Fluorine-19 Nuclear Magnetic Resonance Studies of Lipid Fatty Acyl Chain Order and Dynamics in *Acholeplasma laidlawii* B Membranes. Gel-State Disorder in the Presence of Methyl Iso- and Anteiso-Branched-Chain Substituents†

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ABSTRACT: The hydrocarbon chain orientational order parameters of membranes of *Acholeplasma laidlawii* B enriched with large quantities of a linear saturated, a methyl iso-branched, or a methyl anteiso-branched fatty acid plus small quantities of various isomeric monofluoropalmitic acid probes were determined via fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F NMR) over a range of temperatures spanning the gel to liquid-crystalline phase transitions (determined by differential scanning calorimetry). Membrane orientational order profiles in the liquid-crystalline state were generally similar regardless of the particular fatty acyl structure, showing a region of relatively constant order preceding a region of progressive decline in order toward the methyl terminus of the acyl chain. In the gel state, the order profile of the linear saturated fatty acid enriched membranes was characteristically flat, with little head to tail gradation of order. In contrast, the methyl iso-branched and the methyl anteiso-branched enriched membranes exhibited a local disordering in the gel phase reflected in a very pronounced head to tail gradient of order, which remained at temperatures below the lipid phase transition. In addition, the methyl iso- and anteiso-branched fatty acid enriched membranes were overall more disordered than the membrane containing only linear saturated fatty acyl groups. Thus, at a constant value of reduced temperature below the lipid phase transition, overall order decreased in the progression 15:0 > 16:0i > 16:0ai, suggesting that these methyl-branched substituents lower the lipid phase transition by disrupting the gel phase lipid chain packing. At a constant value of reduced temperature above the lipid phase transition, overall order decreased in the progression 16:0ai > 16:0i > 15:0. However, the apparently more highly ordered liquid-crystalline state of the branched-chain lipids may simply be the result of an absolute temperature effect rather than reflecting any real differences in chain packing above the lipid phase transition.

The methyl iso- and anteiso-branched saturated fatty acids are found as components of the membrane lipids of many prokaryotic microorganisms and some higher organisms and are the predominant fatty acyl species in a variety of bacteria (Polgar, 1971; Kaneda, 1977). Branched-chain fatty acids are able to support the growth of several unsaturated fatty acid auxotrophic bacteria apparently by mimicking the properties of unsaturated fatty acids (Rodwell & Peterson, 1971; Silbert et al., 1973; Silvius & McElhaney, 1978). Both model membrane (Silvius & McElhaney, 1979, 1980a) and natural membrane (McElhaney, 1974; Blume et al., 1978; Silvius et al., 1980) systems exhibit decreased gel to liquid-crystalline phase transition temperatures, relative to membranes containing straight-chain saturated fatty acids, when they are enriched in branched-chain fatty acyl species. Despite their widespread occurrence and their probable role as membrane "fluidizing" agents analogous to the cis-unsaturated fatty acids, it is only recently that the physical properties of methyl-branched fatty acids have begun to receive attention. Monolayer studies of phosphatidylcholines (PC's)[1] containing methyl branched-chain fatty acids indicate that iso branching and particularly anteiso branching reduce the temperature of the liquid-expanded to liquid-condensed transition and also increase the area occupied per molecule in the liquid-condensed state (Kannenburg et al., 1983). Differential scanning calorimetry (DSC) studies have demonstrated that PC's containing methyl-branched fatty acids can exhibit complex phase behavior (Lewis et al., 1985). The multiple endothermic events observed calorimetrically correspond to solid–solid as well as to solid–liquid thermotropic transitions of the methyl branched chain containing PC's. Fourier transform infrared (FTIR) spectroscopic studies indicate that these solid–solid thermotropic transitions may have properties in common with both the subtransition and pretransition exhibited by linear saturated PC's (Mantsch et al., 1985). Fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectroscopic studies of membranes of *Acholeplasma laidlawii* B enriched with methyl branched-chain fatty acids have shown that, in the liquid-crystalline state, these fatty acyl species have an apparent overall ordering effect on a monofluoropalmitoyl nuclear spin probe (Macdonald et al., 1983). To date, the effects of methyl-branched acyl chains on conformation in the hydrocarbon interior of the lipid bilayer in both the gel state and the liquid-crystalline state have not been investigated.

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[1] Abbreviations: NMR, nuclear magnetic resonance; PC, phosphatidylcholine; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; FID, free induction decay; MFPA, monofluoropalmitic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Toward this end, we initiated ^{19}F NMR studies of *A. laidlawii* B membranes highly enriched in either a straight-chain saturated, a methyl iso-branched, or a methyl anteiso-branched fatty acid plus small quantities of various isomeric monofluoropalmitic acids as nuclear spin probes. ^{19}F NMR of monofluoropalmitic acids (MFPA) offers several distinct advantages for the study of membrane lipid physical properties. NMR techniques in general are sensitive to both the conformation and dynamics of the system components. Monofluorinated fatty acids are relatively nonperturbing, as has been demonstrated by using a number of biological, biochemical, and biophysical criteria (McDonough et al., 1983), and report a picture of the conformational state of membrane lipid acyl chains which is both qualitatively (Macdonald et al., 1983) and quantitatively (Macdonald et al., 1984) similar to that provided by use of ^{2}H NMR techniques. The sensitivity of the fluorine nucleus in the NMR experiment permits a single series of monofluorinated fatty acids to be used in small amounts to survey the effects of a wide range of fatty acyl structural substituents upon membrane lipid conformational order (Macdonald et al., 1983, 1984, 1985a,b). The ^{19}F NMR spectrum apparently reflects axially symmetric motions of the membrane lipids in both the liquid-crystalline *and* gel states, so that in both phase states the orientational order of a particular methylene segment may be described in terms of a single order parameter (Macdonald et al., 1984). In addition, the wide range of chemical shifts undergone by fluorine permits the simultaneous and independent monitoring of the physical state of both membrane proteins and lipids when the two are fluorine labeled (Dettman et al., 1984).

We have chosen to perform these studies in membranes of *A. laidlawii* B primarily because this organism is capable of biosynthetically incorporating a variety of exogenously supplied fatty acids into its membrane glyco- and phospholipids (McElhaney, 1974), and when de novo fatty acid biosynthesis is inhibited with the biotin-binding protein avidin, these membranes can be made virtually homogeneous with respect to the particular fatty acid provided (Silvius & McElhaney, 1978). Thus, a membranous venue for these studies, in which the lipid fatty acid content and physical properties can be manipulated over a wide range almost at will, is readily obtained biologically. Moreover, this organism with no cell wall possesses only a single membranous structure, the plasma membrane, so that acquiring relatively homogeneous membrane preparations is particularly facile (Razin, 1975). Not of least importance is the fact that parallel studies using ^{19}F NMR in *A. laidlawii* B membranes (Macdonald et al., 1983, 1984, 1985a,b) will provide a basis for a comparison of the effects of branched acyl chains with those of a variety of alternate fatty acyl structures.

Materials and Methods

Materials. The synthesis of the various isomeric monofluoropalmitic acids has been described previously (McDonough et al., 1983). Pentadecanoic acid (15:0), 14-methylpentadecanoic acid (16:0i), and 13-methylpentadecanoic acid (16:0ai) were purchased from Analabs Co. (North Haven, CT) or synthesized as described elsewhere (Silvius & McElhaney, 1979, 1980a). All fatty acids were greater than 99.9% pure as judged from analytical thin-layer and gas chromatographic results.

Cell Culture, Membrane Isolation, Lipid Analysis, and Differential Scanning Calorimetry. The growth medium and conditions used for culturing *A. laidlawii* B with fatty acids plus avidin have been described previously (Silvius & McElhaney, 1978). Membranes were prepared from late log phase cultures of this organism by osmotic lysis essentially as previously described (Silvius et al., 1977). The extraction and purification of total membrane polar lipids and the preparation of methyl esters and their analysis by gas–liquid chromatography have been detailed elsewhere (Saito & McElhaney, 1977). Differential scanning calorimetry (DSC) on total membrane polar lipids was performed as described formerly (Macdonald et al., 1984).

Table I: Fatty Acid Composition of *A. laidlawii* B Membrane Lipids Enriched with 15:0, 16:0i, or 16:0ai plus Various Monofluoropalmitic Acids

	mol % fatty acid				
supplement	15:0	16:0i	16:0ai	*x*F16:0	other
85% 15:0					
+15% 6F16:0	93.3			6.3	0.4
+15% 8F16:0	92.3			7.2	0.5
+15% 10F16:0	95.9			4.0	0.1
+15% 12F16:0	97.0			2.7	0.3
+15% 14F16:0	94.4			5.2	0.4
85% 16:0i					
+15% 6F16:0		87.7		12.0	0.3
+15% 8F16:0		92.2		7.5	0.3
+15% 10F16:0		90.8		8.8	0.4
+15% 12F16:0		92.8		6.7	0.5
+15% 14F16:0		87.7		11.9	0.4
85% 16:0ai					
+15% 6F16:0			89.2	10.5	0.3
+15% 8F16:0			87.0	12.8	0.2
+15% 10F16:0			89.4	10.1	0.5
+15% 12F16:0			89.0	10.7	0.3
+15% 14F16:0			88.4	11.1	0.5

Nuclear Magnetic Resonance. *A. laidlawii* membranes were suspended in buffer (0.154 M NaCl, 0.05 M Tris-HCl, and 20 mM β-mercaptoethanol, pH 7.4) which had been diluted 20-fold with 95% deuterium oxide. ^{19}F NMR spectra were collected at 254.025 MHz on a Bruker HXS-270 spectrometer equipped with a ^{2}H lock, operating in the Fourier transform mode and using quadrature detection, at a spectral width of ±50 000 Hz. Bessel filters with a filter width of 100 000 Hz were employed. The probe was maintained at the specified temperature to within ±1 °C. Membrane samples were equilibrated at a particular temperature for 30 min prior to data acquisition. Fluorine nuclei were subjected to a 15-μs (~75°) pulse followed by a 10-μs delay and a 20-ms acquisition time. The recycle time was 200 ms. Typically, 20K scans were accumulated for samples in which the temperature was above the main lipid phase transition and 100K scans for samples at lower temperatures. The distortion of the first three points of the free induction decay (FID), which is associated with the receiver dead time, was corrected by a smooth extrapolation of the FID back to time zero such that the signal intensity of the early portion of the FID closely approximated a t^2 time dependence in the in-phase channel and a t dependence in the out-of-phase channel (Bloom et al., 1978). The signal to noise ratio was enhanced with an exponential multiplication which corresponded to a line broadening of 50 Hz, and the FID was Fourier transformed to 2K data points in the real domain. The integrity of the spectrum and the flatness of the base line were taken as indications that the extrapolation procedure had been performed correctly.

Results and Discussion

Fatty Acid Composition of A. laidlawii B Membrane Polar Lipids. Table I lists the fatty acid composition for each case of enrichment with either 15:0, 16:0i, or 16:0ai plus a particular isomeric monofluoropalmitic acid. In all cases, the fatty acids provided exogenously accounted for greater than 99% of the membrane lipid fatty acids. The products of de novo fatty acid

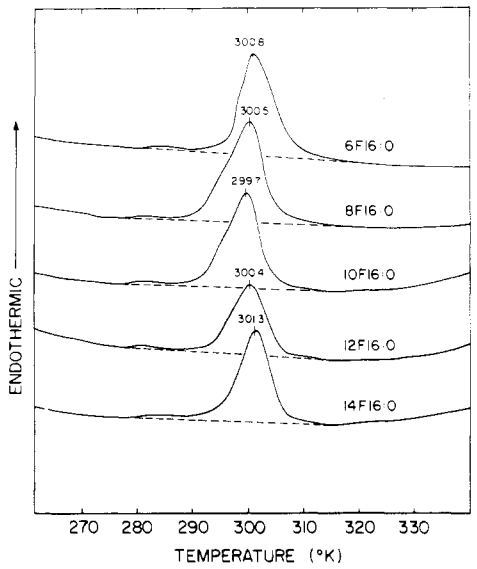

FIGURE 1: Lipid phase transition endotherms obtained by differential scanning calorimetry of the membrane polar lipid fraction from *A. laidlawii* B grown in the presence of 85 mol % 16:0i plus 15 mol % various isomeric monofluoropalmitic acids. The scan rate was 5 °C/min. The dashed line corresponds to the interpolated base line.

biosynthesis in *A. laidlawii* B (12:0, 14:0, 16:0, and 18:0; Saito et al., 1977) were conspicuously absent as expected for cells grown in the presence of avidin, each individually accounting for no greater than 0.1 mol % of the total membrane fatty acids. With the exception of cells enriched with 15:0, the ratio of the two fatty acids provided in the supplement was carried over reasonably well into the membrane lipids, indicating little if any selectivity toward either of the two fatty acyl species. However, in the case of enrichment with 15:0, the mole percent of monofluoropalmitic acid found in the membrane lipids was significantly less than that provided exogenously. Since 15:0 represents the upper chain-length limit of the linear, saturated fatty acyl family which will support the growth of *A. laidlawii* B in the presence of avidin (Silvius & McElhany, 1978), extensive incorporation of longer linear saturated fatty acids such as monofluoropalmitic acids would be detrimental to the maintenance of a properly fluid membrane and hence to cell growth and viability. Therefore, the organism may selectively incorporate proportionately fewer monofluoropalmitic acids. Regardless, the levels of monofluoropalmitic acid incorporation were adequate in all cases for the acquisition and analysis of ^{19}F NMR spectra.

Thermotropic Behavior of A. laidlawii B Membrane Polar Lipids. Any comparison of orientational order between different fatty acyl structures must necessarily consider the effects of those structures on the thermotropic properties of the lipid bilayer. We have obtained DSC endotherms of the *A. laidlawii* B membrane polar lipid fraction for each case of enrichment with a particular isomeric MFPA plus either 15:0, 16:0i, or 16:0ai. Figure 1 illustrates the DSC endotherms obtained in the case of enrichment with 16:0i for each of the isomeric MFPA's employed. The endotherms were typically broad, somewhat asymmetric, and generally similar to those reported previously by this laboratory for *A. laidlawii* B membrane lipids [see, for example, McDonough et al. (1983) and Macdonald et al. (1984)]. The variation in T_m from isomer to isomer of MFPA was minimal, generally being less than ±1 °C from the average. Table II summarizes the calorimetric data for all cases of enrichment studied here and provides, for comparative purposes, previously reported thermotropic data for PC's (Silvius & McElhaney, 1979, 1980a) and *A. laidlawii* B membrane lipids (Silvius et al., 1980) containing 15:0, 16:0i, and 16:0ai. It is evident from this table that the gel to liquid-crystalline phase transition temperature was somewhat higher for the *A. laidlawii* B membrane lipids than the corresponding phase transition temperature in the PC. The presence of approximately 10 mol % MFPA in the *A. laidlawii* B membrane lipids further increased the phase transition temperature of the *A. laidlawii* B membrane lipids as expected for these higher melting fatty acids. Nevertheless, the same trend of progressively decreasing phase transition temperatures from 15:0 to 16:0i to 16:0ai was evident in PC's, *A. laidlawii* B fatty acid homogeneous membrane lipids, and *A. laidlawii* B membrane lipids containing MFPA's.

Table II: Thermotropic Properties of *A. laidlawii* B Membrane Polar Lipids Enriched with 15:0, 16:0i, or 16:0ai plus Various Monofluoropalmitic Acids

supplement	T_m (°C)	ΔT_{10-90} (°C)[a]	T_m(*A. laidlawii* homogenous) (°C)[b]	T_m(PC) (°C)[c]
85% 15:0				
+15% 6F16:0	38.11	8.75		
+15% 8F16:0	41.34	12.20		
+15% 10F16:0	39.99	8.51		
+15% 12F16:0	40.27	9.07		
+15% 14F16:0	40.91	10.73		
	40.12[d]	9.85[d]	36.7[d]	34.2[d]
85% 16:0i				
+15% 6F16:0	27.84	9.43		
+15% 8F16:0	27.48	11.07		
+15% 10F16:0	26.68	9.98		
+15% 12F16:0	27.35	10.32		
+15% 14F16:0	28.30	10.01		
	27.53[d]	10.18[d]	21.8[d]	22.0[d]
85% 16:0ai				
+15% 6F16:0	11.07	13.51		
+15% 8F16:0	11.40	11.38		
+15% 10F16:0	12.40	12.81		
+15% 12F16:0	11.84	10.94		
+15% 14F16:0	13.18	11.60		
	11.98[d]	12.05[d]	4.1[d]	−3.0[d]

[a] ΔT_{10-90} corresponds to the temperature range over which the transition passes from 10% to 90% of completion. [b] Data from Silvius et al. (1980). [c] Data from Silvius & McElhaney (1979, 1980a). [d] Average.

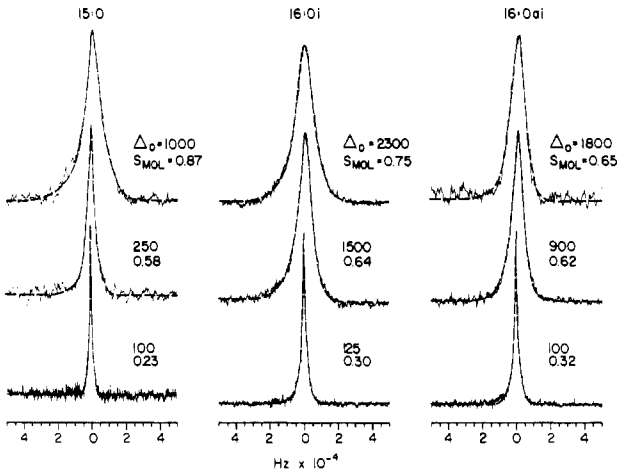

FIGURE 2: Experimental and simulated ^{19}F NMR spectra of membranes of *A. laidlawii* B grown in the presence of 15 mol % 6F16:0 plus 85 mol % 15:0 (286, 301, and 310 K), 16:0i (269, 284, and 301 K), or 16:0ai (254, 270, and 286 K). The values of the computer input parameters Δ_0 (interchain dipolar broadening) and S_{mol} (molecular order parameter) are indicated for the simulated spectra (dashed lines).

^{19}F NMR Analysis of A. laidlawii B Membranes. Examples of experimentally obtained ^{19}F NMR spectra of *A. laidlawii* B membrane samples as a function of temperature are shown in Figure 2, along with the corresponding computer-generated simulated spectra, for the cases of enrichment with 15 mol % 6F16:0 plus 85 mol % either 15:0, 16:0i, or 16:0ai. At a temperature above the particular lipid phase transition, the fluorine spectra consisted of a single, narrow, somewhat asymmetric resonance line, reflecting the extensive and rapid motions of the fatty acyl chains characteristic of a lipid bilayer in the liquid-crystalline state. With decreasing temperature and altered lipid phase state, the fluorine spectra progressively broadened, responding to the increasingly restricted fatty acyl chain motions which are a consequence of the transition to the gel state. An apparently superlorentzian line shape was observed at all temperatures, suggesting that, in *A. laidlawii* B membranes, considerable motional averaging occurs on the ^{19}F NMR time scale even in the gel state.

In the region of the phase transition, ^{2}H-labeled [e.g., see Davis (1983)] and ^{13}C-labeled [e.g., see Wittebort et al. (1981)] lipid NMR spectra clearly consist of distinct contributions from individual gel and liquid-crystalline components. Thus, in the temperature region where domains of gel and liquid-crystalline lipid coexist, any exchange of lipid molecules between phase domains can be considered to be slow on the NMR time scale. Although the fluorine spectra in the coexistence region do not obviously consist of separate gel and fluid spectra components, such spectra can be readily simulated by the addition of 100% fluid and 100% gel spectra (Macdonald, 1984) and must, therefore, be considered superpositions of individual gel and liquid-crystalline state spectra components.

The ^{19}F NMR line shape of a MFPA is influenced by both chemical shift anisotropic and dipole–dipole interactions which are averaged by the anisotropic motions particular to the lipid molecules of a lipid bilayer. The superposition quality of these two interactions, the relative magnitudes of these interactions, and the axially symmetric molecular motions in the lipid bilayer lead to the superlorentzian line shapes shown in Figure 2. Since there is no one spectral parameter which can be readily and unequivocally related to the orientational order of the bilayer lipids, we have resorted to spectra simulations to extract this information.

The mathematical model employed to simulate the fluorine spectra has been described in detail elsewhere (Macdonald et al., 1983, 1984). Briefly, the model assumes that the motions of the lipid molecules in the lipid bilayer are of sufficient rapidity to provide effective axial symmetry on the ^{19}F NMR time scale. Hence, the fluorine chemical shift anisotropy and F–H dipolar interaction are ascribed a $\langle 3\cos^2\theta - 1\rangle$ orientation dependence, where θ represents the angle between the external magnetic field and the perpendicular to the plane of the lipid bilayer.

Under conditions of axially symmetric motional averaging, the geometric constraints on the amplitudes of the hydrocarbon chain motions may be completely described in terms of a single orientational order parameter, S_{mol}. This parameter quantitates the amplitude of the angular excursions of the fatty acyl chains away from an axis perpendicular to the plane of the bilayer. Both tilting of the entire fatty acyl chain with respect to the bilayer normal and also trans/gauche isomerization about individual carbon–carbon bonds could contribute to a decrease in the value of S_{mol} from its maximum of unity to its minimum of zero. S_{mol} is defined as (Seelig, 1977)

$$S_{mol} = \tfrac{1}{2}\langle\cos^2\beta - 1\rangle$$

where β represents the angle between the segment direction and the bilayer normal, where the segment direction is the normal to the H–C–H plane. The bar represents an average over time.

In practice, the maximum chemical shift anisotropy and the maximum F–H dipolar interaction were first estimated for a monofluoropalmitate-containing lipid whose fatty acyl chains had assumed an all-trans configuration aligned parallel to the bilayer normal and were experiencing rapid (on the ^{19}F NMR time scale) rotations about their long axis, i.e., a situation in which S_{mol} equals unity. The decrease in the observed chemical shift anisotropy and intrachain F–H dipolar interaction from their estimated maxima was quantitated via the introduction of the orientational order parameter, S_{mol}. The validity of the model, and hence of the extracted values of S_{mol}, hinges upon the accuracy of the estimated values of the maximum chemical shift anisotropy and intrachain dipolar interaction as well as the veracity of the assumption of effectively axially symmetric motions of the lipid molecules.

The value of the maximum chemical shift anisotropy for a monofluoropalmitic acid under conditions of axially symmetric motional averaging was estimated to be 82.2 ppm (Macdonald et al., 1983) from a consideration of the fluorine chemical shift tensor elements reported for Teflon (Mehring, 1971). If this value were an over- or underestimate, the extracted values of S_{mol} would be inversely under- or overestimated. In the absence of a direct measurement of the chemical shift tensor elements of a monofluoro-type compound, we have consistently used the above estimated value as the maximum. Thus, these data will be internally consistent, and our conclusions regarding the effects of various fatty acyl structural substituents upon orientational ordering will be valid regardless of whether or not the estimated maximum chemical shift anisotropy corresponds exactly to the actual value. Furthermore, we consider it unlikely that the values of the fluorine chemical shift tensor elements or the orientation of the tensor principal axes system will alter under the different conditions of label position, temperature, etc. to which we have subjected the nuclear spin probes, and therefore, we attribute the observed spectral variations to changes in S_{mol}.

The second model parameter of importance, the maximum F–H intrachain dipolar interaction, was estimated to equal approximately 20 000 Hz from an examination of the field strength dependence of the fluorine spectrum of a MFPA incorporated into a lipid bilayer (Macdonald et al., 1983). This value was in good agreement with that estimated theoretically from a consideration of the geometry of a monofluoro-substituted methylene segment within an acyl chain (Macdonald, 1984).

Of singular significance to our spectral simulations is the assumption of effectively axially symmetric motions of the bilayer lipid molecules. In the absence of long axis rotational motions of the whole lipid or torsional motions of the fatty acyl chains of sufficient rapidity to satisfy the criterion for effective axial symmetry on the ^{19}F NMR time scale, it would no longer be possible to completely describe the orientational order of the monofluoromethylene segment in terms of a single order parameter. In the liquid-crystalline phase, the ^{2}H NMR spectrum is characteristic of axially symmetric lipid motions, but this is not so in the gel state [e.g., see Seelig (1977)]. Thus, the restricted rotations of the lipids at temperatures below the phase transitions are insufficiently rapid to provide axial symmetry *on the ^{2}H NMR time scale.* On the other hand, ^{13}C NMR spectra are characteristic of axial symmetry even to temperatures 40 °C below the lipid phase transition [e.g.,

see Wittebort et al. (1981)]. This difference between the characters of gel-state ^2H and ^{13}C spectra can be attributed to the approximately 1 order of magnitude difference in the time scales relevant to the two procedures (Davis, 1983). The time scale relevant to the fluorine spectrum most closely corresponds to that of ^{13}C (Macdonald et al., 1984) so that on theoretical grounds one would predict effective axial symmetry to be manifest in both the gel- and liquid-crystalline-state fluorine spectra. In the absence of axially symmetric motions, the fluorine spectrum assumes a broad Gaussian line shape (Macdonald et al., 1984) which contrasts with the superlorentzian line shapes obtained at temperatures below the lipid phase transition, as illustrated in Figure 2. Further to this point, it is apparent that the spectral simulations, which explicitly assume axial symmetry, more than adequately reproduce the experimental ^{19}F NMR line shapes in both the liquid-crystalline and the gel state. Although these various points do not, of themselves, prove that on the ^{19}F NMR time scale axial symmetry exists in the gel state, when taken together, the theoretical considerations, the superlorentzian character of gel-state fluorine spectra, and the successful spectral simulations assuming axial symmetry strongly suggest that this assumption is valid on the ^{19}F NMR time scale.

Having fixed the maximum values of the chemical shift anisotropy and the H–F intrachain dipolar interaction under conditions of axially symmetric motions, the remaining parameters which the model permits to be varied are the order parameter, S_{mol}, and the interchain H–F dipolar interaction, Δ_0. The latter becomes of significance only in the gel state where increasingly larger values of Δ_0 must be entered (in hertz) in order to simulate the line shape. In the liquid-crystalline state, the rapid lateral and rotational diffusion of the lipid molecules averages their interchain dipolar interactions to a residual minimum. This situation is evident in Figure 2 where the value of Δ_0 increases from 100 Hz at temperatures above the lipid phase transition to greater than 1000 Hz at temperatures below. Over the same temperature range, the value of S_{mol} increased from approximately 0.23 to 0.87 in the case of enrichment with 15:0. Thus, values of the orientational order parameter approach the theoretical maximum at temperatures below the lipid phase transition, at least in the presence of a straight chain saturated fatty acid. It is equally evident that, in the presence of the iso- and anteiso-methyl-branched fatty acids at comparable temperatures below their respective phase transitions, the degree of orientational order achieved by the MFPA probes was substantially lower than that observed in the presence of the straight chain saturated fatty acid.

With regard to the values of the order parameter estimated from spectral simulation at temperatures within the boundaries of the lipid phase transition, several further comments are necessary. Although the fluorine spectra in the region where gel- and liquid-crystalline-state lipids coexist do not overtly display distinct gel and fluid components, they must, nevertheless, be considered as consisting of superimposed spectral contributions from gel-state and liquid-crystalline-state domains. Since a single order parameter suffices to describe fluorine spectra acquired at a temperature within the coexistence region, this value of S_{mol} must be considered an average of the order parameters of the two distinct spectral components. Furthermore, this "average" order parameter is a function of both the relative percent gel-state and percent liquid-crystalline-state lipid pertinent to a particular temperature within the coexistence region as well as the temperature dependence of orientational order within any one

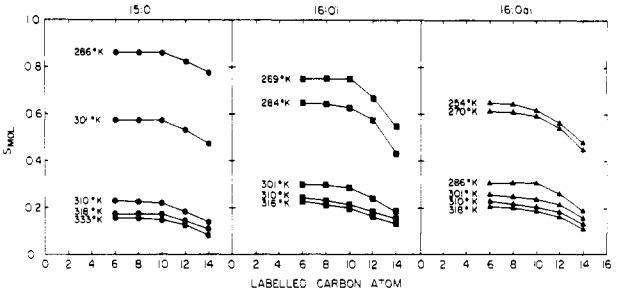

FIGURE 3: ^{19}F NMR orientational order profiles of membranes of *A. laidlawii* B grown in the presence of 85 mol % 15:0, 16:0i, or 16:0ai plus 15 mol % various isomeric monofluoropalmitic acids.

phase domain over the range of the phase transition.

^{19}F NMR Order Profiles. The variation of S_{mol} with the position of the monofluoro substituent provides an order profile for the fatty acyl chain in a given experimental situation. Figure 3 depicts the ^{19}F NMR order profiles of *A. laidlawii* B membranes enriched with either 15:0, 16:0i, or 16:0ai obtained over a range of temperatures spanning the gel to liquid-crystalline phase transitions. These temperatures were chosen to permit comparisons at certain values of absolute temperature as well as at certain values of the reduced temperature (see below) above or below the particular phase transition temperature. In the liquid-crystalline state, ^2H NMR results in both model (Seelig, 1977; Seelig & Seelig, 1980) and biological (Stockton et al., 1977; Galley et al., 1979) membranes show that there is a region of approximately constant order along the fatty acyl chain preceding a region of progressively decreasing order toward the methyl terminus. This gradient of orientational order has come to be recognized as the "signature" of a lipid bilayer.

In the liquid-crystalline state, the ^{19}F NMR order profiles demonstrate the presence of a similar region of relatively constant order followed by a decline in orientational order toward the methyl end of the fatty acyl chain. The effects of methyl iso- or anteiso branching on the ^{19}F NMR order profile were marginal in the liquid-crystalline state, although a local ordering effect of methyl branching could be discerned as a decrease in the steepness of the terminal order gradient by comparison with the linear saturated situation, as reported previously (Macdonald et al., 1983). The resilience of the fatty acyl chains in the liquid-crystalline state should permit them to accommodate the presence of structural substituents such as a methyl iso or anteiso branch without drastically altering the character of the orientational order profile. It has become increasingly apparent that the order profile is relatively refractory to change in the liquid-crystalline state despite alterations to fatty acyl chain chemistry. Previous ^{19}F NMR results have demonstrated that the hydrocarbon chain orientational order profiles in membranes of *A. laidlawii* B are highly similar in the liquid-crystalline state whether those membranes were enriched with straight chain saturated fatty acids such as 15:0 (Macdonald et al., 1983) or 16:0 (Macdonald et al., 1984) or any of an isomeric series of *cis*-octadecenoic acids (Macdonald et al., 1985a) or *trans*-octadecenoic acids (Macdonald et al., 1985b) or with methyl-branched fatty acids such as 16:0i or 16:0ai (Macdonald et al., 1983). Results obtained with specifically deuterated fatty acids via ^2H NMR also indicate that the liquid-crystalline order profile is relatively invariant despite changes in fatty acyl structure. The orientational order profiles of PC's containing cis-unsaturated fatty acids were very similar to those obtained with trans-unsaturated fatty acids after correction for geometric considerations, and these were in turn very similar to the order profiles of

straight chain saturated fatty acids (Seelig & Waespe-Šarčevič, 1978). These results were confirmed by ^{2}H NMR studies in *Escherichia coli* (Gally et al., 1979) and *A. laidlawii* (Stockton et al., 1977; Rance et al., 1980). The one exception to this generalization appears to be the cyclopropyl ring substituted fatty acids where, even after correction for geometric considerations, the ^{2}H NMR order profiles in both model (DuFourc et al., 1983) and biological membranes (Jarrell et al., 1983) indicate that the cyclopropyl substituent experiences a far greater degree of orientational ordering than any other portion of the fatty acid chain.

With decreasing temperature, the orientational order of the MFPA's increased slowly and the character of the order profiles remained relatively constant until the lipid phase transition was traversed. As the proportion of gel-state lipid increased, overall order increased profoundly, and the order profiles in the presence of different fatty acyl chain structures began to acquire distinct dissimilarities. With the straight-chain acid 15:0, a high degree of ordering was achieved with individual values of S_{mol} approaching the theoretical maximum. In addition, it can be discerned that the gradient of order so prominent in the liquid-crystalline state (i.e., at 333 K, the value of S_{mol} at the 14-position was approximately 50% of the value at the 6-position) was nearly absent in the gel state (i.e., at 280 K, the value of S_{mol} at the 14-position was over 90% of the value at the 6-position). Consequently, the configuration of the MFPA chain in the presence of 15:0 in the gel state may be described as highly ordered, approaching but not as yet achieving an all-trans conformation, with any residual gauche rotational isomers distributed with approximately equal probability along most of the length of the fatty acyl chain. ^{19}F NMR gel-state order profiles of *A. laidlawii* membranes highly enriched with 16:0 were also characteristically flat with individual values of the order parameter approaching the theoretical maximum (Macdonald et al., 1984). Allegrini et al. (1983) studied the gel-state ^{2}H NMR spectra of specifically deuterated palmitic acids mixed equimolar with 1-palmitoyllyso-PC. These compounds together assume a bilayer structure, most probably via the formation of a functional dimer of lysophospholipid and free fatty acid. The free deuterated palmitic acid experiences a higher degree of rotational freedom in the gel state than would DPPC, which permits the characterization of its configuration in the gel state in terms of a single orientational order parameter. The values of S_{mol} obtained in the gel state nearly approached unity for positions C2 to C13 and thereafter somewhat decreased toward the acyl chain methyl terminus. Fluorine and deuterium techniques apparently agree regarding the conformation assumed by a linear saturated fatty acyl chain in the gel state.

The inclusion of a methyl-branch substituent had two immediately apparent consequences for the gel-state order profile of MFPA. In the presence of either a methyl iso- or a methyl anteiso-branched fatty acid in the gel state, a large head to tail gradient of orientational order remained, and the overall order achieved at comparable temperatures below the lipid phase transition was significantly less than was observed in the presence of the linear, saturated 15:0.

The gradient of order which remained in the gel state in the presence of methyl-branched-chain substituents indicates that these structures are capable of disrupting the gel-state acyl chain packing in their immediate vicinity. A similar gradient of order was observed via ^{19}F NMR in the gel state in the presence of isomers of *cis*-octadecenoic acid with the site of unsaturation near the methyl terminus of the acyl chain (Macdonald et al., 1985a). Since the ^{19}F NMR order profiles

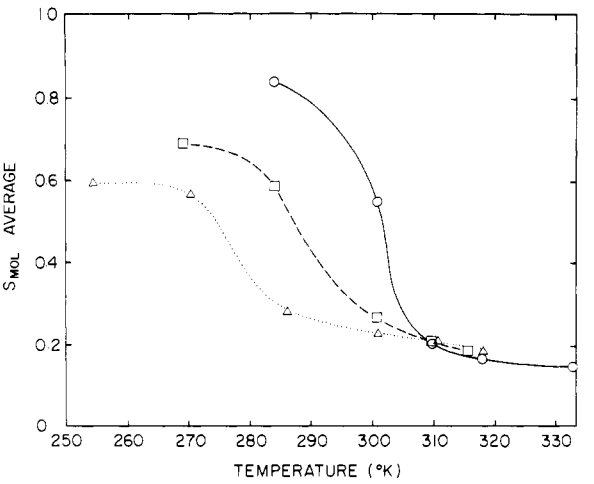

FIGURE 4: Chain average orientational order vs. acquisition temperature. The chain average order was calculated as the numerical average of the values of S_{mol} obtained for the five isomeric monofluoropalmitic acids in any one case of enrichment with either 15:0, 16:0i, or 16:0ai at any one temperature. Circles, 15:0; squares, 16:0i; triangles, 16:0ai.

in the gel state in the presence of isomers of *cis*-octadecenoic acid with the site of unsaturation near the carbonyl head group resembled those obtained in the gel state in the presence of linear, saturated fatty acids, it was concluded that, even in the gel state, a head to tail gradient in the stringency of packing restrictions was still present. The results obtained here with methyl iso- and anteiso-branched fatty acyl species confirm that structural substituents located near the fatty acyl chain methyl terminus are capable of locally disrupting gel-state chain packing.

The second consequence of the inclusion of methyl-branched structures, which is that the overall order achieved in the gel state was far less than that achieved in the presence of 15:0, is more readily apparent when the chain average order parameters are plotted vs. the acquisition temperature for the three cases of enrichment with either 15:0, 16:0i, or 16:0ai, as shown in Figure 4. The chain average order was simply the numerical average of the five values of S_{mol} obtained at any one temperature for any one case of enrichment. While such a data reduction will obscure differences in the characters of the order profiles, it does provide a convenient measure of overall orientational order in a given situation. Several important features are illustrated in Figure 4. At a temperature which was above the lipid phase transition of all three enriched membranes, the overall order was generally very similar regardless of the particular fatty acyl chain structure examined. For any one measuring temperature at which all fatty acyl chain structures are in the liquid-crystalline state, overall order can be shown to be approximately a linear function of $T - T_m$, and therefore to be relatively independent of the particular structure examined (Macdonald et al., 1985a). With increasing temperature and, again, provided that it is exclusively the liquid-crystalline state which is being observed, the linear dependence of overall order on $T - T_m$ decreases until at sufficiently high temperatures (>310 K in *A. laidlawii* B) relative order becomes virtually independent of effects arising due to proximity to the phase transition. For example, at 318 K, where in each case of enrichment the membrane lipids would have assumed exclusively the liquid-crystalline state, the overall order was nearly identical in the presence of 15:0, 16:0i, or 16:0ai.

As the acquisition temperature was lowered, the overall orientational order increased profoundly in a given case of

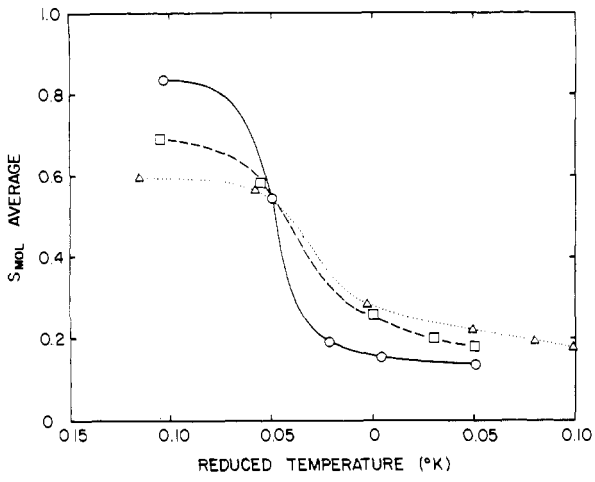

FIGURE 5: Chain average orientational order vs. reduced temperature. T_R was calculated as described in the text. The chain average order was calculated as in Figure 4. Circles, 15:0; squares, 16:0i; triangles, 16:0ai.

enrichment when, and not until, the particular lipid phase transition was encountered. Clearly, the lipid phase transition was the greatest single affector of orientational order regardless of the particular fatty acyl chain structure or the absolute temperature of the phase transition. Nevertheless, membranes containing methyl branched-chain fatty acids appeared to be incapable of assuming a gel state as highly ordered as that observed in the presence of 15:0.

This effect is most apparent when the orientational order data are normalized with respect to the particular phase transition temperature via the introduction of a reduced temperature, T_R, where $T_R = (T - T_m)/T_m$. T is the measuring temperature, and T_m is the phase transition temperature for a particular case of enrichment with one fatty acid, both in degrees kelvin (Seelig & Browning, 1978). This calculation is intended to eliminate or at least minimize effects arising solely from differences in the lipid phase transition temperatures. Thus, measurements made at equal values of T_R should sample equal and coinciding states with respect to the lipid phase transition regardless of the absolute measuring temperature.

Figure 5 depicts the chain average order parameter as a function of the reduced temperature, T_R, for each case of enrichment with either 15:0, 16:0i, or 16:0ai. The T_m (*A. laidlawii* homogeneous plus MFPA) data of Table II were used to calculate T_R. All fatty acids showed a marked increase in orientational order as the proportion of gel-state lipid increased, indicating again that the lipid phase transition was the preeminent affecter of overall orientational order. Nevertheless, at equal values of T_R below the lipid phase transition, orientational order decreased in the progression 15:0 > 16:0i > 16:0ai. Evidently, these methyl-branched substituents were capable of disrupting gel-state chain packing in more than a local sense and, in fact, prevented the assumption of the overall highly ordered, nearly all-trans state characteristic of straight-chain saturated fatty acids in the gel state. It is informative to note that the temperatures of the lipid phase transitions in the presence of these fatty acids decrease in the same progression as their relative overall order in the gel state, that is, 15:0 > 16:0i > 16:0ai. The implication is then that gel-state disordering or instability leads to a lower lipid phase transition temperature and that alterations to gel-state stability can be produced by altering fatty acyl chain chemistry.

There are a number of lines of evidence which indicate that methyl branched-chain fatty acids assume a loosely packed gel state. Natural membranes enriched with branched-chain fatty acids exhibit unusual X-ray diffraction properties below their phase transition temperatures (Haest et al., 1974; Legendre et al., 1980). The sharp 4.2-Å reflection, which is associated with reflections from the closely packed hydrocarbon chains in gel-state lipid, is replaced by a broader reflection with a spacing of 4.3–4.4 Å in these membranes. Pig pancreatic phospholipase A_2, which cannot hydrolyze gel-state phosphatidylglycerol in *A. laidlawii* membranes enriched with straight-chain saturated or unsaturated fatty acids, can attack this phospholipid in membranes enriched in branched-chain fatty acids at temperatures well below the lipid phase transition (Bouvier et al., 1981). Moreover, the lateral segregation of integral membrane proteins into protein-rich domains, which is normally observed by freeze–fracture electron microscopy at temperatures below the lipid phase transition, does not occur in *A. laidlawii* B membranes artificially enriched in branched-chain fatty acids (Haest et al., 1974; Silvius & McElhaney, 1980b). Finally, monolayer studies indicate that PC's containing methyl iso- and anteiso-branched fatty acids reduce the temperature of the liquid-expanded to liquid-condensed transition and increase the molecular area occupied per PC in the liquid-condensed state (Kannenburg et al., 1983). Thus, the results obtained in the present study confirm those obtained in earlier work.

It is evident in Figure 5 that, at values of T_R above the lipid phase transition, the overall order decreases in the progression 16:0ai > 16:0i > 15:0. This observation suggests that these methyl branched-chain fatty acids assume a more highly ordered liquid-crystalline state than those a linear saturated fatty acid. We have previously attempted to rationalize this result in terms of fatty acyl chain packing effects arising from differences in the cross-sectional area occupied by these different chain structures (Macdonald et al., 1983). Recent monolayer studies by Kannenburg et al. (1983) indicate, however, that methyl-branched and linear saturated fatty acids occupy approximately equal cross-sectional areas in the monolayer in the liquid-expanded state. Packing considerations seem insufficient to explain the more highly ordered liquid-crystalline state assumed by these methyl-branched structures at equal values of T_R. The simplest explanation may be that at equal values of $T_R > 0$, one is actually making comparisons across a wide range of absolute temperature. From Figure 4, it can be seen that, at 310 °C, 15:0, 16:0i, and 16:0ai are almost equally ordered. As the temperature is decreased, the orientational order of first 15:0 and then 16:0i rises dramatically as the lipid phase transition is encountered. However, over the same temperature range and in the absence of any phase change, the orientational order in the presence of 16:0ai increases progressively and significantly. This dependence of orientational order on absolute temperature in the liquid-crystalline state offers the most probable explanation for the apparently higher ordering of methyl branched-chain fatty acids at constant values of $T_R > 0$.

Registry No. 15:0, 1002-84-2; 16:0i, 4669-02-7; 16:0ai, 20121-96-4.

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