AN ALTERNATIVE INTERPRETATION OF NUCLEAR MAGNETIC RESONANCE OBSERVATIONS IN THE GEL STATE OF LIPID BILAYERS

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ABSTRACT In the established interpretation of nuclear magnetic resonance (NMR) spectra of phospholipid bilayers in the gel state, the molecules are assumed to perform rotational diffusion about their long axis. Here we present an alternative model of the molecular mobility in this phase, which considers the positions of the lipid molecules in the two-dimensional bilayer lattice as fixed within the NMR timescale. Instead we assume an intramolecular two-site hopping of the hydrocarbon chains about their long axis. It is shown that deuterium NMR spectra of chain-labeled compounds are very sensitive to the precise angle of this flip-flop motion near 90°, so that the diversity of these gel-phase spectra is easily explained by slight variations of this angle. In addition, it is argued that the axial symmetry of ^{13}C spectra of carbonyl-labeled phospholipids might also result from this intramolecular mobility.

INTRODUCTION

The thermotropic properties of lipid bilayers have been studied with a variety of spectroscopic methods. Among these, nuclear magnetic resonance (NMR) has played an important role in the elucidation of molecular mobility of these systems (for a review see references 1–7). Until quite recently these investigations were limited to the fluid Lα phase, where the spectra are significantly narrowed by motional averaging. In particular, NMR measurements on compounds that were labeled with appropriate isotopes at specific molecular sites yielded a wealth of information about the motional structure. Recently with the employment of solid state NMR techniques, the less mobile gel states of lipid bilayers could also be studied in some detail. Now, in the interpretation of these spectra, a crucial puzzle has appeared. The data seem to contradict one another; some data seem to indicate rotational motion of the molecule, whereas other data give evidence of motions without axial symmetry. In previous interpretations this contrast is attributed to the different timescales of the respective measurements.

The purpose of this article is to draw attention to an alternative model that considers only intramolecular motions. Originally, this model was derived to explain ^1H- and ^{13}C-NMR results on lecithins of natural isotope abundance, which were made in our laboratory (8, 9). It will be shown here that, with a slight modification, this model also explains the puzzling solid state NMR observations made in the gel phase of various specifically labeled phospholipids, i.e., in particular, lecithins and cephalines. The intermediate phase Pβ', observed in lecithins below the main transition temperature (10), seems to be an exception in that there is evidence of both solid and fluid bilayer regions in this state (11–14). Although we believe that the basic features of our gel-phase model also hold true for at least the solid regions of this intermediate phase, the following discussion will be restricted to the lamellar phases Lβ and Lβ' for the sake of simplicity.

EXPERIMENTAL EVIDENCE AND PREVIOUS INTERPRETATIONS

The chemical shift tensors of the phosphorous nucleus in the headgroup of phospholipids and both ^{13}C nuclei at the carbonyl linkages of the two hydrocarbon chains to the glycerol moiety are clearly asymmetric in the rigid limit with asymmetry parameters η of ~0.5 and ~0.2, respectively (2, 12, 13, 16–22). In the gel phase of fully hydrated bilayers, however, axial symmetric spectra with reduced anisotropy are observed. In the same state the electric field gradient (EFG) tensor of the C-^2H bonds in the hydrocarbon chains, which is known to be axial symmetric in the rigid case, gives rise to lineshapes that appear asymmetric. In addition, the shapes of the ^2H spectra vary with each lipid, and also change substantially when the temperature is varied within the gel-phase range (13, 14, 16, 23–29). Also in this respect the deuterium NMR results of the hydrocarbon-chain region are unlike the findings at other nuclei, such as ^{13}C or ^{31}P (12–16, 18–22), which are known to vary only moderately. To explain these surprising results consistently, it is usually assumed that (*a*) the lipid molecules undergo rotational diffusion about their long axis with a rate between the frequencies of the interactions, i.e.,

the anisotropy of the ^{13}C and ^{31}P chemical shift on one side, and the C-^{2}H EFG on the other. (*b*) The hydrocarbon chains perform asymmetric *trans-gauche* isomerizations, which are even slower than the rotational diffusion. Under these conditions the overall molecular rotation (*a*) will affect only the weaker ^{13}C and ^{31}P interactions in the fast limit and result in averaged chemical shift tensors of axial symmetry. For the stronger quadrupolar coupling in the C-^{2}H bonds of the hydrocarbon chains, both types of motion (*a* and *b*) appear in the intermediate time domain. It is well known that motion in this time regime has a pronounced effect on the spectral shapes. The variance of these lineshapes is simulated in computer calculations by adjusting the correlation times of both motions, and the relative population of the *gauche* bonds.

This interpretation is consistent, but there are some objections. (*a*) In some cases ^{2}H spectra of the gel phase exhibit their maximum intensity at the central frequency, i.e., their asymmetry seems to approach values close to 1 (e.g., in DPPE at 11°C [13, 15], or throughout the Lβ' phase of DPPC [14]. Within the frame of this model this requires a relative abundance of *gauche* conformers near 0.5 per bond, a value that is much larger than estimates done on the basis of infrared and Raman spectroscopy studies of the gel phase (30–33). If more realistic *gauche* probabilities of 0.02–0.07 per bond are used, the resulting simulations do not reflect the characteristic patterns of the experimental spectra.

(*b*) The idea of a fast axial diffusion of the lipid molecules contradicts the existence of a hexagonal or orthorhombic lattice of the hydrocarbon chains (34–40). One is forced to assume a special kind of defect diffusion, where an empty lattice site is occupied by an adjacent chain. Since two neighboring chains belong to one lipid molecule, this diffusion step links a 60°-rotational jump of the molecule with a lateral motion of its gravitational center, about half the lattice constant, i.e., ~2 Å. So, the value of the lateral self-diffusion constant in the gel phase provides a crucial test for this model. Unfortunately, however, it has not been determined for the Lβ or Lβ' phase. Measurements of the lateral diffusion constant in the Pβ' phase of DPPC have yielded two values, one parallel ($\sim 10^{-11}$ cm^2/s) and one perpendicular ($\leq 10^{-17}$ cm^2/s) to the periodic bilayer corrugations observed in this particular phase (41). The higher diffusion constant is thought to be present within the distortions of the bilayer

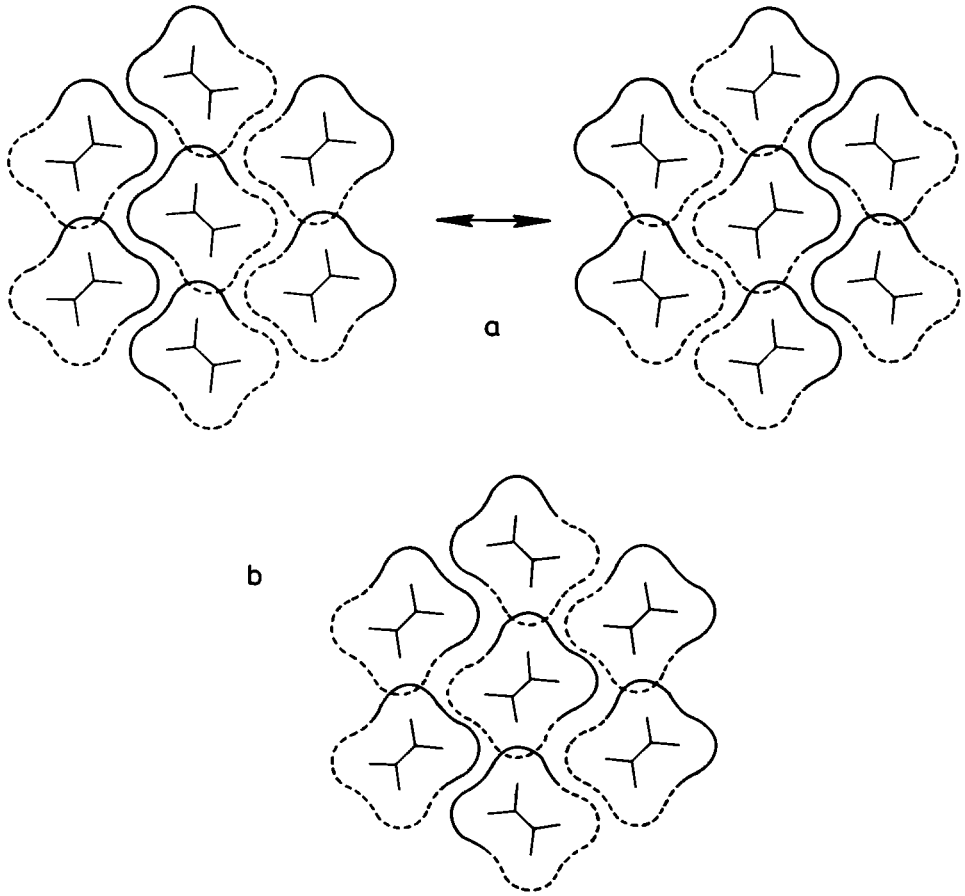

FIGURE 1 (*a*) The two states of the cooperative flip-flop motion with a hopping angle $\beta = 90°$. The cross-sectional area is perpendicular to the hexagonal chain lattice. Intermolecular contours of atoms that are not in this plane are indicated by dashed lines. (*b*) A 90°-jump of the central chain without cooperativity of the neighboring chains.

lattice along the ripples, whereas the lower diffusion constant refers to the diffusion within the undisturbed gel domains of the bilayer. So the latter value can be taken as an estimate of the lateral diffusion constant in the Lβ or Lβ' gel phase of lipids, and it follows that the corresponding rotational diffusion is by far too slow to affect NMR spectra.

(*c*) Finally it must be emphasized that the gap between the time windows of the investigated interactions is very small. In ^{31}P NMR a static tensor of ~200 ppm total width is averaged by axial diffusion to an axial symmetric tensor. At a 120-MHz operating frequency this requires a diffusion rate of ≥25 kHz. The EFG tensor, which, according to this model, is also subjected to this axial diffusion has a width of ~180 kHz in the rigid limit. Thus the difference between the two time constants is not more than a factor of 7. It is difficult to imagine that the rotational diffusion rate remains in the fast limit for ^{31}P and ^{13}C throughout the entire gel phase range, whereas its influence on the variation of the deuterium spectra is striking.

AN ALTERNATIVE MODEL

The alternative model presented here considers the gravitational centers of the lipid molecules as fixed in the two-dimensional lattice. The extended hydrocarbon chains are assumed to perform a two-site hopping about their long axes. If this chain flip-flop occurs in cooperativity with the microscopic environment, as illustrated in Fig. 1 *a*, the two states of the microcrystal are mirrorlike images of each other. With this symmetry their relative population must be 0.5. However, this cooperativity is not a necessary condition of this motion. A distortion of the herringbone chain packing is possible without steric restraints. This is illustrated in Fig. 1 *b*, which shows that the van der Waals contour of a distorted chain does not overlap its neighbors. This freedom has its origin in the relatively loose packing of the hydrocarbon chains in the hexagonal or nearly hexagonal lattice of the lamellar gel phase. For lecithin a phase transition to a denser, orthorhombic packing is observed at lower temperatures (36–38). In addition to this paraffin-like chain lattice, this low temperature state (referred to as Lσ' in our works [9, 22, 40] and L_c or L_ϵ by other authors [38, 42]) is characterized by a drastic reduction of the chain mobility (9). It seems reasonable to assume that the driving force of the lattice expansion at the phase transition L$\sigma' \rightarrow$ Lβ' is the suggested chain flip-flop.

The remaining open parameters of this motion are the rate and the hopping angle β, which determine the intrinsic linewidth of the ^{2}H spectra and the principal values of the resulting averaged EFG tensor, respectively. From the lineshape of the ^{2}H spectra as well as from ^{1}H-$T_{1\rho}$ investigations (8), it is clear that the correlation times of the chain motion in the gel phase lie in the intermediate time regime with respect to the ^{2}H-quadrupole splitting, i.e., ($\sim 10^{-5} - 10^{-6}$ s. To simplify the following discussion, it will nevertheless be argued in terms of the fast limit. This treatment is based on the view that it is the most important task to reproduce the basic experimental features in these simplified spectra.

In our original work (8, 9) we assumed a constant flip-flop angle of 90° for the Lβ' phase of lecithins. It was shown that the total second moments of the ^{1}H-NMR lines are not very sensitive to the precise value of β. This is basically because proton lines are broadened by many dipolar interactions, which, in their superposition, do not display the geometry of the averaging motion very well. The modification of our previous model, presented here, allows for deviations of β from 90°. Now the symmetry of this jump is reduced from four-fold to two-fold. It is well known that such a reduction of symmetry can be reflected in the asymmetry of the motional average of a single interaction (43, 44).

The Impact of this Model on ^{2}H-NMR of Acyl Chains

Unlike proton spectra, ^{2}H-NMR lineshapes of labeled hydrocarbon chains are governed by only one single interaction, the coupling of the electric quadrupole with the EFG tensor, which has its unique axis aligned along the C-^{2}H bond. The two-site hopping occurs about the long axis of the chain, perpendicular to this. The geometry of this motion is shown in Fig. 2, where the two orientations 1, 2, 3 and 1, 2', 3' of the static EFG tensor with the principal values $V_{11} = V_{22} = -V_{33}/2$ are given in a molecule fixed reference frame x, y, z. The directions of these axes coincide with the crystalline a, b, and c axes as well as with the principal axes of the resulting averaged tensor. In terms of the static value, V_{33}, and the flip-flop angle, β, the three principal components of the residual averaged tensor are given by

$$V_{xx} = \left(1 - \frac{3}{2}\cos^2\frac{\beta}{2}\right) V_{33}$$

$$V_{yy} = \left(\frac{3}{2}\cos^2\frac{\beta}{2} - \frac{1}{2}\right) V_{33}$$

$$V_{zz} = -\frac{1}{2} V_{33}. \qquad (1)$$

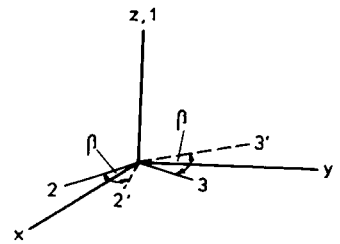

FIGURE 2 Flip-flop motion of the C-^{2}H EFG tensor in a molecule fixed reference frame x, y, z. The two positions of the rigid tensor are noted by the indices 1, 2, 3, and 1, 2', 3'. The orientations of the principal axes of the averaged tensor coincide with the chosen fixed frame.

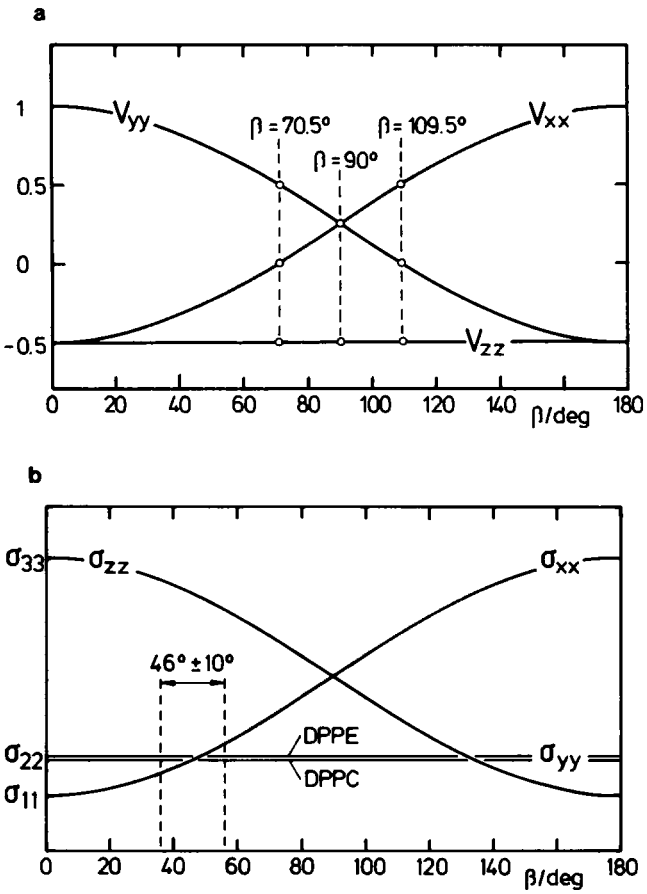

FIGURE 3 (*a*) Reduction of the principal values of the residual EFG tensor V_{xx}, V_{yy}, and V_{zz} by the two-site jump about z. The scale of the vertical axis is in units of V_{33}, the principal value of the static tensor. The three special cases with vanishing (β = 90°) and maximum (β = 70.5°, 109.5°) asymmetry are particularly marked. (*b*) Reduction of the principal values of the residual chemical shift tensor σ_{xx}, σ_{yy}, and σ_{zz} by a two-site jump about y. The different static values of σ_{11}, σ_{22}, and σ_{33}, which were found experimentally for DPPC and DPPE, are considered by a normalization of σ_{33}–σ_{11} in the vertical scale. With this representation only the intermediate principal components σ_{22} appear as two distinct values in this figure. The range, within which $\eta \leq 0.1$, is particularly marked.

These three functions are sketched in Fig. 3 *a*. It now becomes evident that around β = 90° the shape of the ^{2}H spectra is extremely sensitive to slight variations of β. At this particular value the tensor is axial symmetric (η = 0), i.e., the averaging effect of this type of motion on the intramolecular C-^{2}H EFG tensor is identical to the result of rotational diffusion (8). A deviation of β from 90° of about less than 20° results in a tensor of total asymmetry η = 1.

This model explains the ^{2}H-NMR gel-phase spectra without any further postulates. With 70° < β < 120° the three principal values of the residual C-^{2}H EFG tensor are within the limits of $\pm V_{33}/2$. As a result the entire spectral intensity of all of the gel phase spectra is expected to be concentrated in the range between $\pm V_{33}/2 \simeq \pm 60$ kHz, in accordance with the reported experimental evidence. The particular shape is a function of the precise value of the flip-flop angle β and the temporal rate of this motion. With these two input paramters a lineshape simulation could provide the quantitative analysis of this mobility.

Even though this detailed treatment is outside the scope of this article, it should be emphasized that the proposed model appears to be able to cover the remarkable diversity of the deuterium spectra. In particular, the significant differences in the ^{2}H spectra of various chain deuterated lipids and their substantial changes within the temperature range of the gel phase are easily understood as the result of slight changes in the flip-flop angle β. This angle is assumed to depend on two types of interactions. One is due to the attachment of the hydrocarbon chains to the glycerol moiety by covalent chemical bonds. This results in a spacing of the two chains, which, in turn, depends on the structure of the headgroup and its interaction with the water interface. The second interaction is given by the lattice constraints of the two-dimensional crystal. It is well known that the lattice constants vary slightly with different lipids and within the temperature range of the gel state (34–40, 45). In particular, the orthorhombic distortion, increasing towards lower temperatures within the Lβ' phase of lecithins, might cause a slight change of β. As a result, these weak crystallographic effects can have a pronounced effect on the ^{2}H-NMR spectral shape.

Speculation on the Impact of the Model on the $^{13}C{=}O$ Resonance

We still must account for the axial symmetry of the chemical shift tensors of ^{13}C at the two carbonyl positions and ^{31}P in the headgroup. The latter case is easily explained as a result of intramolecular rotation of the headgroup about an internal chemical bond, such as the P–O linkage to the glycerol moiety. Due to the chemical structure of the carbonyl group, such an internal molecular rotation cannot occur at this site.

At first thought the observation of an axial symmetric chemical shift tensor at this position seems to be an evidence of rotational diffusion of the whole molecule. However, note that there is a multitude of other motions that result in axial symmetry. One example was already given in the previous discussion; a two-site hopping about one principal axis of a tensor with the angle β = 90° leads to a convergence of the other two principal values, i.e., to axial symmetry. However, the molecular geometry at the carbonyl site is not the same and certainly not as simple as in the case of the C-^{2}H EFG tensor. Unfortunately, neither the precise orientation of the carbonyl chemical shift tensor in the molecular frame nor the exact crystallographic structure of this moiety in fully hydrated lipids is known. Therefore, a detailed discussion of the impact of the chain flip-flop on the carbonyl resonance is speculative. Nevertheless, it might be worthwhile to treat this problem in some detail to demonstrate that within the proposed model it is not unlikely to obtain axial symmetric spectra at the carbonyl position.

Recall the experimental evidence of the ^{13}C-carbonyl spectra. For two phospholipids, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE), labeled at the sn-2-chain, both static and averaged chemical shift tensors have been determined (13, 16). The static tensors, which were measured at very low tempertures or at low hydration states, are clearly asymmetric with η = 0.24 and 0.27, respectively. In the lamellar Lβ' or Lβ phases this asymmetry vanishes within the spectral resolution, leaving two principal values $\sigma_\parallel$ and $\sigma_\perp$. A remarkable coincidence, which has attracted little attention up to now, is that in both cases $\sigma_\perp \simeq \sigma_{22}$, where σ_{22} is the intermediate principal value of the static tensor.

The geometry of the carbonyl moiety according to x-ray studies on dihydrated lipids (46) is sketched in Fig. 4. From comparison with related compounds it is usually assumed that axis 3 of the carbonyl tensor points into the direction of the C = O bond, while the axes 1 and 2 are oriented perpendicular to it, within and outside the ester group plane, respectively (12, 47). An intramolecular chain flip-flop of ~90° implies that also the torsional angles of the covalent bonds of the ester group jump between two configurations. Due to its vicinity to the relatively stiff glycerol moiety, the hopping angle β at the carbonyl site should be <90°. The hopping axis might point in the direction of the central carbon of the glycerol backbone, i.e., the orientation of the axis 2 of the chemical shift tensor.

The averaging effect of such a two-site hopping is described by a relation similar to Eq. 1

$$\sigma_{xx} = \sigma_{33} - (\sigma_{33} - \sigma_{11}) \cos^2\beta/2$$

$$\sigma_{yy} = \sigma_{22}$$

$$\sigma_{zz} = \sigma_{11} + (\sigma_{33} - \sigma_{11}) \cos^2\beta/2. \qquad (2)$$

These functions are sketched in Fig. 3 *b* for the two cases of DPPC and DPPE. It demonstrates that the outer two principal values, σ_{xx} and σ_{zz}, converge with β increasing to 90°. At $\beta \approx 46°$ there is an intersection of σ_{xx} with σ_{yy} leading to the axial symmetric spectrum with $\sigma_\perp = \sigma_{22}$. This motion accounts for not only the axial symmetry of the gel phase carbonyl spectra but also for the particular reduction of the anisotropy with respect to the static values. Although details of the geometry of the two-site jump might differ slightly from that depicted in Fig. 4, the given example is attractive due to its simplicity. Without the precise knowledge of the molecular geometry, a more elaborate treatment of this problem is not useful. But note that the number of conformations within the two-site hopping model is even considerably extended by the limited accuracy of the reported ^{13}C spectra. Also with the given resolution slight asymmetries with $\eta \leq 0.1$ could be easily overlooked.

Note that in the case of glycolipids the gel-phase spectra are not axial symmetric (4), an indication that the vanishing of η in the carbonyl resonance is not a general feature of the gel phase of lipids. In terms of the proposed model, this observation simply indicates that the molecular geometry and/or the principal values of the static chemical shift tensor are different in these compounds. This is a much simpler explanation than the view that two classes of lipids exhibit two different types of gel phases, one with (phospholipids) and one without (glycolipids) the presence of axial molecular diffusion.

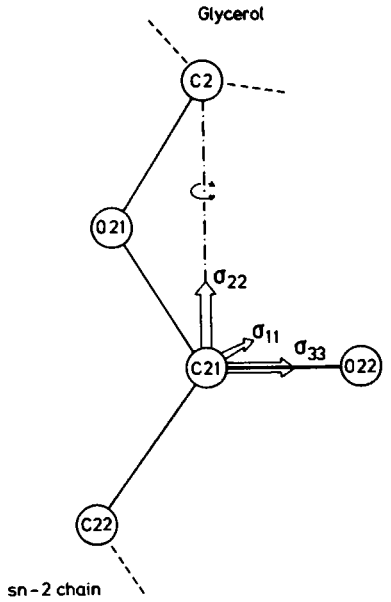

FIGURE 4 The ester linkage of the sn-2 chain to the glycerol backbone. According to one of the configurations suggested by Pearson and Pascher (46), the five nuclei C2, O21, C21, O22, C22 are assumed to be in the plane. The adjacent bonds, indicated by dashed lines, point out of the plane, as well as the average direction of the hydrocarbon chain.

Note also that another problem with the carbonyl resonance explanation is resolved once we no longer assume there is rotational diffusion in the gel state. In the fluid Lα phase these spectra collapse to an almost isotropic line (12–15). Within our model this is simply the result of the fast rotational diffusion about an axis inclined ~55° with respect to the unique axis of the C = O chemical shift tensor. In this fluid state this type of molecular motion about the bilayer normal is expected. On the other hand, if one assumes that rotational motion exists already in the gel phase, then an additional postulate becomes necessary to explain this significant spectral change at the main transition to the Lα phase. Usually a conformational change in the glycerol moiety is assumed, which changes the orientation of the carbonyl tensor about an angle of at least 30°. However, no other independent evidence of this postulated process has been reported.

CONCLUSION

In summary, the model of a chain flip-flop, as introduced in our earlier publications (8, 9), is the key to a consistent view of the gel phase of lipid bilayers. If the flip-flop angle is allowed to deviate slightly from 90°, the asymmetric

shapes of the gel phase ^{2}H-NMR spectra of phospholipids are a natural consequence of this particular motion. The axial symmetry of the corresponding carbonyl spectra does not contradict this interpretation. On the contrary, the particular reduction of the chemical shift anisotropy could be the direct result of the hopping motion. Also the collapse of the anisotropy at the main phase transition is understood without further postulations. Finally, the proposed model is in agreement with results obtained by other methods, which deal with the existence of a two-dimensional lattice (34–40, 45), the *gauche* populations in the hydrocarbon chains (29–31), and the translational molecular mobility (41).

In some remotely related systems similar situations were found; we regard this as a support of our model. Also in the smectic-E phase of thermotropic liquid crystals, herringbone molecular packing seems to be linked with different types of intramolecular motion including a two-site hopping (48). Yet another case has been reported for the model bilayer $(C_{10}H_{21}NH_3)_2CdCl_4$ (49). In this system the molecular head is fixed in a two-dimensional lattice, and the chains perform a two-site flip-flop about their long axis.

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