Figure 5). It should, however, be noted that the distributions $P(E_T)$ and $P^*(E_T)$ based on such a model underestimate the translational energy of the photofragments.

Conclusions

The photodissociation of formaldazine at 193 nm has been investigated under collisionless conditions using photofragment translational spectroscopy. The only primary dissociation reaction is the production of two H_2CN radicals in coincidence. No evidence was found for H atom elimination, formation of methylene and azomethane, or unimolecular disproportionation. Recoil anisotropy measurements of the primary photofragments clearly show that dissociation occurs promptly, i.e., within a rotational period of the parent molecule, and therefore a long-lived excited formaldazine molecule, which has been proposed previously,[17] can be excluded. Furthermore, the N-N bond breaking process is indicated to proceed via a predissociation mechanism as evidenced by the surprisingly low fragment translational energy release.

The decay of a molecule into two chemically identical fragments is an interesting process owing to the symmetry with respect to fragment interchange. Detailed studies of H_2O_2[37] and $(CN)_2$[38] have been carried out by spectroscopic probing of the diatomic photofragments OH and CN, respectively. Formaldazine is a more complicated system and spectroscopic detection of nascent H_2CN photofragments by laser-induced fluorescence techniques is apparently impeded by the predissociation of the upper electronic states.[39] Under these conditions the method of photofragment translational spectroscopy turned out to be particularly useful: in addition to establishing that cleavage of the N-N bond is the exclusive primary reaction, it allowed us a coarse mapping of the internal energy pair distribution $P(E_A,E_B)$. Furthermore, it could be shown that approximately 80% of the primary photofragments contain enough internal energy for a secondary decay to HCN and H atoms. Hence, photodissociation of formaldazine at 193 nm produces mainly H atoms and HCN. For efficient generation of stable H_2CN radicals the high internal energy of H_2CN can be reduced by using a longer wavelength photolysis (e.g. 248 nm) or collisional energy transfer to a buffer gas.

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Characterization of the Orientation and Ordering of Fatty Acid Pyrrolidine Nitroxyls in Lipid Bilayers

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The electron spin resonance (ESR) spectra of a series of fatty acids bearing the paramagnetic pyrrolidin-*N*-oxyl ring have been studied in oriented bilayers of dimyristoylphosphatidylcholine:cholesterol (70:30 mol/mol) at pH 9.0. Nitroxyl fatty acids with the alkylcarboxy and alkyl substituents located respectively at the 2,2-, 3,2-, 2,4-, or 2,5-positions of the pyrrolidine ring were used. These disubstitutions correspond to different modes of incorporation of the nitroxyl ring into the chain. The order parameter of the nitroxyl *z* axis (principal hyperfine tensor element A_{zz} = 32 G), S_{NO}, was determined from the angular dependence of ESR spectra recorded at temperatures that were demonstrated to be in the motional narrowing regime. Combination of S_{NO} with the segmental order parameters of the chain axis, S_{mol}, allowed determination of the orientation, θ, of the nitroxyl *z* axis with respect to the chain diffusion axis in fluid lipid bilayers. The values of S_{mol} were obtained from the ESR spectra of the appropriate positional isomers of *n*-(4,4-dimethyloxazolidin-*N*-oxyl)stearic acid, for which $\theta = 0$. Values of $\theta = 0$, 90, 40 ± 5, and 65 ± 5° were obtained for the 2,2-, 2,4-, 2,5-, and 3,2-disubstituted pyrrolidine fatty acids, respectively. Comparison with molecular modeling suggests that the rotational diffusion axis lies parallel to the fatty acid trans-chain axis for all molecular conformations except that of the 2,5-disubstituted fatty acid. This spectroscopic characterization of the pyrrolidine-based fatty acids is necessary for their use as lipid spin label probes in biological membranes.

Introduction

Electron spin resonance (ESR) spectroscopy of spin-labeled lipid molecules has proved to be a very useful means of studying molecular dynamics in biological membranes.[1-4] Commonly used lipid spin labels are the 4,4-dimethyloxazolidin-*N*-oxyl derivatives in which the nitroxyl ring is attached stereospecifically at the 2,2 position to a methylene segment of the hydrocarbon chain. These spin labels have the property that the nitroxyl *z* axis lies parallel to the chain segment axis, and hence the spectra are optimally sensitive to trans-gauche rotational isomerism in the lipid chains. The spin label group does, however, project out from the lipid chain in these labels, which potentially could give rise to unfavorable steric interactions.

A type of "minimal perturbation" spin-labeled fatty acid probe has been introduced by Lee et al.,[5] in which the nitroxyl ring is

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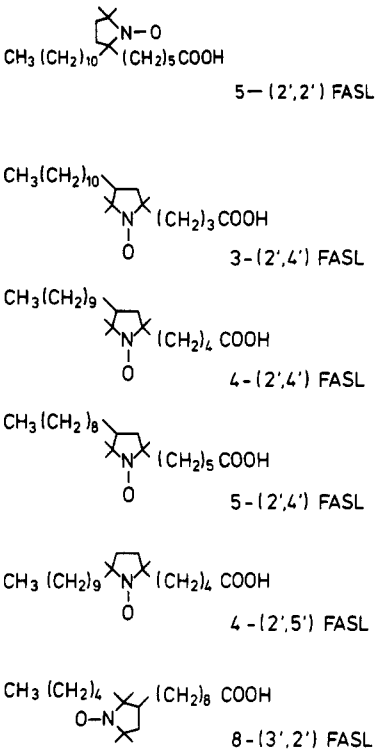

Figure 1. Structures of the pyrrolidine-based nitroxyl fatty acids used in this study.

incorporated as an integral part of the hydrocarbon chain. These azethoxyl fatty acids contain the nitroxyl ring by attachment of the chain at the 2 and 5 positions of the pyrrolidine ring [*n*''-(2',5')FASL in the abbreviated nomenclature used here]. The different orientation of the nitroxyl ring also means that these labels are sensitive to different modes of chain rotation from those of the more common oxazolidine labels.[6]

In the present work, we have characterized the ESR spectroscopic behavior of a series of six different pyrrolidine-based fatty acid spin labels in oriented lipid bilayer model membranes. The nitroxyl fatty acids investigated are the 2,2-, 2,4-, 2,5-, and 3,2-disubstituted alkyl derivatives of 2,2,5,5-tetramethylpyrrolidin-*N*-oxyl (see Figure 1). These differ in the manner in which the nitroxyl ring is incorporated in the chain and also, in the case of the 2,4 substituents, in the length of the linkage between the ring and the carboxyl anchor group. Previously, these spin label probes have been shown to be well suited for the study of lipid–protein interactions in biological membranes.[7,8] The advantages lie in the different ways in which the nitroxyl group is integrated in the chain. This makes it possible to verify that the lipid–protein interactions observed are not influenced unduly by the structure of the spin label reporter group and also to investigate the effects of lipid–protein interactions on different modes of chain motion.

A knowledge of the spectral and dynamic properties of these spin labels, in particular of the orientation of the nitroxyl group relative to the chain diffusion axis, is a necessary requirement for the detailed interpretation of experiments on biological systems. This characterization is provided by the ESR studies on oriented model membranes reported here. The orientation of the nitroxyl *z* axis relative to the chain axis has been determined in fluid lipid bilayer membranes by comparison of the order parameters with those of the corresponding positional isomers of the oxazolidine-based spin labels for which the orientation is known. This strategy is necessary to provide data relevant to natural membrane systems, since it has been demonstrated previously that the nitroxyl orientation can be sensitive to the host environment of the probe.[6] Data obtained at low temperatures, however, suggest that the same nitroxyl orientation as determined in the fluid phase is also preserved in more rigid lipid bilayers, for at least some of the spin labels studied here. Finally, comparison with molecular modeling allows estimation of the orientation of the principal diffusion axis relative to the molecular axes of the fatty acid chain.

Experimental Section

Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland) and cholesterol was from Merck (Darmstadt, F.R.G.). The oxazolidine-based positional isomers of nitroxyl stearic acid, *n*-(4,4-dimethyloxazolidin-*N*-oxyl)stearic acid (*n*-SASL), were synthesized essentially as described in ref 9. The pyrrolidine-based nitroxyl fatty acids 5,5-dimethyl-2-undecyl-2-(5-carboxypentyl)pyrrolidin-*N*-oxyl [5-(2',2')FASL], 2,5,5-trimethyl-4-undecyl-2-(3-carboxypropyl)pyrrolidin-*N*-oxyl [3-(2',4')FASL], 2,5,5-trimethyl-4-decyl-2-(4-carboxybutyl)pyrrolidin-*N*-oxyl [4-(2',4')FASL], 2,5,5-trimethyl-4-nonyl-2-(5-carboxypentyl)pyrrolidin-*N*-oxyl [5-(2',4')FASL], 2,5-dimethyl-5-decyl-2-(4-carboxybutyl)-pyrrolidin-*N*-oxyl [4-(2',5')FASL], and 2,5,5-trimethyl-2-pentyl-3-(8-carboxyoctyl)pyrrolidin-*N*-oxyl [8-(3',2')FASL] were synthesized as described in refs 10 and 11. The two stereoisomers of the pyrrolidine-based fatty acids were not specifically resolved, although examination of the synthetic routes involved indicates that they should yield predominantly the trans isomer. The analysis given here of the ESR spectra of these nitroxyl fatty acids in oriented lipid bilayers suggests that they are characteristic of the trans isomer. Additionally, comparison has been made previously with a shorter chain analogue of the 5-(2',4')FASL fatty acid, for which the stereoisomers could be resolved. The trans isomer of 2,5,5-trimethyl-4-hexyl-2-(5-carboxypentyl)pyrrolidin-*N*-oxyl yielded ESR spectra that were very similar to those obtained for the 5-(2',5')FASL fatty acid in the same system.[7,8]

Sample Preparation. Oriented lipid bilayers containing the various nitroxyl fatty acids were prepared by depositing a solution of the desired lipid mixture (containing 1 mol % nitroxyl fatty acid) in CH_2Cl_2 in a specially fabricated open flat quartz cell (inner dimensions 29 × 7 × 0.5 mm). The solution was gently evaporated in a stream of dry nitrogen and subsequently dried under vacuum for ≥3 h. The dry film was then carefully hydrated with a buffer containing 0.1 M KCl, 2 mM boric acid, pH 9.0. This latter operation is critical for maintaining a well-oriented lipid bilayer. Excess buffer was then removed, and the cell sealed with a flat quartz plate. Random lipid bilayer dispersions were prepared by suspending the dried lipid in the buffer and vortex mixing above the phase transition temperature. Working at pH 9.0 ensures that the fatty acid ($pK_a \approx 6.7$ in DMPC/cholesterol bilayers[12]) is solely in the deprotonated state.

ESR Measurements. ESR spectra were recorded on a Varian E-Line 9-GHz spectrometer with a horizontally mounted rectangular TE102 microwave cavity. The entire cavity was thermostated by nitrogen gas flow. The angular orientation of the plane of the sample with respect to the magnetic field was determined by using a coaxial vernier system attached to the sample cell. Spectra recorded at high temperature were simulated by using the program described in ref 13, which applies to fast anisotropic rotation in an orienting potential.

The order parameter of the nitroxyl group for ESR spectra in the fast motional regime was calculated from the following expression:[1,2,9]

$$S_{NO} = (A_{\parallel} - A_{\perp})/[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})](a_0'/a_0) \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the hyperfine splitting constants measured from the baseline crossing points in the spectra of the oriented

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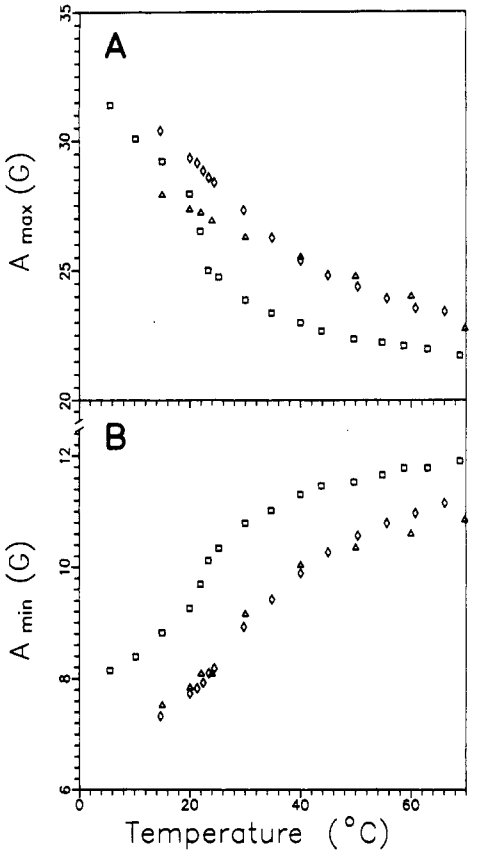

Figure 2. Temperature dependence of (A) the maximum and (B) the minimum hyperfine splittings (A_{max} and A_{min}, respectively) of 7-SASL nitroxylstearic acid in unoriented dispersions of DMPC (□) and of DMPC:cholesterol 70:30 mol/mol (◇), compared with that of the hyperfine splitting constants determined from oriented DMPC:cholesterol (70:30 mol/mol) bilayers (△) with the magnetic field directed (A) parallel and (B) perpendicular to the bilayer normal.

samples with the magnetic field direction parallel and perpendicular, respectively, to the bilayer normal. The hyperfine tensors of the nitroxyl group were taken from doxylpropane[14] [(A_{xx}, A_{yy}, A_{zz}) = (5.9, 5.4, 32.9 G)] for the *n*-SASL nitroxylstearic acids and from 3-oxo-2,2,5,5-tetramethylpyrrolidin-1-oxyl[15] [(A_{xx}, A_{yy}, A_{zz}) = (4.7, 4.7, 31.0 G)] for the pyrrolidine-based nitroxyl fatty acids. The *z* principal axis of the nitroxyl group lies along the nitrogen 2pπ orbital of the NO group and the *x* principal axis along the N–O bond.[15,16] The isotropic hyperfine splitting constant in the lipid bilayer is $a_0 = {}^1/_3(A_\parallel + 2A_\perp)$, and that in the crystalline host in which the hyperfine tensors were determined is $a_0' = {}^1/_3(A_{xx} + A_{yy} + A_{zz})$.

Results and Discussion

Motional Narrowing and Degree of Orientation. The temperature dependence of the principal hyperfine splittings of 7-SASL nitroxylstearic acid in oriented bilayers and in random bilayer dispersions of DMPC + 30 mol % cholesterol are given in Figure 2. Measurements on random dispersions of DMPC are also included for comparison. These experiments were performed to provide an experimental check both on the degree of orientation of the oriented bilayers and on the temperature required for achieving the fast motional limit of conventional nitroxyl ESR spectroscopy in this system. (A theoretical test is provided later by spectral simulation.) The values of A_{max} correspond to half the maximum hyperfine splitting between the outer peaks in the ESR spectrum of the random dispersion and to half the separation between the baseline crossing points of the $m_I = \pm 1$ manifolds in the spectrum of the oriented bilayers with the magnetic field parallel to the bilayer normal. In the fast motional regime, these values should both correspond to the $A_\parallel$ principal component of the motionally averaged axial hyperfine tensor. If the spectra are not in the fast motional limit, the slow motional effects will influence the spectra from oriented bilayers and random dispersions in different ways and the two values of A_{max} will not coincide. Additionally, if the bilayers are not well oriented, the misoriented parts will cause distortions in the baseline crossing positions, resulting in a lack of agreement between the two values of A_{max}. The agreement of the two values at the higher temperatures (Figure 2A) therefore indicates both that the bilayers are well ordered and that the motions are in the fast regime in this temperature range. Similar results were obtained from comparison of the temperature dependence of the spectra of the pyrrolidine nitroxyl fatty acids in oriented bilayers and in random dispersions (data not shown). Therefore, the measurements reported subsequently were performed at 70 °C, so as to be in the fast motional regime. Essentially similar results are found from the measurements of A_{min}, which corresponds to half the separation between the inner hyperfine splitting in the unoriented sample and between the baseline crossing points in the oriented sample with the magnetic field oriented perpendicular to the bilayer normal. In the latter case, A_{min} corresponds to the $A_\perp$ principal hyperfine tensor element, but in the former case a correction is necessary[1,2,9] that accounts for the lack of exact agreement in Figure 2B.

TABLE I: Angular Orientation, θ, of the Nitroxyl *z* Axis with Respect to the Rotational Diffusion Axis for the FASL Pyrrolidine Fatty Acids, Deduced from Comparison with the Positional Isomers of *n*-SASL, and the Euler Angles (Φ, θ, ψ) Used in the Spectral Simulation

nitroxyl	equivalent *n*	θ, deg	(Φ, θ, ψ), deg
5-(2′,2′)FASL	7	0	
3-(2′,4′)FASL	5 ± 1	90	(162, 90, 18)
4-(2′,4′)FASL	6 ± 1	90	(162, 90, 18)
5-(2′,4′)FASL	7 ± 1	90	(162, 90, 18)
4-(2′,5′)FASL	6 ± 1	40 ± 5	(0, 42, 0)
8-(3′,2′)FASL	11 ± 1	65 ± 5	(0, 65, 0)

Oriented ESR Spectra and Simulations. Representative ESR spectra of three of the pyrrolidine nitroxyl fatty acids in oriented bilayers of DMPC + 30 mol % cholesterol at 70 °C are given in Figure 3. Cholesterol was included in the bilayers in order to increase the molecular ordering of the lipid chains and hence also the spectral anisotropy (cf. Figure 2), so as to improve the precision with which the orientation of the nitroxyl axes could be determined. The different orientations of the nitroxyl *z* axes relative to the rotational diffusion axis for the three pyrrolidine fatty acids are immediately clear from the figure. The 4-(2′,5′)FASL fatty acid (Figure 3A) has a larger hyperfine splitting with the magnetic field oriented parallel to the bilayer normal than with the magnetic field oriented perpendicular to the bilayer normal. This means that the nitroxyl *z* axis is preferentially oriented toward the chain diffusion axis for this fatty acid. For the 4-(2′,4′)FASL fatty acid (Figure 3C), the opposite is the case, indicating that the nitroxyl *z* axis is oriented preferentially perpendicular to the chain diffusion axis. For the 8-(3′,2′)FASL fatty acid (Figure 3B) the hyperfine splittings are rather similar for both orientations of the magnetic field, suggesting that the nitroxyl *z* axis is oriented close to the magic angle [$(3\cos^2\theta - 1) \approx 0$, i.e., $\theta \approx 54.7°$].

The dashed lines in Figure 3 are simulations of the spectra using motional narrowing theory for anisotropic nitroxyl rotation in an orienting potential[13] and assuming a zero orientational spread for the director axis. That the spectra can be simulated reasonably well with this model indicates both that the spectra are in the fast motional regime and that the orientational spread of the bilayers is rather small, i.e., that they are well oriented. This result is in full accord with the experimental test given above. Simulation of the spectra requires a knowledge of the Euler angles (Φ,θ,ψ)[17]

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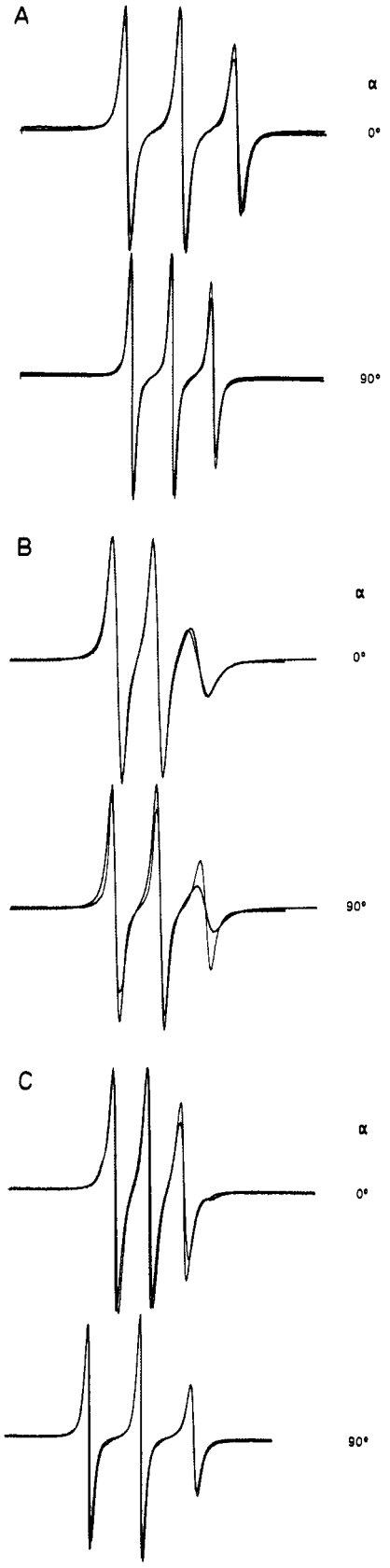

Figure 3. ESR spectra recorded at 70 °C of (A) 4-(2′,5′)FASL, (B) 8-(3′,2′)-FASL, and (C) 4-(2′,4′)FASL nitroxyl fatty acids in oriented bilayers of DMPC:cholesterol (70:30 mol/mol) at pH 9.0. Upper and lower rows correspond to spectra for the magnetic field oriented parallel ($\alpha = 0°$) or perpendicular ($\alpha = 90°$), respectively, to the bilayer normal, in each case. Full lines, experimental spectra; dotted lines, spectra simulated by using motional narrowing theory[13] with the Euler angles for the nitroxyl orientation given in Table I. Maximum spectral width = 100 G.

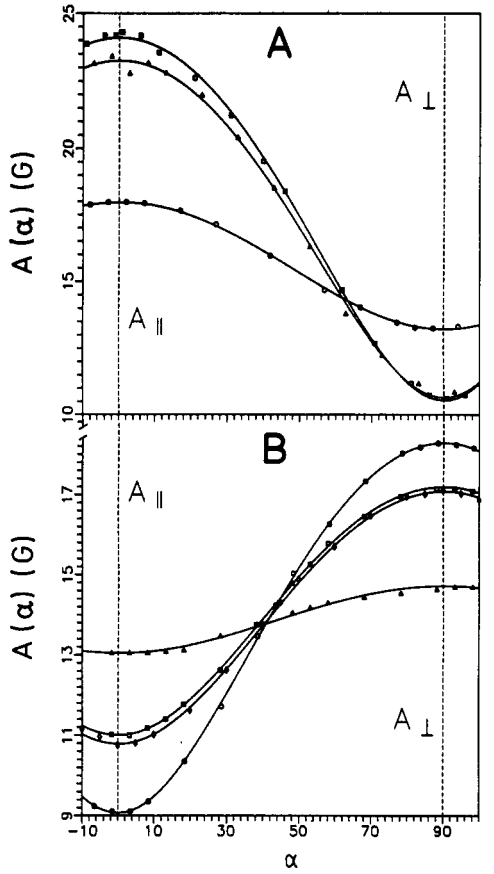

Figure 4. Angular dependence of the hyperfine splitting, $A(\alpha)$, of the different nitroxyl fatty acids in oriented bilayers of DMPC:cholesterol (70:30 mol/mol) pH 9.0 at 70 °C. α is the angle between the magnetic field and the bilayer normal. A: (○) 4-(2′,5′)FASL; (□) 5-(2′,2′)FASL; (△) 7-SASL. (B) (○) 3-(2′,4′)FASL; (□) 4-(2′,4′)FASL; (◇) 5-(2′,4′)FASL; (△) 8-(3′,2′)FASL. Full lines are the angular dependences calculated according to eq 2.

relating the nitroxyl principal axes to the chain diffusion axis. These were obtained as described below and are listed in Table I, given also below. The refinement of these values by simulation further supports the determination of the orientation of the nitroxyl axes given later.

Dependence on Magnetic Field Orientation. The angular dependences of the hyperfine splitting, $A(\alpha)$, for the six different pyrrolidine-based nitroxyl fatty acids and for 7-SASL, in oriented bilayers of DMPC + 30 mol % cholesterol at 70 °C, are given in Figure 4. It is seen that the dependence with respect to the orientation of the magnetic field, α, relative to the bilayer normal is very well described by the expression predicted theoretically for motional narrowing:[1,18]

$$A(\alpha) = (A_{\parallel}^2 \cos^2 \alpha + A_{\perp}^2 \sin^2 \alpha)^{1/2} \qquad (2)$$

This good agreement gives further evidence that the bilayers are well ordered and that all the nitroxyl fatty acid ESR spectra are in the fast motional regime at 70 °C. The angular dependence shows that the various nitroxyl fatty acids can be divided into two groups according to the orientation of the nitroxyl axes, as represented by parts A and B of Figure 4, respectively. One group comprises the fatty acids 4-(2′,5′)FASL, 5-(2′,2′)FASL, and 7-SASL for which $A_{\parallel} > A_{\perp}$, corresponding to an orientation of the nitroxyl z axis with $\theta < 54.7°$ (Figure 4A). The other group comprises the fatty acids 3-(2′,4′)FASL, 4-(2′,4′)FASL, 5-(2′,4′)FASL, and 8-(3′,2′)FASL for which $A_{\parallel} < A_{\perp}$, corresponding to an orientation of the nitroxyl z axis with $\theta > 54.7°$ (Figure 4B).

The result that $A_{\parallel} > A_{\perp}$ for 4-(2′,5′)FASL confirms that this nitroxyl fatty acid corresponds to the trans isomer. Previous studies

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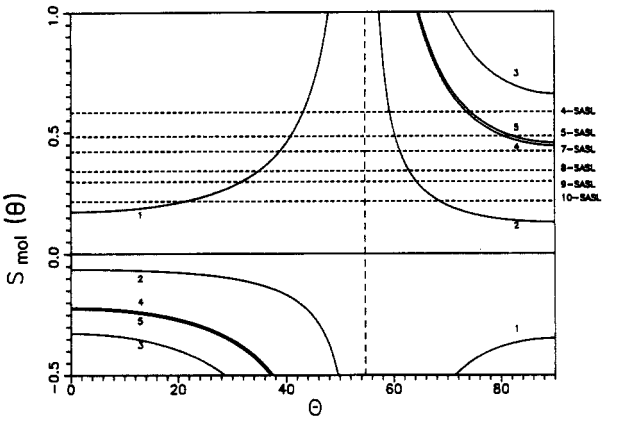

Figure 5. Chain order parameter, S_{mol}, calculated by using eq 3 from the measured hyperfine splittings of the pyrrolidine-based fatty acids, as a function of the angle, θ, between the nitroxyl z axis and the chain diffusion axis. Oriented bilayers of DMPC:cholesterol (70:30 mol/mol) pH 9.0 at 70 °C. Full lines: (1) 4-(2′,5′)FASL; (2) 8-(3′,2′)FASL; (3) 3-(2′,4′)FASL; (4) 4-(2′,4′)FASL; (5) 5-(2′,4′)FASL. Dashed horizontal lines give the data from the nitroxylstearic acids, n-SASL, assuming a constant $\theta = 0°$; from top to bottom: 4-SASL, 5-SASL, 7-SASL, 8-SASL, 9-SASL, 10-SASL.

with similar analogues in oriented egg phosphatidylcholine bilayers have shown that $A_{\parallel} > A_{\perp}$ for the trans isomer, whereas $A_{\parallel} < A_{\perp}$ for the cis isomer.[6]

Order Parameters and Nitroxyl Orientation. The principal values, $A_{\parallel}$ and $A_{\perp}$, of the motionally averaged axial hyperfine tensor obtained from Figure 4 at 70 °C can be used to determine the order parameter of the nitroxyl z axis, S_{NO}, by means of eq 1. The segmental order parameter, S_{mol}, of the chain axis is related to this by[3,19]

$$S_{mol} = S_{NO}/[\tfrac{1}{2}(3\cos^2\theta - 1)] \qquad (3)$$

where θ is the angle between the nitroxyl z axis and the chain diffusion axis, and axial symmetry of the nitroxyl order tensor is assumed. The values of S_{mol} calculated as a function of θ for the different nitroxyl fatty acids in DMPC + 30 mol % cholesterol are given by the full lines in Figure 5. The segmental order parameters of different chain positional isomers of n-SASL (for which $\theta = 0$) are indicated by the dashed horizontal lines in the figure, for comparison. This allows determination of the range of values of θ for the different pyrrolidine-based fatty acids that give consistent values of S_{mol} with those determined from the n-SASL fatty acids. In spite of uncertainties as to the exact positional isomer of n-SASL to which the various pyrrolidine fatty acids correspond, the steepness of the dependence of S_{mol} on θ and the limiting values of S_{mol} in Figure 5 allow the orientation of the nitroxyl axes of the pyrrolidine fatty acids to be calibrated from the n-SASL data with a reasonably high degree of precision.

The rigid nature of the pyrrolidine ring makes it most natural to associate the value of n corresponding to the n-SASL fatty acids with the carbon atom to which the pyrrolidine ring is attached on the carboxy side. These equivalent values of n are listed in Table I. An uncertainty of approximately ±1 is included to allow for on one hand the possibility that the ring hinders rotation about the C–C bond linking the ring to the chain and on the other hand that the ring itself is probably located deeper in the bilayer than the point of attachment. In this way, it is seen that 4-(2′,4′)FASL and 5-(2′,4′)FASL must possess the limiting values of $\theta = 90°$ and by analogy that this must also hold true for 3-(2′,4′)FASL. That all three of these pyrrolidine fatty acids have the same orientation of the nitroxyl z axis relative to the chain diffusion axis is reasonable in view of the similarity in their structures (cf. Figure 1). The equivalent value of n for 4-(2′,5′)FASL is 6 ± 1, and since the calculated value of S_{mol} has a rather steep dependence on θ in this region, the orientation of the nitroxyl axes can be determined reasonably accurately: $\theta \approx 40 \pm 5°$. Similarly for 8-(3′,2′)FASL, the nitroxyl orientation is estimated as $\theta \approx 65 \pm 5°$. As noted previously, the lower limit for the latter lies close to the magic angle ($\theta = 54.7°$).

These values deduced for the orientation of the nitroxyl z axis in the various pyrrolidine-based fatty acids are summarized in Table I. Data for 5-(2′,2′)FASL is also included in the table. This pyrrolidine derivative has exactly the same mode and position of attachment of the nitroxyl ring as for 7-SASL (i.e., $n = 7$), and the near coincidence of the angular dependences of the hyperfine splittings for both (Figure 4A) confirms that $\theta = 0°$ for 5-(2′,2′)FASL. The values of S_{mol} and θ deduced from Figure 5 form the basis for the spectral simulations presented in Figure 3. The values for the Euler angles defining the orientation of the nitroxyl axes relative to the chain diffusion axis are given in Table I. The agreement between the experimental and simulated spectra provide further support for the values of θ deduced from Figure 5.

A further check on the values deduced for the nitroxyl orientation, θ, is that they should be independent of temperature, whereas the values of S_{mol} should decrease with increasing temperature, corresponding to the increasing segmental flexibility of the chains. The validity of this prediction is demonstrated in Figure 6. The temperature dependence of the order parameter of the nitroxyl axes, S_{NO}, is given in Figure 6A, and the temperature dependences of S_{mol} deduced from eq 3 with the fixed values of θ given in Table I are compared with those from the n-SASL isomers in Figure 6B. Whereas S_{NO} may take positive or negative values, depending of the orientation of the nitroxyl axes of the particular pyrrolidine fatty acid, the values deduced for S_{mol} are all positive, as expected on physical grounds. Further, the temperature dependences of S_{mol} deduced from the pyrrolidine-based fatty acids agree quite closely with those deduced from the corresponding positional isomers of n-SASL, throughout the higher temperature range for which the motional narrowing limit holds (cf. Figure 2). These results confirm that spectra of the pyrrolidine-based fatty acids are characterized by the temperature-independent values of θ given in Table I.

Low-Temperature Orientation. The above determinations of the nitroxyl orientation refer to the fluid lipid bilayer state. Different orientations have been observed for n''-(2′,5′)FASL analogues in fluid lipid bilayers from those in occlusion crystals.[6] Therefore, it must not necessarily be the case that the same orientation as in fluid bilayers is preserved at lower temperatures for which the lipid environment is more similar to that of gel-phase bilayers. This has been checked for the $\theta = 90°$ and $\theta = 0°$ nitroxyl fatty acids that might be expected to display well-defined oriented spectra in the absence of rapid rotational diffusion about the chain axis, if the orientation is the same as that at higher temperatures.

The temperature dependence of the ESR spectra of 4-(2′,4′)-FASL in oriented DMPC + 30 mol % cholesterol bilayers is given in Figure 7. At 40–70 °C, the ESR spectra with the magnetic field oriented perpendicular to the bilayer normal (Figure 7A) consist of three first-derivative lines, corresponding to axial averaging of the hyperfine tensor anisotropy. At 15 °C, the spectrum is no longer characteristic of axial averaging of the principal elements of the hyperfine tensor. With the magnetic field oriented perpendicular to the bilayer normal, the spectrum is a two-dimensional powder pattern with a maximum outer hyperfine splitting of ≈62 G, which indicates that the nitroxyl z axis lies in the plane of the bilayer.[20] Therefore, for this particular fatty acid, the $\theta = 90°$ orientation is still maintained at low temperatures, for which the chain motion is slow on the conventional nitroxyl ESR time scale. The ESR spectra with the magnetic field oriented parallel to the bilayer normal (Figure 7B) are also consistent with this interpretation. As the temperature is de-

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(20) It should be noted that the chain tilt present in gel-phase phosphatidylcholine bilayers is removed on the addition of cholesterol (Ladbrooke, B. D.; Williams, R. M.; Chapman, D. *Biochim. Biophys. Acta* **1968**, *150*, 333).

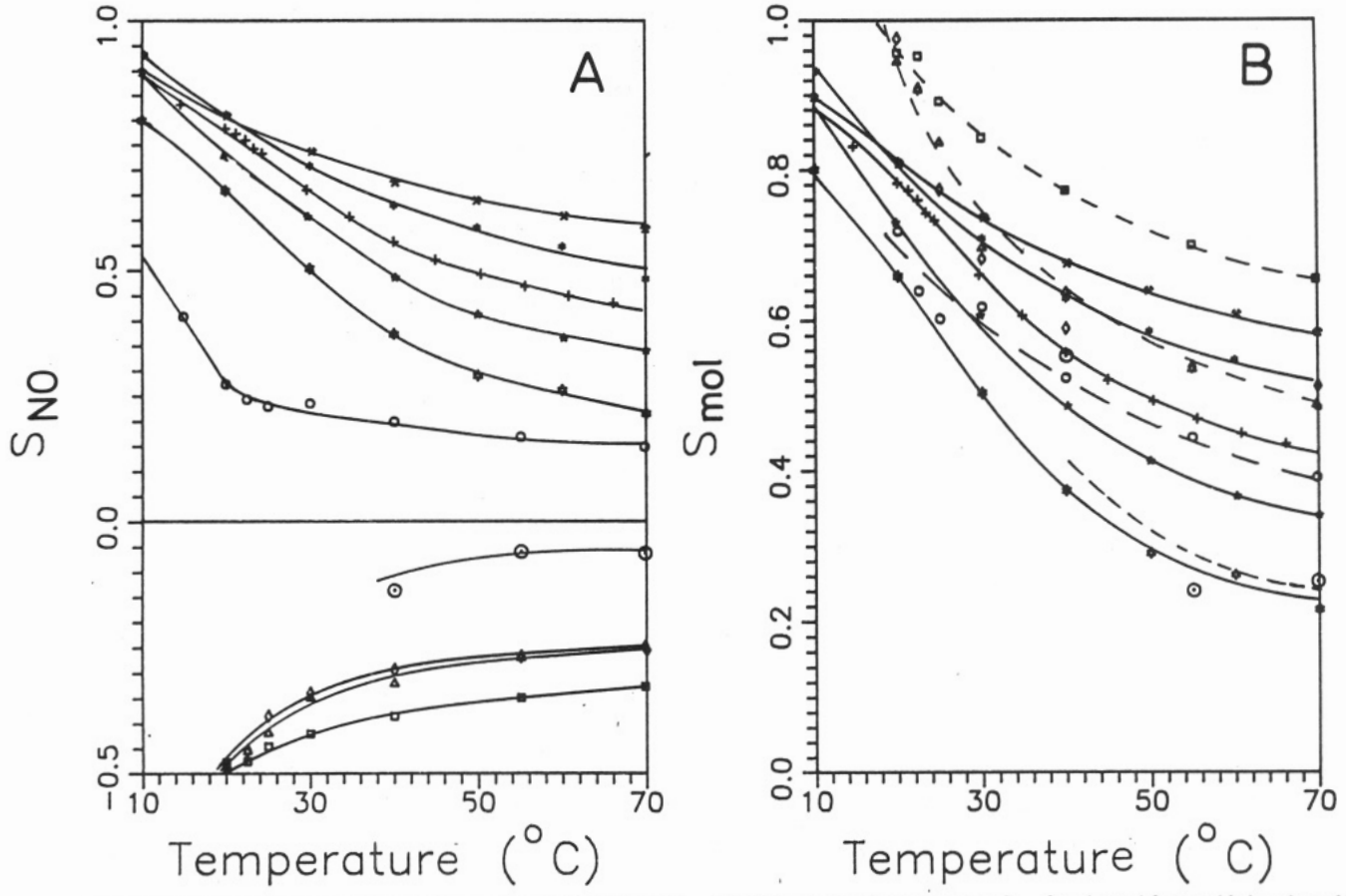

Figure 6. Temperature dependence of (A) nitroxyl group order parameter, S_{NO}, and (B) chain order parameter, S_{mol}, for nitroxyl fatty acids in oriented bilayers of DMPC:cholesterol (70:30 mol/mol) at pH 9.0. The values of S_{mol} for the pyrrolidine-based fatty acids are calculated from those of S_{NO} by using eq 3 and the values for the orientation, θ, between the chain diffusion axis and the nitroxyl z axis given in Table I. For the n-SASL nitroxyl fatty acids, $S_{mol} = S_{NO}$ ($\theta = 0°$). (□) 3-(2′,4′)FASL; (△) 4-(2′,4′)FASL; (◇) 5-(2′,4′)FASL; (⊙) 8-(3′,2′)FASL; (○) 4-(2′,5′)FASL; (✱) 4-SASL; (★) 5-SASL; (+) 7-SASL; (☆) 8-SASL; (✡) 10-SASL.

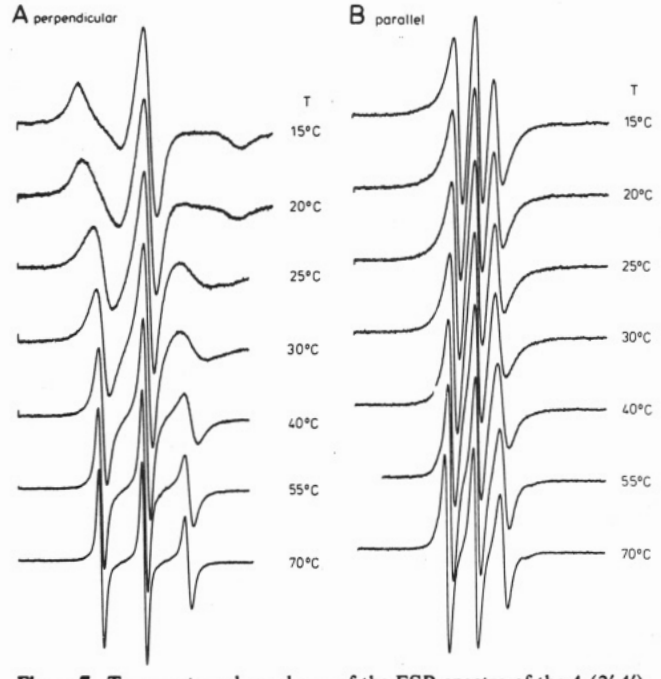

Figure 7. Temperature dependence of the ESR spectra of the 4-(2′,4′)-FASL nitroxyl fatty acid in oriented bilayers of DMPC:cholesterol (70:30 mol/mol) at pH 9.0: (A) magnetic field oriented perpendicular to the bilayer normal; (B) magnetic field oriented parallel to the bilayer normal. Maximum spectral width = 100 G.

creased, the spectrum retains its first-derivative form, and the hyperfine splitting gradually reduces to a value corresponding to the minor tensor element (A_{yy} = 4.7 G). Experiments with θ =

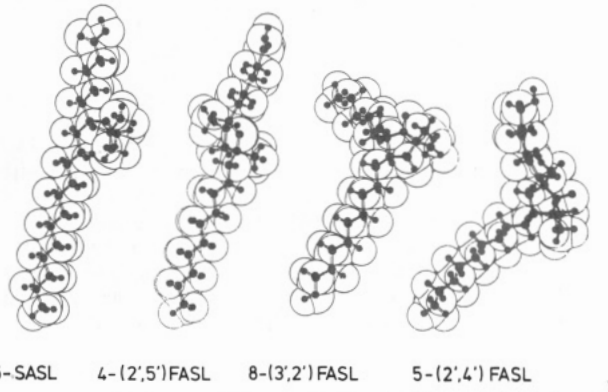

Figure 8. Simulated space-filling and ball-and-stick models of nitroxyl fatty acids: 6-SASL, 4-(2′,5′)FASL, 8-(3′,2′)FASL, and 5-(2′,4′)FASL. Conformations were calculated by using semiempirical interatomic potentials.[21]

0° nitroxyl fatty acids indicate only relatively small changes at low temperature in the spectra with the magnetic field oriented perpendicular to the bilayer normal (data not shown). This indicates that the nitroxyl x and y axes, for which the principal hyperfine elements are very similar ($A_{xx} \approx A_{yy}$) remain oriented in the bilayer plane. Thus the fluid phase orientation (θ = 0°) is preserved for these fatty acids also at low temperature in cholesterol-containing lipid mixtures.

Molecular Modeling. The values obtained for the orientation, θ, of the nitroxyl axis relative to the chain diffusion axis (Table I) can be compared with the molecular conformations of the nitroxyl fatty acids. The structures of the different nitroxyl fatty acids have been calculated in vacuo by using semiempirical interatomic potentials[21] (see Figure 8). The resulting minimum

energy conformations consist of stretches of all-trans chain attached to the pyrrolidine ring. The tetrahedral geometry of the C–C linkages in the fatty acid chain dictates that the only allowed orientations of a methylene chain segment relative to the all-trans chain axis are 0, 60, and 90°.[22] The orientations of the nitroxyl z axis of 5-(2′,2′)FASL, 3-(2′,4′)FASL, 4-(2′,4′)FASL, 5-(2′,4′)FASL, and 8-(3′,2′)FASL correspond to these values, suggesting that the diffusion axis is collinear with the axis of the chain joining the pyrrolidine ring to the carboxyl group for these fatty acids. With the exception of 5-(2′,2′)FASL, this would require one or more gauche conformations in the chain section immediately following the ring, in order to preserve an approximate cylindrical symmetry consistent with bilayer chain packing and axial rotation (cf. Figure 8). In the case of 4-(2′,5′)FASL, the value of $\theta = 40°$ implies that the rotational diffusion axis does not lie parallel to the trans-chain axis but is tilted at an angle of approximately 20° to this axis, possibly as a result of the slightly noncylindrical shape of the molecule (cf. Figure 8).

Conclusion

The methods employed here have allowed the determination of the orientation the nitroxyl group for six different pyrrolidine-based fatty acid spin labels in fluid lipid model membranes, in a manner consistent with the segmental flexibility of the chain. The orientations so derived indicate that the ESR spectra of all these nitroxyl fatty acids will be sensitive, to different extents, to rotation about the chain axis, in addition to trans–gauche isomerization, when they are incorporated as spin label probes in biological membranes. This accounts for the previously demonstrated sensitivity of the ESR spectra to lipid–protein interactions and provides the data necessary for a detailed characterization of the chain motional modes that are inhibited on interaction with integral membrane proteins.

Registry No. DMPC, 13699-48-4; 5-(2′,2′)FASL, 60113-93-1; 3-(2′,4′)FASL, 107643-05-0; 4-(2′,4′)FASL, 130905-30-5; 5-(2′,4′)FASL, 130905-31-6; 4-(2′,5′)FASL, 130905-32-7; 8-(3′,2′)FASL, 130905-33-8; 4-SASL, 35545-52-9; 5-SASL, 29545-48-0; 7-SASL, 40951-82-4; 8-SASL, 35375-98-5; 10-SASL, 50613-98-4; cholesterol, 57-88-5.

(21) (a) Program MMP2 Rev. 6.0, Molecular Design Ltd., San Leandro, CA. (b) Allinger, N. L.; Burkert, V. *Molecular Mechanics*; American Chemical Society: Washington, 1982.

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Femtosecond–Picosecond Laser Photolysis Studies on the Dynamics of Excited Charge-Transfer Complexes: Aromatic Hydrocarbon–Acid Anhydride, –Tetracyanoethylene, and –Tetracyanoquinodimethane Systems in Acetonitrile Solutions

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Formation processes of contact ion pairs (CIP) from the excited Franck–Condon (FC) state of charge-transfer (CT) complexes of aromatic hydrocarbons with acid anhydride as well as cyano compound acceptors in acetonitrile solution and charge recombination (CR) rates (k_{CR}^{CIP}) of produced CIP states have been investigated by femtosecond and picosecond laser photolysis and time-resolved absorption spectral measurements covering a wide range of free energy gap–$\Delta G°_{ip}$ between the ion pair and the ground state. It has been confirmed that the CIP formation becomes faster and k_{CR}^{CIP} of the produced CIP increases with increase of the strengths of the electron donor (D) and acceptor (A) in the complex, i.e., with decrease of the $-\Delta G°_{ip}$ value. This peculiar energy gap dependence of k_{CR}^{CIP}, quite different from the bell-shaped one observed in the case of the solvent-separated ion pairs (SSIP) or loose ion pairs (LIP) formed by encounter between fluorescer and quencher in the fluorescence quenching reaction, has been interpreted by assuming the change of electronic and geometrical structures of CIP depending on the strengths of D and A.

Introduction

It is generally believed[1] that the rate of the photoinduced CS (charge separation) and that of the CR (charge recombination) of the produced CT (charge transfer) or geminate IP (ion pair) state are regulated by the magnitude of the electronic interaction responsible for the ET (electron transfer) between D (electron donor) and A (electron acceptor) groups, the FC (Franck–Condon) factor which is related to the energy gap for the ET reaction, the reorganization energies of D and A as well as the surrounding solvent, and various solute–solvent interactions including the solvent orientation dynamics[2] and solvent-induced electronic structure change of strongly interacting D,A systems[3] in the course of ET or CT in polar solvent.

On the other hand, experimental observations on the photoinduced CS between D and A molecules, and CR of produced CT or IP state in polar solutions, have been made in the following cases:[1b] (i) CS at encounter between fluorescer and electron donating or accepting quencher in strongly polar solvent leading to the formation of geminate IP which undergoes CR and dissociation into free ions (in this case, the electronic interaction between D and A responsible for ET may be relatively weak); (ii) intramolecular photoinduced CS and CR of the produced intra-

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