Bent Fatty Acid Chains in Lecithin Bilayers

(phospholipid spin labels/lipid bilayers)

BETTY GAFFNEY McFARLAND AND HARDEN M. McCONNELL

Department of Chemistry, Stanford University, Stanford, California 94305

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ABSTRACT An analysis of the paramagnetic resonance spectra of a number of phospholipid spin labels in multilamellar arrays of lecithin bilayers has been carried out in terms of a distribution of label orientations, and the amplitude and time dependence of high-frequency random motions about these orientations. This analysis indicates that there is a long-lived (>10⁻⁸ sec) average bending of the fatty acid chains such that near the polar head groups the chains are comparatively rigid and tilted at about 30° relative to a normal to the bilayer plane, whereas near the terminal methyl groups the chains are flexible and on the average parallel to this normal. This bent conformation of the chains permits the packing of chains into planar lamellar arrays even when different segments of the chain have a di ferent motional freedom.

Studies of the paramagnetic resonance spectra of a number of spin labels (1) incorporated in intact biological membranes have provided evidence that (a) some membranes contain phospholipid bilayers (2-7) and (b) the hydrophobic fatty acid chains in these membrane bilayers exhibit rapid anisotropic motion, where the anisotropy of motion decreases rapidly with increasing distance from the polar head group (4, 5). Thus, the amount of local methylene-chain motion near the center of the bilayer can approach that of an isotropic liquid hydrocarbon, whereas in the same bilayer, the amount of local methylene-chain motion near the polar head group region can approach that of a solid, crystalline hydrocarbon (4, 5). Conclusion (a) has been based on a comparison of the observed orientations and motions of a number of spin labels in biological membranes (1-8) and in pure phospholipid bilayers (1-10). Conclusion (b) has been based on the exponential (or even more rapid) decrease of the order-parameter S as a function of n, the number of methylene groups in a polymethylene chain separating a paramagnetic oxazolidine ring from the carboxy group in the fatty acid spin labels I(m,n) for in the phospholipid spin labels II(m,n) when

one or the other of these labels is incorporated in smectic liquid crystals (11), in lecithin bilayers (5, 9, 11, 12), or in various biological membranes (4-8). The order-parameter S is a measure of the mean rotational amplitude of the paramagnetic oxazolidine ring: S = 1 when this ring has a fixed orientation in space, and S = 0 when this ring undergoes isotropic rotational motion with a correlation time that is less than about 10^{-9} sec. These conclusions are, of course, based on the behavior of nitroxide spin labels in model and biological membranes in which the paramagnetic nitroxide group necessarily represents a perturbation on the local membrane structure. However, several different labels with different structures do lead to the same conclusions. [For example, in the present work, labels I(m,n) and II(m,n) give semiquantitatively similar results.] Also, preliminary studies of proton relaxation in aqueous dispersions of (unlabeled) phospholipids appear to be consistent with these conclusions (S.I. Chan, Dept. of Chemistry, California Inst. of Technology, private communication). Furthermore, our results are entirely consistent with what is known about bilayers and membranes from x-ray diffraction (13, 14). We have therefore felt justified in proceeding to what appears to be the next problem in bilayer structure.

The rapidly increasing disorder of the fatty acid chains toward the center of phospholipid bilayers in biological membranes, and in multilamellar arrays of phospholipid bilayers, poses an interesting problem in the *packing* of phospholipids in planar membranes, where the bilayer is extended in two dimensions. One would expect that as the rotational freedom of each methylene group increases, it should occupy a larger effective volume. It is clear that in an essentially infinite two-dimensional lamellar array of phospholipids one could not have *parallel* arrays of fatty acid chains that were tightly packed in one region and "fluid" in another unless



the extra space in the tightly-packed region were taken up by other molecules, such as water. A more plausible solution to this packing problem involves a bending of the fatty acid chains, as suggested schematically in Fig. 1. Here a systematic bending of the chains yields a larger average interchain distance when the chains are perpendicular to the surface of the bilayer and a smaller interchain distance near the polar head groups.

This bending of the fatty acid chains can be achieved in several ways. *Gauche* conformations about carbon-carbon bonds as well as small partial rotations about carbon-carbon bonds can produce one or more changes in the direction of a saturated fatty acid chain. Unsaturated fatty acid chains with *cis* carbon-carbon double bonds have an intrinsic bend. It must be emphasized that the drawing in Fig. 1 is schematic, and that the chain bending envisioned here is only a statistical bending corresponding to an appropriate short-time average over isomeric conformations of the chains.

The possibility that there might exist an average or statistical bending of the fatty acid chains leads to two expectations. First, the intermolecular interaction giving rise to this bending must involve cooperative bending among a relatively large number of lipid molecules and thus, the lifetime of a bent state might very well be long, at least long compared to $\sim 10^{-8}$ sec. Secondly, it would be expected that spin labels such as II(m,n) should provide a qualitative or even semiquantitative measure of this chain bend. The second expectation is dependent on the first. If the lifetime of the direction of the bent state were too short—i.e., less than $\sim 10^{-8}$ sec—, then a bend would not be directly observable from the paramagnetic resonance of spin labels such as II(m, n), since rapid bending in various directions would average out the otherwise observable average tilt. In the work reported here, we have found that the paramagnetic resonance of labels such as I(m, n) and II(m, n) in oriented multilamellar films of lecithin can be interpreted in terms of a bend such as the one sketched in Fig. 1. That is, spin labels of this type show that the plane of the oxazolidine ring is parallel to the bilayer surface when this ring is near the terminal methyl group, whereas the plane of the oxazolidine ring is tilted at about 30° when the ring is near the polar head group. (The plane of the oxazolidine ring is perpendicular to the polymethylene chain when the chain is in its extended, all-trans conformation.) Subsequent sections of the present paper give a very brief summary of the technical details whereby we have extracted this information on the orientation of the oxazolidine ring in labels I(m, n) and II(m, n) when they are incorporated in planar multilayers of lecithin. A more detailed description of our analysis will be given in subsequent publications. In the last section of the present paper we consider briefly the biophysical implications of our results with respect to membrane structure.

MATERIALS

Methyl 5-ketopalmitate-4,4,6,6-d4

For the introduction of deuterium into methyl 5-ketopalmitate, 2 g of the ester was heated under reflux for 3 days in 50 ml of CH₃OD containing 1 ml of 38% DCl in D₂O. Evaporation to dryness gave methyl 5-ketopalmitate-4,4,6,6-d₄ (MW 288 by mass spectrometry).

2-Amino-2-methylpropanol-d₁₁

 α -Aminoisobutyric-d₆ acid was prepared from acetone-d₆ by the procedure employed for the undeuterated analog (15).



FIG. 1. Schematic, idealized representation of the packing of fatty acid chains in a small section of one half of a planar lecithin bilayer. This packing allows greater motional freedom at the end of the methylene ohains near the terminal methyl groups.

The acid was esterified with methanol and anhydrous hydrochloric acid. 2-Amino-2-methylpropanol- d_{11} was obtained by the addition of solid methyl α -aminoisobutyrate- d_6 hydrochloride to a 2.5-fold excess of lithium aluminum deuteride in anhydrous tetrahydrofuran. After 2 hr, sodium deuteroxide in deuterium oxide was added and the reaction was worked up in the usual manner.

The spin-labeled fatty acid methyl ester, deuterated at all carbon atoms β to the nitroxide, was prepared from 1 g of methyl 5-ketopalmitate-4,4,6,6-d₄ and 2 g of 2-amino-2-methylpropanol-d₁₁ as described earlier for the unlabeled analog (5). However, water was removed in this case by the addition of dicyclohexylcarbodiimide. The methyl ester, purified by preparative thin-layer chromatography (12), gave: C, 65.80; N, 3.71. Calcd for C₂₁H₂₈D₁₂O₄N: C, 65.93; N, 3.66. Mass-spectrometric analysis indicated a minimum of 89% deuterium incorporation at the β positions. The methyl ester was hydrolyzed as described elsewhere (5).

Acylation of egg lysolecithin with I(1,14) anhydride

Lysolecithin from egg yolk lecithin was esterified as previously described (5) with I(1,14) anhydride. 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl was from Syva Associates, Palo Alto. Multilamellar arrays of hydrated lecithin (containing approximately 25% water) were prepared by pressing about 5 mg of the sample between two optically flat quartz plates. An isotropic distribution of multilayers was obtained using two matched hemispherical Teflon surfaces. Label/lecithin mole ratios were $\sim 1/200$.

SPECTRAL ANALYSIS

Isotropic spectra

An analysis of the paramagnetic resonance line shapes of isotropic distributions of spin labels I(m, n) and II(m, n) in lecithin has been given previously (5). Here we briefly summarize this earlier analysis and indicate how it has been improved in the present work.

In standard notation, the spin Hamiltonian for the para-

magnetic oxazolidine ring in I(m, n) or II(m, n) is

$$\mathfrak{K} = |\beta| \mathbf{S} \cdot \mathbf{g} \cdot \mathbf{H}_0 + h \mathbf{S} \cdot \mathbf{T} \cdot \mathbf{I} - g_n \beta_n \mathbf{I} \cdot \mathbf{H}_0 \qquad (1)$$

The principal axes of the g-factor tensor \mathbf{g} and hyperfine tensor **T** coincide, and are designated z, x, y, where z is the axis corresponding to the largest hyperfine splitting and the smallest g-factor. The z-axis is perpendicular to the plane of the oxazolidine ring and is parallel to the polymethylene chain when this chain is extended (all-trans conformation). The principal axes z, x, y are molecule-fixed and the Hamiltonian 3C is time-dependent in the presence of molecular motion, since the applied field vector $\mathbf{H}_0 = H_0 \mathbf{h}$ is laboratory-fixed. The spectra of labels I(m,n) and II(m,n) in isotropic distributions of membranes can be approximated using an axially symmetric effective Hamiltonian x'. First approximations to the elements of the tensors $\mathbf{T'}$ and $\mathbf{g'}$ can be obtained directly from the observed isotropic spectra, and more refined estimates of the elements can be obtained from trial-and-error comparisons between observed spectra and theoretical computer-calculated spectra. In these comparisons, it was found that this agreement was good, but not perfect (5). The discrepancy was attributed to the Lorentzian line shape parameters having a line width independent of nuclear spin quantum number M, and independent of ϑ' , the angle between z'and **h**, where z' is the symmetry axis of \mathcal{K}' . In the present work we have attempted to improve on this approximation by using Lorentzian (derivative curve) peak-to-peak line-width parameters $\Delta(M)$ dependent on M and ϑ' , as follows.

$$\Delta(M) = \sum_{q} B_{q}(M) \left| \mathbf{h} \cdot \mathbf{T}'_{q} \cdot \mathbf{h} \right|^{2}$$
(2)

Here the \mathbf{T}'_q (or $\mathbf{h} \cdot \mathbf{T}_q \cdot \mathbf{h}$) are irreducible representations of the rotation group, of order two, and are defined below. The rationale for this choice is also given below.

Orientation distribution parameters

The effective Hamiltonian \mathfrak{K}' is anisotropic and axially symmetric. The symmetry axis is z' and the resonance spectrum of a given label depends on the angle ϑ' between z' and the direction of the applied field h. The composite resonance spectrum of a statistical ensemble then depends on the distribution of values of ϑ' . In the present work, the spin labels are contained in highly ordered multilamellar arrays of lecithin between two optically flat quartz plates. This method of sample preparation, as well as the complete optical extinction between crossed polaroids, shows that the distribution function $\rho(\vartheta)$ must be axially symmetric, where ϑ is the angle between z' and **N**, the normal to the quartz plates (and to the bilayer surfaces), and $\rho(\vartheta)d\vartheta$ is the probability that z' be found between ϑ and ϑ + d ϑ . For the present analysis we have assumed the following cylindrically symmetric distribution function.

$$\rho(\vartheta) \sim \sin \vartheta \exp\left\{-(\vartheta - \bar{\vartheta})^2/2\vartheta_0^2\right\}$$
(3)

Previous studies of the amphiphilic spin labels I(m, n) in biological membranes (4) and in lecithin multilayers (9), have shown the principal axis z' to be oriented more nearly perpendicular than parallel to the membrane surface. The distribution function $\rho(\vartheta)$ includes the parameter $\overline{\vartheta}$ to allow for a net tilt of z' relative to the normal **N** to the membrane surface, and also includes the *disorder parameter* ϑ_0 to allow for a spread in this tilt. [In an early discussion of the spectra of spin labels in multilamellar arrays of bilayers, Libertini *et al.* have used a distribution function similar to that in Eq. (3), except they did not include a tilt $(\bar{\vartheta})$ term (9). Also, this discussion did not treat high-frequency motions, either in producing an effective Hamiltonian, or in giving rise to angular-dependent relaxation.] With these parameters, the *most probable angle* between z' and **N** is ϑ_{mp} , which is the solution to the equation

$$\tan \vartheta_{\rm mp} = \vartheta_0^2 / (\vartheta_{\rm mp} - \overline{\vartheta}) \tag{4}$$

Contributions to ϑ_0 can be expected from heterogeneity of lipid composition (e.g., unsaturation of fatty acid chains).

Relaxation line-shape parameters

In the present approach to the calculation of the resonance spectra of spin labels in membranes, the exact time-dependent Hamiltonian is broken up into two parts, a time-independent axially symmetric effective Hamiltonian \mathfrak{K}' , and a time-dependent perturbation, $\{\mathfrak{K}(t) - \mathfrak{K}'\}$.

$$\mathfrak{K}(t) = \mathfrak{K}' + \{\mathfrak{K}(t) - \mathfrak{K}'\}$$
(5)

The perturbation $\{\mathfrak{K}(t) - \mathfrak{K}'\}$ broadens transitions between the stationary eigenstates of \mathfrak{K}' . We assume the correlation times describing the time-dependent fluctuations of $\{\mathfrak{K}(t) - \mathfrak{K}'\}$ to be sufficiently short that this perturbation can be treated by Bloch–Wangsness and Redfield theory (16). Our approach to the calculation of the relaxation parameters describing line broadening is thus similar to that used by Marshall and Glarum (17) for treating resonance spectra of free radicals in nematic liquid crystals, except that our calculation is necessarily more general since our Lorentzian linewidth parameter T_2^{-1} is anisotropic.

$$T_{2}^{-1} = (2\pi)^{2} \sum_{\substack{q = 0, \\ \pm 1, \pm 2}} (|\overline{D_{oq}}|^{2} - |\overline{D}_{oq}|^{2})|^{1}/_{2}\nu_{e}G_{0}(\mathbf{h} \cdot \mathbf{T}'_{q} \cdot \mathbf{h}) + A_{0}(\mathbf{h} \cdot \mathbf{T}'_{q} \cdot \mathbf{k}'')M|^{2}\tau_{oq} + (2\pi)^{2} \sum_{\substack{m \neq 0, q = 0, \\ \pm 1, \pm 2}} G_{m}^{2} |\overline{D_{mq}}|^{2} (1/_{4}\nu_{e}^{2})|\mathbf{h} \cdot \mathbf{T}'_{q} \cdot \mathbf{h}|^{2}\tau_{mq}$$
(6)

In the above equation, $D_{mq} \equiv D^{(2)}{}_{mq}(\alpha \beta \gamma)$ are the Wigner rotation matrices that transform the *instantaneous* molecular axes in $\mathcal{K}(t)$ to the time-average axes in \mathcal{K}' , and $|\overline{D}_{mq}|^2$ and $|\overline{D}_{mq}|^2$ denote appropriate time averages. The $\mathbf{T'}_{mq}$ (distinct from the hyperfine tensor \mathbf{T}') are irreducible tensors referred to the principal axes $\mathbf{k'}$, $\mathbf{j'}$, $\mathbf{i'}$ of $\mathbf{T'}$ and $\mathbf{g'}$ in \mathcal{K}' :

$$\mathbf{T'}_{\pm 2} = (\mathbf{i'} \pm i\mathbf{j'})(\mathbf{i'} \pm i\mathbf{j'})$$
(7)
$$\mathbf{i} = (-1)^{1/2}$$

$$\mathbf{T'}_{\pm 1} = \pm \mathbf{k'}(\mathbf{i'} \pm \mathbf{ij'}) \pm (\mathbf{i'} \pm \mathbf{ij'})\mathbf{k'}$$
(8)

$$\mathbf{T'_0} = (^2/_3)^{1/2} (3 \mathbf{k'} \mathbf{k'} - \mathbf{U})$$
(9)

The quantities G_m and A_m are measures of the anisotropy of the **g** and **T** tensors. In the present problem only A_0 , G_0 , and $G_{\pm 2}$ are non-zero:

$$A_0 = 6^{-1/2} (T_{zz} - 1/2 (T_{xx} + T_{yy}))$$
(10)

$$G_{\pm 2} = \frac{1}{4}(g_x - g_y) \tag{11}$$

In Eq. 6, **h** and \mathbf{k}'' are unit vectors in the external field direction and in the direction of nuclear spin quantization, respectively. The unit vector \mathbf{k}'' lies in the plane of **h** and \mathbf{k}' , and $\mathbf{h} \cdot \mathbf{k}'' = \cos \vartheta''$, where

$$\cos\vartheta'' = \frac{\cos^2\vartheta'T'_{\parallel} + \sin^2\vartheta'T'_{\perp}}{(\cos^2\vartheta'T'_{\parallel}^2 + \sin^2\vartheta'T'_{\perp}^2)^{1/2}} \qquad (12)$$

The τ_{mq} are correlation times for the functions $D_{mq} - \overline{D}_{mq}$. The angular dependencies of T_2^{-1} arise from the terms involving $\mathbf{h} \cdot \mathbf{T'}_q \cdot \mathbf{h}$ and $\mathbf{h} \cdot \mathbf{T'}_q \cdot \mathbf{k''}$. In our present calculations we have neglected the difference between $\mathbf{h} \cdot \mathbf{T'}_q \cdot \mathbf{h}$ and $\mathbf{h} \cdot \mathbf{T'}_q \cdot \mathbf{k''}$ in Eq. 2, and have neglected nonsecular and pseudo-secular contributions to the electron resonance line-widths. These approximations can be tested by resonance experiments at different field strengths.

Spectral analysis summary

The parameters giving the best fit of the calculated spectra to the observed spectra are summarized in Table 1, except for the line-width parameters, which will be given in a later publication devoted to the analysis of line shape. By varying all the parameters, we could obtain almost perfect agreement between calculated and observed spectra (including both the isotropic distributions of orientations, and spectra arising from all possible directions of the applied field relative to the lamellar arrays.) There appeared to be no strong "interaction" between the parameters of Table 1 and the line-shape parameters B_q . In other words, for various choices of the B_q within the range of acceptable calculated spectra, one obtains essentially the same range of parameters for Table 1. This is fortunate, since as yet we have no rigorous check on the adequacy of the line-shape parameters (see Eq. 2), other than the agreement between observed and calculated spectra. As indicated above, these line-width parameters are appropriate to an axially symmetric spin Hamiltonian together with high-frequency random time-dependent perturbations that can be accounted for using the general methods of Bloch-Wangsness and Redfield theory (16). It is very likely that this approximation is excellent for label II(1, 14), which shows three sharp ¹⁴N hyperfine lines at all orientations of the applied field relative to the bilayer plane. Indeed, the linewidth of each hyperfine component in the resonance spectrum of II(1, 14) was found to be proportional to a constant + $B[1/2(3\cos^2\vartheta'-1)]^2$. Thus, the line-width parameters given in Eq. 2 are entirely satisfactory for this label. For the other labels, where there are significant contributions to the overall line shapes arising from $\overline{\vartheta}$ and ϑ_0 effects, it is difficult to say whether or not Eq. 2 is a totally adequate representation of the line-shape parameters for the outer hyperfine lines. The line-shape parameters in Eq. 2 might be incomplete in some instances if (i) unresolved proton hyperfine structure were to contribute to the line widths, or if (ii) the approximation of using an axially symmetric Hamiltonian and a high frequency random perturbation were invalid, or if (iii) the pseudosecular terms were large. In cases (i) and (ii), one would have to include terms linear in $\mathbf{h} \cdot \mathbf{T'}_{q} \cdot \mathbf{h}$ in addition to the quadratic terms already included. An experiment with I(10, 3) fully deuterated at all carbon atoms β to the nitroxide showed that in this case the contribution of unresolved proton hyperfine structure to the line-width was negligible. Possible contributions of the type (ii) and (iii) can best be tested with resonance spectra at different field strengths. Approximation (ii) is certainly valid for I(1, 14) and II(1, 14). On the other hand, it is difficult to see how (ii) could be entirely valid for II(10, 3), which has such a well-defined tilted orientation of the oxazolidine ring (see Table 1). However, approximation (ii) is really not essential for the most important features of the resonance spectrum, namely the outer wings, which do not depend on (small) deviations of \mathcal{K} or \mathcal{K}' from axial symmetry.

TABLE 1. Spectral parameters for phospholipid spin labels II(m, n) in egg lecithin bilayers

(m, n)	T'_{ii}	T'_{\perp}	$g'_{ } - g'_{\perp}$	\boldsymbol{S}	v	θo	_{Յաթ}
(10, 3)	27.2	8.9	-0.0036	0.68	25-30°	5–10°	29-32°
(7, 6)	24.0	10.5	-0.0034	0.50	0-30°	30–25°	28-31°
(5, 10)	20.0	11.5	-0.0024	0.33	0-10°	15–20°	14–25°
(1, 14)	16.8	12.8	-0.0013	0.16	0°	5°	5°

 $T'_{\,\,\rm l}{},\,T'_{\,\,\rm L}$ are in gauss. The various symbols are explained in the text.

BIOPHYSICAL CONCLUSIONS

As pointed out above, the nonuniform rotational freedom of the methylene groups of the fatty acid chains in phospholipid bilayers poses a problem in the packing of these chains. This problem could be resolved if the average methylene-chain direction near the terminal methyl groups were normal to the bilayer surface whereas the average chain direction were tilted relative to this normal near the polar head groups. This expectation is borne out in the present work to the extent that in the phospholipid spin labels II(m, n) with (m, n) = (1, 14), (5, 10), (7, 6), (10, 3), the most probable angles between the plane of the oxazolidine ring and the bilayer plane were found to be approximately 5°, 20°, 30°, and 31° (Table 1). Because the spin labels II(m, n) are certainly not identical to the corresponding unlabeled phospholipids, we can only say that our results support the idea of statistically bent chains (illustrated schematically in Fig. 1), and it can hardly be claimed that we have demonstrated this bending conclusively for unlabeled chains. On the other hand, a tilted conformation of the labels II(m, n) is established by the present work, and this does show conclusively that the polar head-group region has a "collective" structure with a lifetime long compared to ca. 10^{-8} sec; otherwise the resonance experiment would only detect an average orientation of the oxazolidine ring which would necessarily be parallel to the bilayer surface. A tilt of 30° near the polar head groups produces a carbon atom density that is $\sim 12\%$ higher than the carbon atom density near the terminal methyl groups, and this is just the order of magnitude of the difference in density between liquid hexadecane (density = 0.77 g/ml at 20°C) and solid paraffin (0.88), or between liquid methyl arachidonate (0.901) and crystalline methyl stearate (1.009) determined from unit cell dimensions (19).

A tilted conformation of the fatty acid chains in the polar head group region has at least two significant biophysical implications. First, there is an evident mechanism for a cooperative interaction between amphiphilic molecules that bind to bilayers. These molecules may disrupt this tilted structure locally at the site of binding, and by a "domino effect" produce a relatively long-range interaction between distant binding sites. Second, it is also clear that a bilayer is suited to the binding of an amphiphilic molecule that partially penetrates the bilayer. Under these circumstances, the fatty acid chains could then be on the average perpendicular to the bilayer surface throughout their length and thus provide extra space in the head-group region. It will also be seen that if some of the fatty acid chains in Fig. 1 are rotated by 180° about an axis perpendicular to the bilayer surface, it is possible to create amphiphilic as well as purely hydrophobic pockets in the bilayer. These pockets might serve to bind

amphiphilic as well as hydrophobic molecules such as proteins.

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