± 2°
1S, 3R, 2RS§	0.7 ± 0.1	0.2 ± 0.1	28 ± 2°

*23°C.
‡Error in each entry covers the range of S values calculated using torsion and bond angles that differ from published values (10,30) by ±5°.
¶*sn*-1-chain.

where ν_Q, $S_{z'z'}$ are as defined previously, and $a^i_{z'z'} = 3/2 \cos^2 \beta' - 1/2$ with β' being the angle between the directions of the C—^2H_i bond and the principal molecular axis (z'). It is possible to show that

$$\cos \beta' = \sin \beta \sin \alpha \sin \gamma + \cos \beta \cos \gamma, \quad (6)$$

where γ is as defined, and β, α are the polar and azimuthal angles expressing the orientation of the C—^2H_i bond in the arbitrarily chosen molecular frame (x, y, z).

Substitution of Eq. 6 into Eq. 5 yields

$$\bar{\nu}^i_Q = \nu_Q [3/2 (\sin \beta \sin \alpha \sin \gamma + \cos \beta \cos \gamma)^2 - 1/2] S_{z'z'} \quad (7)$$

If a similar expression is written for either one of the 2-R,S sites on the *sn*-1 chain (site j), then the ratio of the observed quadrupole splittings for the ith and jth sites is given by

$$\frac{\bar{\nu}^i_Q}{\bar{\nu}^j_Q} = \frac{[3/2 (\sin \beta_i \sin \alpha_i \sin \gamma + \cos \beta_i \cos \gamma)^2 - 1/2]}{[3/2 (\sin \beta_j \sin \alpha_j \sin \gamma + \cos \beta_j \cos \gamma)^2 - 1/2]}.$$

After conversion of the direction cosines in Table I to polar and azimuthal angles, experimentally determined $\bar{\nu}_Q$ values can be used in the above equation to calculate a γ for each C—^2H_i bond. Substitution of each γ into Eq. 7 gives a corresponding $S_{z'z'}$ for each site in the glycerol backbone. Such a treatment gave a set of γ and $S_{z'z'}$ values which were in agreement with each other and with the data in Table II.

DISCUSSION

^{31}P NMR spectra of oriented phosphatidylcholine multibilayers dispersed in excess water have been interpreted in terms of the head group undergoing relatively unhindered rotation about the C(2)—C(3) bond of the glycerol backbone (46–48). At the same time, there is evidence from ^2H NMR and ESR that, in the L_α phase, the phosphatidylcholine molecule undergoes rapid rotation about its long molecular axis (36, 49). If these two motions occur simultaneously, the two quadrupole splittings from the 3R and 3S sites on the glycerol should be equivalent, which is not observed. Gally et al. (26), have shown by stereospecific labeling that the two splittings observed for the 3R and 3S glycerol sites in several phospholipids, arise from motional inequivalence of the individual deuterons and not from the existence of two relatively long-lived conformations. Thus, a model of motional averaging, in which the whole phosphatidylcholine molecule undergoes rapid rotation about its long axis, and at the same time there is rapid intramolecular rotation about the glycerol C(2)—C(3) bond, is clearly inconsistent with the ^2H NMR results.

Consequently, we question the assumption that, in phosphatidylcholine bilayers, there is rapid rotation about the glycerol C(2)—C(3) bond. Because two different conformations about the C(2)—C(3) bond have been observed in x-ray studies of glycerophospholipids in the solid state, does not mean that there is rotational freedom about that bond in the L_α phase (24). The axially symmetrical powder patterns observed in the ^{31}P and ^2H NMR spectra of phospholipid bilayers, labeled in the head group, can readily be accounted for by other types of intramolecular motion in the headgroup (e.g., rotation about the P—O(C), C—O(P), C—C bonds) and by rotational motion of the molecule as a whole. Indeed, Kohler and Klein (50) have proposed a model that includes rotations about the P—O (glycerol) bond and the long molecular axis, and a wobble of the molecule about the bilayer normal, in order to account for the ^{31}P NMR spectral line-shapes. In this model, no rotation occurs about the C(2)—C(3) bond of the glycerol. Such a model is consistent with the experimental results and calculations reported in this paper.

The inequivalence of the 1R and 1S sites in the ^2H NMR spectra of phospholipid bilayer membranes has been accounted for by a model in which there is an asymmetric two-site jump process about the glycerol C(1)—C(2) bond (26). This model, which quantitatively predicts the experimental quadrupole splittings at the glycerol 1R and 1S sites, was developed to be consistent with ^1H NMR studies of micellar systems that showed intrinsic flexibility about the C(1)—C(2) bond (2), and with single-crystal x-ray data that showed this bond to occur in both *trans* and *gauche* conformations (10). However, planar DMPC bilayers in excess water are quite a different system from micelles of DPPC in an organic solvent. Also, as stated above, because two conformations of DMPC are found in the solid state it does not follow that their interconversion will necessarily be an energetically favorable process in the presence of excess water. Thus, a model that is developed to account for motional averaging in the L_α phase of a planar bilayer membrane need not be the same as that observed in the micellar and solid states.

We propose that another explanation for the spectral data from the 1R,S sites is that there is limited flexibility about the C(1)—C(2) bond in the glycerol backbone. It follows that if there is also limited flexibility about the C(2)—C(3) bond then the glycerol backbone is essentially rigid over the time-scale of the NMR measurement. It is appropriate to speculate on why the glycerol moiety may adopt a rigid conformation in the bilayer membrane. High resolution ^1H NMR studies of dehexanoylphosphatidylcholine dissolved in methanol indicate that about the C(1)—C(2) and C(2)—C(3) bonds of the glycerol backbone, all three staggered conformations exist (2). For the same compound present in the form of micelles in an aqueous medium, a restriction in headgroup motion is reflected by a decrease in the fractional population of conformers in which the acyl chains are *trans*. It is reasonable to expect that this decrease may continue into the planar bilayer membrane, but this cannot be confirmed because of the impossibility of performing high resolution ^1H NMR studies on these liquid crystalline systems. To a much greater extent than micellar systems, bilayer mem-

branes represent a condensed phase in which conformational interconversion could be energetically less favorable because of the close proximity of neighboring molecules.

It is known that the polar headgroup, and probably the glycerol backbone, in phosphatidylcholine bilayer membranes in excess water are strongly hydrated. If water molecules in the L_α phase, assume particular sites within the polar headgroup structure, as they are observed to do in the single-crystal structure of DMPC dihydrate (10), then it is possible that they could stabilize a certain geometry of the glycerol moiety. Of course, the ^2H NMR spectra of phosphatidylcholine-^2H_2O systems indicate that the exchange rates between these headgroup sites and the bulk water environment would need to be rapid on the NMR time scale (51).

If the assumption that the glycerol moiety is almost rigid, is correct, then it is appropriate to apply the molecular frame and order matrix approach to the analysis of ^2H NMR quadrupole splittings from multiple sites in the rigid section. Using this analysis we have found good agreement among the $S_{p'q'}$ and γ values calculated using different sets of glycerol sites, for a conformation that is very similar to that of conformer B in the single-crystal structure of DMPC dihydrate (10). Far better agreement is found between the various calculated $S_{p'q'}$ values for this conformation than for any other conformer that we tested. It is quite remarkable that the conformer that gives a self-consistent set of quadrupole splittings from the glycerol region, is one of the structures found in the solid state. This supports the validity of the original assumption that the glycerol backbone is rigid. The value of $S_{z'z'} = 0.6 \pm 0.1$ is in good agreement with $S_{z'z'}$ values determined in analogous thermotropic liquid crystals. The small value of $S_{x'x'} - S_{y'y'}$ indicates that there is almost completely free or unhindered rotation of the most ordered axis in the molecule about the director axis. A small value for this order parameter is not unexpected because of the approximately cylindrical shape of the DMPC molecule. The assumption of free-rotation about the director axis, which has been made by most other workers in their analyses of ^2H NMR spectra, is therefore shown to be reasonable.

There are further assumptions in our analysis that should be checked experimentally. That is, that all the deuterated sites in the rigid region share the same principal axis system for the time-averaged quadrupole interaction, and that the principal component of this axis system is parallel to the bilayer normal. To check this experimentally requires an aligned bilayer sample and an angular dependence study of the spectral splittings (20). In order for the principal component of the time averaged quadrupole interaction to be parallel to the bilayer normal it is essential that the most-ordered molecular axis sample most orientations around the bilayer normal within the time scale of the NMR measurement. This could be accomplished either by the lipid molecule sweeping out a cone in one fixed location or by rapid lateral diffusion, with the molecule sampling a different orientation at each position in its lateral movement.

If our analysis is extended to the 2R and 2S positions of the *sn*-2 chain, then their a^i_{pq} values for conformer B of DMPC dihydrate (10) yield a set of $S_{p'q'}$ values that are in poor agreement with the other entries in Table II. The agreement is not improved by trying other possible orientations of the 2S and 2R segments. This poor agreement results from either accumulated errors produced by coordinate transformations over a large number of atoms, or the 2R, 2S segment of the *sn*-2 chain being not rigid with respect to the rigid glycerol region. If the latter is the correct explanation then, we propose that intramolecular movement of the 2R, 2S segment on the *sn*-2 chain with respect to the rigid glycerol segment, occurs principally by rotation about the C(1)—C(2) bond of the *sn*-2 chain. For conformation B, we have calculated that the direction of the carbonyl bond on the *sn*-2 chain is at the same angle as is the glycerol 1S C—^2H bond with respect to the most-ordered molecular axis. Since the latter has a time averaged quadrupole splitting of zero, then the chemical shift anisotropy for the ^{13}C nucleus of the *sn*-2 carbonyl group should be approximately zero, assuming that the principal component of the ^{13}C chemical shift tensor is along the carbonyl carbon-oxygen bond.^{13}C NMR studies in the L_α phase of DPPC that had been ^{13}C labeled at the carbonyl position of the *sn*-2 chain, give a narrow isotropic-like line for the carbonyl carbon with a small chemical shift anisotropy of ~7ppm (25). Thus, the ^{13}C NMR results are consistent with the C=O bond direction being fixed with respect to the glycerol unit. This is further evidence that the glycerol segment in phosphatidylcholine bilayer membrane is almost rigid.

In summary, we have outlined the reasons for assuming that in DMPC bilayer membranes, the glycerol backbone region including the 2 position of the *sn*-1 chain, is rigid. If this assumption is correct, then we have shown the following features of motional averaging in the bilayer. First, there is a most-ordered molecular axis in DMPC whose position can be defined. This axis is not parallel to the direction of the all-*trans* or extended conformation of the *sn*-1 chain but tilted with respect to it. Second, ordering of the most-ordered axis is approximately axially symmetrical about the director axis as indicated by the small value of $S_{x'x'} - S_{y'y'}$. Third, a measure of the ordering of the most-ordered molecular axis about the director axis is provided by $S_{z'z'}$, whose value of 0.6 ± 0.1 is similar in magnitude to that found in analogous thermotropic liquid crystals.

Finally, we should like to point out the potential of this analysis in further studies of molecular ordering in the membrane. First of all, the position of most-ordered axis in the molecule is no longer a model-dependent parameter but is measured directly. Second, by fixing the molecular frame to the glycerol moeity one can determine the relative time-averaged conformation of the flexible region of the

molecule by measuring the a^i_{pq} at different sites. Third, there is more information available on the manner in which the molecule is ordered in the bilayer.

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