^{19}F Nuclear Magnetic Resonance Studies of Lipid Fatty Acyl Chain Order and Dynamics in *Acholeplasma laidlawii* B Membranes. Orientational Order in the Presence of a Series of Positional Isomers of *cis*-Octadecenoic Acid†

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ABSTRACT: The ^{19}F nuclear magnetic resonance (NMR) spectra of membranes of *Acholeplasma laidlawii* B enriched with one of a series of positional isomers of *cis*-octadecenoic acid plus small amounts of one of a number of isomers of monofluoropalmitic acid were interpreted in terms of an orientational order parameter (S_{mol}). The variation of S_{mol} with the position of the fluorine label in the liquid-crystalline state yielded an "order profile" with characteristics similar to those obtained via ^2H NMR and which was relatively invariant regardless of the site of cis unsaturation. In the gel state, values of S_{mol} approached the theoretical maximum, and the order profiles in the presence of different isomeric *cis*-octadecenoic acids displayed distinct dissimilarities. When the cis double bond was located proximal to the methyl terminus of the fatty acyl chain, a gradient of order across the bilayer was still evident in the gel state. When the cis double bond was located near the carbonyl head group, values of S_{mol} were approximately equal at all chain positions. These observations were interpreted as indicating that in the gel state the stringency of packing restrictions is still subject to variation across the width of the bilayer. Relative overall orientational order among all isomers examined (specifically, 18:1cΔ4, Δ5, Δ6, Δ7, Δ8, Δ9, Δ10, Δ11, Δ12, Δ13, Δ14, and Δ15) varied directly as a function of proximity to the lipid gel to liquid-crystalline phase transition (T_m) (determined via differential scanning calorimetry) when compared at a constant temperature. Furthermore, when normalized with respect to the T_m, values of S_{mol} all fell within a relatively narrow band which increased exponentially toward the theoretical maximum at temperatures below the T_m, indicating that the proportion of gel to liquid-crystalline lipid is the primary determinant of the overall orientational order. Nevertheless, isomers with the site of unsaturation near the center of the chain were revealed to be somewhat more ordering in the liquid-crystalline state than others following the normalization procedure, suggesting that structural considerations, although of secondary importance, were still significant.

Despite the recognition that biological membranes are characterized by a heterogeneity of lipid structure and composition, the manner in which many specific structural substituents contribute to the conformational and dynamic properties of lipid membranes requires clarification. It has been established from differential scanning calorimetry (DSC)[1] and differential thermal analysis (DTA) studies that the inclusion of various structural modifications can drastically alter the gel to liquid-crystalline transition temperature relative to that of a saturated lipid, and hence modulate the "fluidity" of a membrane [for a recent review, see McElhaney (1982)]. The concept of fluidity must involve parameters which consider the ordering or conformation of the hydrocarbon chains as well as the rates of particular types of motions. The most detailed insights into membrane lipid orientational order and membrane fluidity have been obtained by using nuclear magnetic resonance (NMR) techniques.

While ^2H NMR of labeled lipids has been the method of choice for the determination of lipid conformational order, the application of the technique is hampered by the necessity—and the accompanying difficulty and not inconsiderable expense—of synthesizing a series of specifically deuterated lipids for each different fatty acyl structure to be studied.

^{19}F NMR of specifically monofluorinated lipids also proffers the opportunity to report in detail upon the molecular order and dynamics of membrane lipids as it is similarly sensitive to the anisotropy of motions and segmental fluctuations inferred from ^2H NMR spectra. 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 lipids which is essentially identical with that elucidated via ^2H NMR (Macdonald et al., 1983). Furthermore, the sensitivity of the ^{19}F nucleus in the NMR experiment confers upon the monofluorinated fatty acyl nuclear spin probes a versatility which permits a single series of specifically monofluorinated fatty acids to be used in small amounts to survey the effects of a range of fatty acyl structural substituents upon lipid conformational order in a membrane (Macdonald et al., 1983). The ^{19}F NMR spectra in both the liquid-crystalline state and the gel state can be simulated by using a model which assumes axially symmetric lipid motions

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[1] Abbreviations: NMR, nuclear magnetic resonance; FID, free induction decay; DSC, differential scanning calorimetry; DTA, differential thermal analysis; MFPA, monofluoropalmitic acid; Tris, tris(hydroxymethyl)aminomethane; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; PEPC, 1-hexadecanoyl-2-*trans*-octadec-9-enoyl-*sn*-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-*cis*-octadec-9-enoyl-*sn*-glycero-3-phosphocholine.

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(Macdonald et al., 1984). Therefore, the orientational order of a particular methylene segment may be described by a single order parameter, S_{mol}, in both the liquid-crystalline and gel states. 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 fluorine-labeled protein and lipid are incorporated together into a lipid bilayer (Dettman et al., 1982, 1984).

The cis double bond occurs widely in biological membranes and has been the subject of recent ^2H NMR studies in both model (Seelig & Seelig, 1977; Seelig & Waespe-Šarčevič, 1978) and biological (Rance et al., 1980) membranes. These studies indicated that conformational order was reduced in the presence of cis-unsaturated acyl chains relative to saturated acyl chains when measured at the same temperature. When compared at the same temperature relative to their respective lipid phase transition points, the cis-unsaturated acyl chains displayed nearly identical or a somewhat greater degree of orientational order than straight-chain saturated acyl chains, suggesting that the conformations available to saturated and unsaturated acyl chains in the liquid-crystalline state were nearly identical. It was concluded that a local "organizational perturbation" induced by the cis double bond determines the proportion of gel and liquid-crystalline lipid. The nature of this perturbation remains ill-defined. As pointed out by Barton & Gunstone (1975), simple gel-state chain-packing considerations seem insufficient to explain the relationship between the temperature of chain melting and the position of a cis double bond along an acyl chain.

The availability of a series of positional isomers of *cis*-octadecenoic acid (Gunstone & Ismail, 1967a), coupled with the ability to rapidly screen a range of fatty acid structures via ^{19}F NMR, led us to undertake a systematic survey of the relationship between lipid orientational order and the position of a cis double bond in membranes of the organism *Acholeplasma laidlawii* B. This organism has only a single membranous structure, the plasma membrane, and lacks a cell wall so that the preparation of pure, homogeneous membranes is rapid and simple. Furthermore, *A. laidlawii* readily incorporates a variety of exogenous fatty acids, and its membrane can be made virtually homogeneous with respect to a particular fatty acid when de novo fatty acid biosynthesis is inhibited with, for example, the protein avidin (Silvius & McElhaney, 1978).

The ^{19}F NMR spectra of membranes of *A. laidlawii* enriched with small amounts of one of a series of positional isomers of monofluoropalmitic acid, plus one of a series of positional isomers of *cis*-octadecenoic acid ranging from Δ4 through Δ15 inclusive, were acquired at a variety of temperatures and analyzed in terms of conformational order parameters. It is demonstrated that while orientational order was very similar in the liquid-crystalline state regardless of double-bond position, significant differences in chain ordering were manifest in the gel state with different sites of unsaturation. These results suggest that, even in the densely packed gel state, regions of the fatty acyl chains differ in their susceptibilities to conformational perturbation. Nevertheless, normalization of the S_{mol} values with respect to the gel to liquid-crystalline phase transition indicates that the lipid phase state remains the preeminent determinant of the overall lipid orientation order.

Experimental Procedures

The growth medium and conditions for culturing *A. laidlawii* B with fatty acids and avidin have been described previously (Silvius & McElhaney, 1978). With the exception of cultures supplemented with the isomers 18:1cΔ8, Δ9, Δ10, and Δ11, which were grown at 32 °C, all other cultures were grown at 37 °C. 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 lipids and the preparation of fatty acyl methyl esters and their analysis by gas–liquid chromatography have been described elsewhere (Saito & McElhaney, 1977).

Membrane samples for NMR analysis were suspended in buffer (0.154 M NaCl, 0.05 M Tris-HCl, and 20 mM β-mercaptoethanol, pH 7.4) diluted 20-fold with 95% deuterium oxide. ^{19}F NMR spectra were collected at 254.025 MHz on a Bruker HXS-270 NMR spectrometer equipped with a ^2H lock, operating in the Fourier-transform mode, using quadrature detection, at a spectral width of ±50 000 Hz. Bessel filters with a filter width of ±100 000 Hz were used. The ^{19}F probe was constructed entirely of fluorine-free materials and was void of any ^{19}F background signal. The temperature of the probe was maintained at the specified temperature to within ±1 °C. Samples were equilibrated at a particular temperature for 30 min prior to data acquisition. ^{19}F-Labeled nuclei were subjected to a 15-μs (~75°) pulse followed by a 10-μs delay and a 20-ms acquisition time. Total recycling time was 200 ms. Typically, 25K scans were accumulated for temperatures at which the samples were above the gel to liquid-crystalline phase transition, and 100K scans were accumulated for temperatures at which the samples were below the gel to liquid-crystalline phase transition. The distortion of the first three points of the free induction decay (FID) was corrected by a smooth extrapolation of the FID back to time zero such that the early portion of the FID closely approximated a t^2 time dependency (Bloom et al., 1978). The distortion is associated with the dead time of the receiver. The signal 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.

Lipid phase transition endotherms were collected on a Perkin-Elmer DSC-2 scanning calorimeter equipped with a subambient temperature accessory and a thermal analysis data station. Total membrane polar lipids were rehydrated in a minimal volume of ethylene glycol–water (1:1 v/v) by gentle vortexing and repeated warming to ~45 °C. Samples were scanned at a heating rate of 5 °C/min with ethylene glycol–water in the reference cell. Ethylene glycol was employed to avoid the water transition endotherm which would obscure the lipid transition of the lower melting isomers. A minimum of two complete scans was obtained for each lipid sample. T_m and ΔT_{10-90} were calculated by using the partial-areas computer program provided with the Perkin-Elmer thermal analysis data station.

The various monofluoropalmitic acids were synthesized as described previously (McDonough et al., 1983). The synthesis of the isomers of *cis*-octadecenoic acid has been described elsewhere (Gunstone & Ismail, 1967a). All fatty acids were purified by silicic acid column chromatography and recrystallized from ethanol prior to use and were greater than 99.9% pure as determined by thin-layer chromatography and gas–liquid chromatography. All other chemicals and reagents used were of reagent grade or better.

Results

Fatty Acid Composition of A. laidlawii B Membranes. The fatty acid compositions of membranes of *A. laidlawii*, enriched with each of the isomeric *cis*-octadecenoic acids studied, are

Table I: Fatty Acid Composition of Membranes of *Acholeplasma laidlawii* B Enriched with 8F-16:0 plus a Particular Isomer of *cis*-Octadecenoic Acid

fatty acid supplement	membrane fatty acid composition (mol %)						
	12:0	14:0	16:0	16:1	18:0	18:1	8F-16:0
15% 8F-16:0 + 85% 18:1cΔ4	1.6	1.0	0.8			85.1	11.3
15% 8F-16:0 + 85% 18:1cΔ5		1.4	3.1			85.7	9.8
15% 8F-16:0 + 85% 18:1cΔ6		0.9	0.7	0.3		83.1	14.9
20% 8F-16:0 + 80% 18:1cΔ7	0.1	1.4	1.0	6.8	0.1	73.4	17.2
20% 8F-16:0 + 80% 18:1cΔ8	1.2	0.6	1.8	6.0		72.4	18.0
20% 8F-16:0 + 80% 18:1cΔ9	0.9	1.2	2.1	8.1		72.1	15.6
20% 8F-16:0 + 80% 18:1cΔ10	0.3	1.0	4.2	5.8	1.0	69.8	17.9
15% 8F-16:0 + 85% 18:1cΔ11		5.4	9.2			64.2	21.2
20% 8F-16:0 + 80% 18:1cΔ12	1.0	0.9	1.9	5.9		72.3	18.0
10% 8F-16:0 + 90% 18:1cΔ13	1.8	1.5	0.8	2.3	1.0	81.2	11.3
15% 8F-16:0 + 85% 18:1cΔ14	1.6	1.0	0.8			84.2	12.3
15% 8F-16:0 + 85% 18:1cΔ15	0.9	0.8	1.3			84.0	12.9

listed in Table I for the typical case of coenrichment with 8F-16:0. When other monofluoropalmitic acids were substituted, similar results were obtained. In all cases, the products of de novo fatty acid biosynthesis in *A. laidlawii*, specifically 12:0, 14:0, 16:0, and 18:0 (Saito et al., 1977), contributed minimally to the membrane fatty acid composition, reflecting the effects of inhibition with avidin (Silvius & McElhaney, 1978). In turn, the combination of fatty acids provided exogenously together generally accounted for more than 90% of the total membrane fatty acids present. Levels of enrichment were greatest when the physical properties of the particular *cis*-octadecenoic acid presented the least challenge to the organism's ability to grow (Silvius & McElhaney, 1978). The membranes enriched with 18:1cΔ7, Δ8, Δ9, Δ10, or Δ12 contained significant quantities of 16:1 in addition to the fatty acids provided in supplement. Since *A. laidlawii* B can neither biosynthesize nor degrade unsaturated fatty acids, it must be assumed that this "contaminant" was concentrated from background levels in the media (Saito et al., 1977).

The ratio 18:1cΔ^X/XF-16:0 present in the membrane lipids generally corresponded to that originally provided exogenously, indicating that, as demonstrated previously (McDonough et al., 1983; Macdonald et al., 1983), the monofluoropalmitic acids are readily incorporated by *A. laidlawii*. The generally high levels of supplement incorporation and low levels of background de novo biosynthesis would indicate that the reported order parameters represent almost exclusively the effect of the *cis*-octadecenoic acid of interest upon the orientational order of the monofluoropalmitic acid probe.

Thermotropic Behavior of Membrane Lipids. The membrane lipid gel to liquid-crystalline phase transition is a major effector of fatty acyl chain orientational order. Any comparison of orientational order among a variety of fatty acyl chain structures must necessarily consider the accompanying variation in the temperature of the chain-melting phase transition (T_m). Utilizing DSC, we have determined the position of the phase transition of the polar lipids extracted from each membrane sample employed. Representative endotherms are illustrated in Figure 1. The endotherms were typically somewhat asymmetric and similar to transition endotherms of *A. laidlawii* membrane lipids reported previously from this laboratory [see, for example, Macdonald et al. (1984)]. For any particular isomer of *cis*-octadecenoic acid, the T_m of the membrane polar lipids varied only 1–2 °C over the range of monofluoropalmitic acids utilized. Although transition enthalpies were not calculated in each case, where estimated, ΔH was approximately 80% of that of the corresponding dioctadecenoylphosphatidylcholine (Barton & Gunstone, 1975). The quantity ΔT_{10-90}, corresponding to the

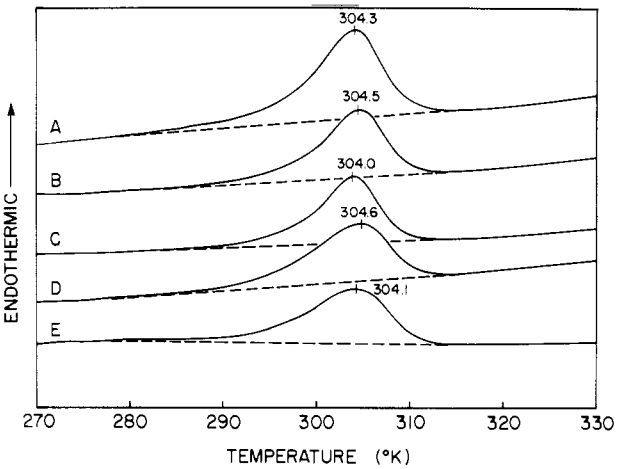

FIGURE 1: Gel to liquid-crystalline phase transition endotherms of total membrane polar lipids extracted from membranes of *A. laidlawii* B enriched with 15% MFPA plus 85% 18:1cΔ4. (A) 6F-16:0; (B) 8F-16:0; (C) 10F-16:0; (D) 12F-16:0; (E) 14F-16:0. The scan rate was 5 °C/min. The dashed lines represent the interpolated base lines.

temperature range over which the phase transition progresses from 10% to 90% of completion, can be used as a measure of the cooperativity of the lipid phase transition in biological membranes. Values of ΔT_{10-90} were in these cases in the range of 6–11 °C, in good agreement with values reported previously from this laboratory for *A. laidlawii* membrane lipids highly enriched with a variety of fatty acids (Silvius et al., 1980).

Figure 2 illustrates the variation of T_m with the position of the cis double bond along the 18-carbon chain. The T_m's of the corresponding synthetic di-*cis*-octadecenoylphosphatidylcholines (Barton & Gunstone, 1975) are included for comparative purposes. In both cases, the T_m's were minimal when the cis double bond was located near the center of the acyl chain and increased progressively as the double bond was relocated toward either the carbonyl head group or the methyl terminus of the acyl chain. The alternation of the values of the melting points exhibited by the fatty acids themselves (Gunstone & Ismail, 1967b) was not observed. Values of T_m for the *A. laidlawii* membrane polar lipids were consistently greater than those for the corresponding di-*cis*-octadecenoylphosphatidylcholines. This effect may be attributed to the presence in the *A. laidlawii* membrane lipids of 10–20% of the generally higher melting point monofluoropalmitic acid as well as to differences arising solely as a result of the glycerolipid head-group composition of *A. laidlawii* membrane lipids. The polar lipids of *A. laidlawii* B consist mainly of five diacyl glycerolipid species, of which two neutral glycolipids, mono- and diglucosyldiacylglycerol, and one phospholipid, phosphatidylglycerol, constitute the

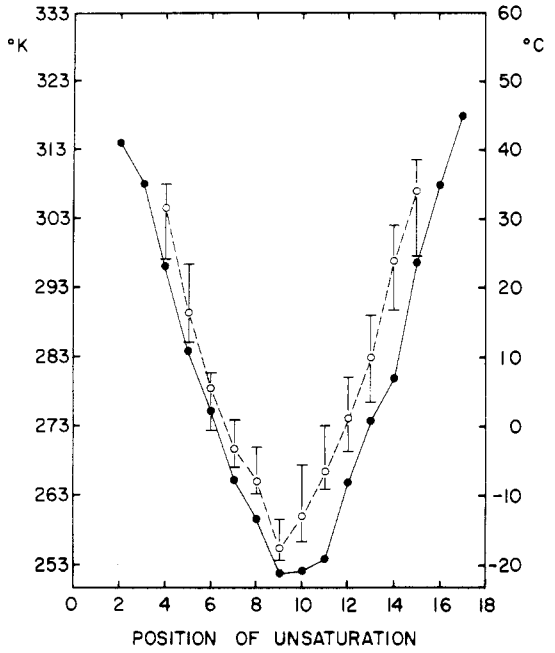

FIGURE 2: Variation of T_m with the position of the site of unsaturation in *A. laidlawii* B total membrane polar lipids. The data on di-*cis*-octadecenoylphosphatidylcholines were taken from Barton & Gunstone (1975). (○) *A. laidlawii* B total membrane polar lipids; (●) 1,2-dioctadecenoyl-*sn*-glycero-3-phosphocholines. The vertical bars represent the values of ΔT_{10-90}.

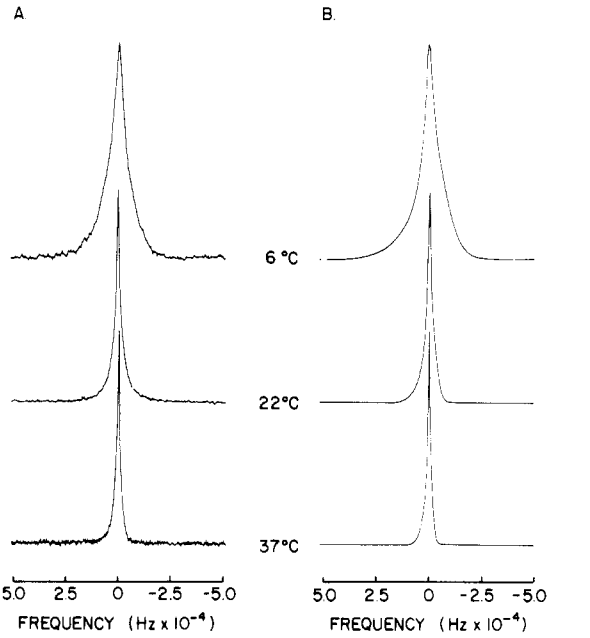

FIGURE 3: ^{19}F NMR spectra of membranes of *A. laidlawii* B enriched with 15% 14F-16:0 plus 85% 18:1cΔ14 (A) and the corresponding computer-simulated spectra (B). The experimental spectra had the following parameters: 6 °C, 100 000 transients and 250-Hz line broadening; 22 °C, 75 000 transients and 150-Hz line broadening; 37 °C, 20 000 transients and 100-Hz line broadening. The simulated spectra had the following parameters: 6 °C, D_0 = 800 Hz, D_1 = 20 000 Hz, S_{mol} = 0.94; 22 °C, D_0 = 150 Hz, D_1 = 20 000 Hz, S_{mol} = 0.36; and 37 °C, D_0 = 100 Hz, D_1 = 20 000 Hz, S_{mol} = 0.20 where D_0 = the intermolecular dipole interaction, D_1 = the intramolecular dipole interaction, and S_{mol} = the orientational order parameter.

major membrane lipid components (Smith, 1971; Wieslander & Rilfors, 1977). When isolated, the two neutral glycolipids generally demonstrate gel to liquid-crystalline phase transition temperatures which are 10–15 °C higher than that for the corresponding diacylphosphatidycholine, while isolated phosphatidylglycerols and phosphatidylcholines have nearly identical T_m's (Silvius et al., 1980). As such, the T_m's of *A. laidlawii* membrane lipids are generally somewhat higher than those of the corresponding phosphatidylcholines (Silvius, 1979).

Typical Experimental and Theoretical Spectra. Examples of experimentally obtained ^{19}F NMR spectra of *A. laidlawii* membrane samples as a function of temperature and the corresponding computer-generated simulated spectra are illustrated in Figure 3 for the case of membranes enriched with 15% 8F-16:0 plus 85% 18:1cΔ14. The ^{19}F NMR spectrum is considered to consist of individual resonance lines corresponding to particular orientations of the motionally averaged ^{19}F chemical shift tensor elements with respect to the magnetic field—each resonance line being subject to broadening due to both intramolecular and intermolecular dipole interactions (Niederberger & Seelig, 1976). The final spectrum can be stimulated, as described previously (Macdonald et al., 1983), by employing a mathematical model which assumes axially symmetric lipid motions and input variables which consider the maximum chemical shift anisotropy, the intramolecular and intermolecular dipole broadening, and also the hydrocarbon chain orientational order parameter S_{mol}, where S_{mol} is defined according to Seelig (1977).

The extensive broadening of the ^{19}F NMR spectrum which accompanies a reduction in temperature and the consequent change in lipid phase state are apparent in Figure 3. The spectral changes are faithfully reproduced in our simulations even for temperatures where the bulk of the membrane lipids are in the gel state. The apparent adequacy of the spectral simulations of gel-state ^{19}F NMR spectra using a model which assumes axial symmetry, and the fact that none of the characteristics of axial asymmetry becomes apparent in ^{13}C (Wittebort et al., 1981) and ^{31}P (Campbell et al., 1979) spectra at temperatures well below the gel to liquid-crystalline phase transition, suggests that, as with these aforementioned nuclei, the assumption of axial symmetry is valid for the ^{19}F NMR spectra of a MFPA at temperatures immediately below the lipid phase transition. Furthermore, as discussed previously (Macdonald et al., 1984), there are theoretical considerations of the time scale of measurement intrinsic to the ^{19}F NMR spectra of MFPA which indicate that a line shape reflecting axially symmetric motions should pertain at temperatures below the gel to liquid-crystalline phase transition.

Representative Order Profiles. ^{19}F NMR order profiles were acquired at a variety of temperatures for each isomer of *cis*-octadecenoic acid from Δ4 through Δ15 inclusive. Representative order profiles are illustrated in Figure 4. At 310 K, all isomers exhibited the progressive decrease of orientational order, with its decline toward the methyl termini of the acyl chains, which is characteristic of the order profiles obtained by other techniques, such as ^2H NMR, at temperatures above the gel to liquid-crystalline phase transition (Seelig, 1977; Stockton et al., 1977). Provided that over the range of temperatures studied the membrane lipids remained in the liquid-crystalline state, the shape of the order profiles remained relatively constant with decreasing temperature, while the overall orientational order increased marginally. This situation can be seen to apply particularly to the cases of the Δ9 and Δ11 isomers, and similar results have been obtained previously by using both ^2H NMR (Seelig & Seelig, 1977) and ^{19}F NMR (Macdonald et al., 1983) techniques. Since the ^{19}F NMR order profiles obtained at 310 K—where in each case the membrane lipids would have assumed the liquid-crystalline state—were highly similar regardless of the position of the site of unsaturation, there was no evidence of the local ordering effect of a cis double bond upon a neighboring saturated acyl chain as reported by Seelig & Seelig (1977). However, since

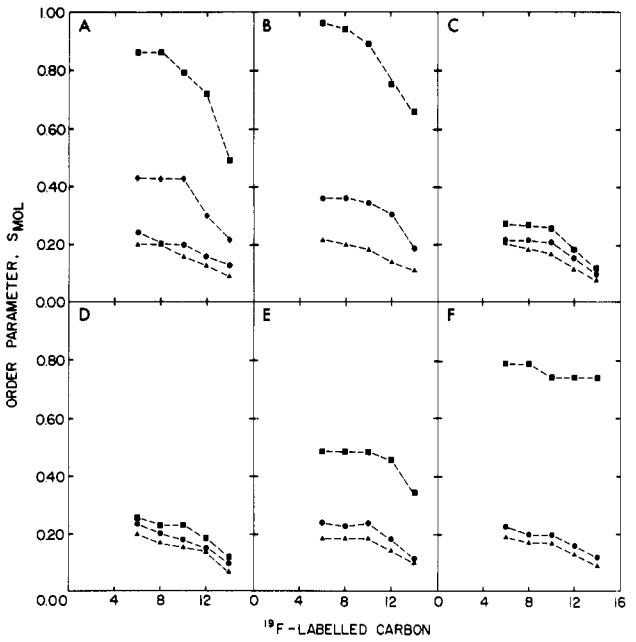

FIGURE 4: Representative ^{19}F NMR order profiles of *A. laidlawii* B membranes enriched with various isomeric *cis*-octadecenoic acids. The acquisition temperatures are listed in parentheses in order, from top to bottom, for a particular case of enrichment. (A) 18:1cΔ15 (279, 295, 310, and 325 K); (B) 18:1cΔ14 (179, 295, and 310 K); (C) 18:1cΔ11 (179, 295, and 310 K); (D) 18:1cΔ9 (279, 289, and 310 K); (E) 18:1cΔ5 (179, 295, and 310 K); (F) 18:1cΔ4 (279, 310, and 315 K).

we report order parameters only for every second methylene segment, this finding is not particularly surprising. When the temperature range which was employed overlapped into or entirely encompassed the gel to liquid-crystalline phase transition, there was a dramatic increase in the overall orientational order with decreasing temperature, and the order profiles for different isomers of *cis*-octadecenoic acid began to acquire distinct dissimilarities. At 279 K, the lowest temperature employed, only four of the *cis*-octadecenoic acid enriched membranes contained large quantities of gel-state lipid, and these corresponded to isomers with positions of unsaturation nearest either the carbonyl head group or the methyl terminus of the acyl chain.

The order profile, in the gel state, of membranes enriched with 18:1cΔ4 was almost flat with individual values of S_{mol} approaching 0.8, indicating a highly ordered state with all methylene segments experiencing an approximately equal degree of disorder. In contrast, the order profile of membranes enriched with 18:1cΔ15, when obtained at 279 K, indicated that a gradient of disorder still existed with S_{mol} varying from 0.86 nearest the carboxyl head group to approximately 0.5 near the fatty acyl chain methyl terminus. A similar gradient of disorder remained at 279 K in membranes enriched with 18:1cΔ14. In addition, a comparison of the order profile at 279 K, in the case of enrichment with 18:1cΔ5, with the order profile obtained at 295 K, in the case of enrichment with 18:1cΔ15, again tends to indicate that the disorder gradient is much more pronounced in the gel state when the site of unsaturation is near the methyl terminus of the acyl chain than when it is near the carboxyl head group, since at these temperatures these two isomers are in approximately equal physical states with respect to the gel to liquid-crystalline phase transition.

Effect of Proximity to T_m. The effect of relative proximity to the gel to liquid-crystalline phase transition upon overall orientational order is illustrated in Figure 5. The chain-average order at a particular temperature for a particular enriched membrane was calculated as the numerical average of the five values of S_{mol} measured for that situation. When the acquisition temperature was 310 K, the overall orientational order increased linearly with increasing proximity to T_m, apparently in a manner independent of the position of the site of unsaturation. At 279 K, a similar yet more pronounced linear increase in the chain-average order was observed with increasing proximity to the phase transition temperature, again apparently independent of the site of unsaturation. As the acquisition temperature of 279 K became equal to and then less than T_m, the overall orientational order increased sharply and eventually appeared to plateau at a high level of orientational order.

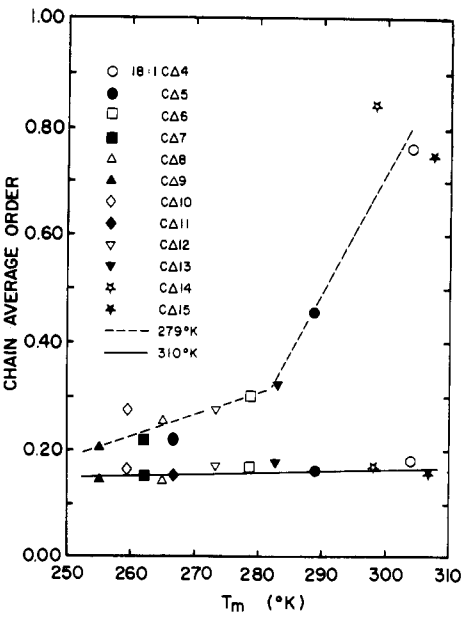

FIGURE 5: Chain average order vs. T_m. Chain average order was calculated as the numerical average of the value of S_{mol} at each position determined for a particular enriched membrane. T_m data were taken from Figure 2.

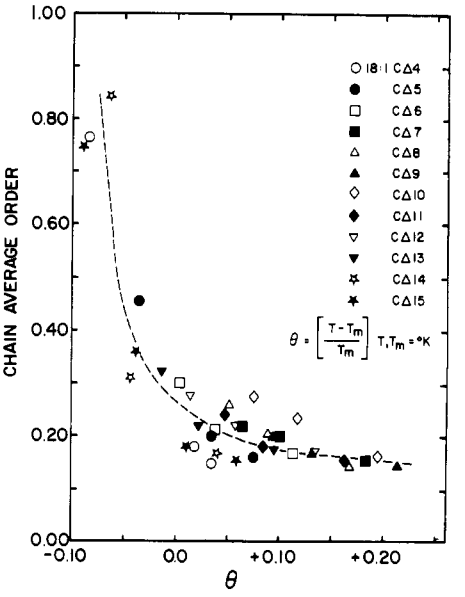

FIGURE 6: Normalization of the chain average order with respect to the T_m.

Normalization with respect to T_m. Figure 6 compares the chain-average order parameters for each isomer of *cis*-octadecenoic acid at the reduced temperature θ. This comparison

is intended to normalize the data with respect to the gel to liquid-crystalline phase transition so that effects attributable to differences in the phase transition temperature can be eliminated or at least minimized (Seelig & Seelig, 1980). The chain-average order parameters in Figure 6 all fall within a relatively narrow band, indicating that all isomers assume approximately similar states of orientational order at equal values of reduced temperature, regardless of the position of the site of unsaturation. The average order increased slowly as the temperature decreased toward the lipid phase transition ($\theta = 0$), began to rise exponentially as that transition was traversed, and plateaued at $S_{mol} \simeq 0.8$. Although a direct comparison of the average order of all isomers at all values of reduced temperature is not possible with these data, it can be discerned that in the region $0 < \theta < +0.12$ isomers with double bonds situated near the center of the fatty acyl chain are generally more ordered than those isomers with sites of unsaturation further toward either the carboxyl head group or the methyl terminus of the fatty acyl chain. In addition, the differences noted earlier in the order profiles of Δ4, Δ5, Δ14, and Δ15 isomers are manifest, particularly at $\theta = -0.4$.

Discussion

The ^{19}F NMR line shape can be interpreted in terms of the orientational order parameter (S_{mol}) of the hydrocarbon long molecular axis. S_{mol} is a quantitative measure of the time-averaged angular excursions of particular methylene segments away from an axis perpendicular to the plane of the bilayer. If S_{mol} was equal to the theoretical maximum of unity for each methylene segment, then the acyl chain would have assumed an all-trans configuration aligned parallel to the normal to the bilayer. Both trans–gauche isomerization at individual methylene segments and tilting of the entire chain with respect to the bilayer normal could contribute to a decrease in the value of S_{mol}. When for each methylene segment the value of S_{mol} had decreased to the minimum, the acyl chains would be experiencing essentially isotropic motion. S_{mol} is defined (Seelig, 1977) such that

$$S_{mol} = (1/2)(3 \langle \cos^2 \theta \rangle - 1)$$

The broken brackets represent an average over time, and θ is the angle between the segment direction and the bilayer normal, where the segment direction is defined as the normal to the plane formed by the H–C–H atoms of a methylene segment.

The effects of cis unsaturation on molecular order in the liquid-crystalline state have been studied in both model and biological membranes. Seelig & Seelig (1977) compared the ordering of the palmitic acyl chain of POPC with its equivalent in DPPC via ^2H NMR. Although a local ordering effect of the cis double bond could be discerned in the order profile of the neighboring palmitic acyl chain, the head to tail distribution of order parameters was generally similar in the presence or absence of an adjacent cis double bond. When compared at the same temperature, the palmitic acyl chains in DPPC were overall more ordered than the palmitic acyl chain in POPC. This result could be directly related to the lower lipid phase transition temperature of POPC (~5 °C) relative to DPPC (41.8 °C). However, when compared at a constant temperature relative to their respective phase transition temperatures, the palmitic acyl chains of DPPC were overall less ordered than those in POPC. Thus, the cis double bond restricted the range of conformations available to the adjacent acyl chain, and this characteristic was evident only when the two systems were compared under conditions where they were subjected to the same average molecular forces.

The quadrupolar splittings of specifically deuterated *sn*-2 oleic acyl chains in POPC exhibited a sharp minimum at the C-10 position, the location of the cis double bond (Seelig & Waespe-Šarčevič, 1978). This observation could be explained in terms of an average inclination of the cis double bond of 7–8° with respect to the bilayer normal. When corrected for this geometric consideration, the order parameter profiles of the unsaturated *sn*-2 acyl chain were very similar to those of the saturated *sn*-1 chain of POPC.

Gally et al. (1979) incorporated specifically deuterated oleic acids into the membrane lipids of a fatty acid auxotroph of *Escherichia coli* and observed the same sharp minimum in the positional dependence of the quadrupolar splittings at the C-10 position that was observed in the model POPC system. Rance et al. (1980) demonstrated that the orientation of the rigid cis double bond inferred from the quadrupolar splittings in POPC and *E. coli* was manifest as well in membranes of *A. laidlawii* enriched with specifically deuterated oleic acids.

The ^2H NMR studies agree then that in the liquid-crystalline state, in both model and biological membranes, the shape of the order profile is relatively constant, whether it be the oleic acyl chain after correction for geometric considerations or a neighboring palmitic acyl chain. Moreover, specifically deuterated elaidic acyl chains incorporated into PEPC exhibit an order profile very similar to POPC or DPPC (Seelig & Waespe-Šarčevič, 1978). Although it is possible to discern the effects of specific structural substituents upon the shape of the order profile in the liquid-crystalline state via ^{19}F NMR (Macdonald et al., 1983) as well as by ^2H NMR (Seelig & Seelig, 1977), these changes are small when compared to the temperature dependence of the order parameters. It seems likely that the plasticity of the liquid-crystalline state is sufficient to accommodate a diversity of fatty acyl structural substituents without seriously affecting the head to tail gradient of configurational probabilities. The present observation that, provided the *A. laidlawii* membrane lipids were in a liquid-crystalline state, the ^{19}F NMR order profiles were similar regardless of the particular isomeric *cis*-octadecenoic acid present lends further credence to this conclusion.

Further to this point, it is instructive to note that the positional dependence of the quadrupolar splittings obtained with specifically deuterated fatty acids incorporated into nematic, smectic, or cholesteric liquid-crystal hosts displays a head to tail gradient of orientational order similar to that observed in a lipid bilayer (Forest & Reeves, 1979; Covello et al., 1983; Davis, 1983; Alcantara et al., 1983). Here the common denominator is a preferred orientation of the molecules along a particular axis, and chain motions which would alter the orientation of the entire acyl chain or a portion thereof are necessarily restricted to those which are concerted or cooperative. An acyl chain experiencing overall isotropic reorientations manifests a gradient of multiple internal rotations (e.g., trans–gauche isomerizations) which accumulate in frequency in a linear fashion from the center of mass (the polar head group) toward the methyl terminus (Brown et al., 1979). It is the interplay of these two forces in the lipid bilayer, restriction due to preferred orientation vs. progressively accumulating internal reorientations, which is manifest in the observed bilayer orientational order profile. In the liquid-crystalline state, trans–gauche isomerizations and tilting of the acyl chains are (relative to the gel state) highly probable and chain-packing restrictions much less severe. Thus, it is not surprising that acyl chains in the liquid-crystalline state are able to accommodate structural substituents in any portion of the chain without markedly affecting the head to tail range

of available conformations when their motional freedom is so great to begin with.

The effect of specific acyl chain structural substituents can be more readily discerned in the liquid-crystalline state via their impact on the relative overall orientational order of adjacent acyl chains. Thus, the palmitic acyl chains of POPC were overall more ordered than those of DPPC (Seelig & Seelig, 1977), and the overall order of MFPA probes was greater in the presence of methyl iso- or anteiso-branched fatty acids than in the presence of a straight-chain saturated fatty acid (Macdonald et al., 1983), when the comparisons were made at a constant temperature above the particular lipid phase transition. This effect can be correlated with the cross-sectional area occupied by acyl chains of different structure (Macdonald et al., 1983), where the larger area occupied by one chain type restricts and therefore orders the adjacent one. The present observations, that MFPA's were more ordered in the presence of *cis*-octadecenoic acids with sites of unsaturation near the center of the acyl chain than when the site of unsaturation was closer to the carbonyl head group or the methyl terminus, when compared at a constant "reduced" temperature, can again be correlated to the greater cross-sectional areas occupied by *cis*-octadecenoic with sites of unsaturation near the center of the chain as determined by monolayer studies (Rakshit et al., 1981).

In the gel state, fatty acyl chain-packing densities increase substantially, and the restriction of acyl chain motional freedom is severe. Thus, the orientational order parameters of straight-chain saturated fatty acids approach the theoretical maximum in the gel state whether measured via ^{2}H NMR (Allegrini et al., 1983) or ^{19}F NMR (Macdonald et al., 1984). One might predict that the inclusion of structural substituents which perturbed the acyl chain packing and relieved the restriction of motional freedom would manifest this effect in the orientational order profile. The gel-state order profiles of membranes enriched with 18:1cΔ14 or 18:1cΔ15 indicate that when a cis double bond is located proximal to the methyl terminus of an acyl chain, it perturbs the chain packing in the gel state sufficiently to permit a substantial increase in motional freedom in its immediate vicinity. This can be most readily appreciated when it is noted that the ^{19}F NMR gel-state order profiles of *A. laidlawii* membranes enriched with palmitic acid are characteristically flat, with any residual disordering being evenly distributed along almost the entire length of the acyl chain (Macdonald et al., 1984). When the cis double bond is located proximal to the acyl chain carbonyl head group, there is little evidence of a local increase in motional freedom, but rather there is a decrease in orientational order at all chain segments relative to the comparable situation in palmitic acid enriched *A. laidlawii* membranes. This observation can be most readily interpreted if it is assumed that the portion of the acyl chain posterior to the cis double bond is tilted with respect to the bilayer normal and experiences an approximately equal and small degree of disordering at each chain position due to trans–gauche isomerization. This circumstance further suggests that the acyl chains are less susceptible to packing perturbations in the region near the carbonyl head group than near the methyl terminus. Therefore, even in the gel state, the stringency of packing restrictions is subject to variation across the width of the bilayer. This conclusion is supported by a ^{2}H NMR study of a mixture of deuterated palmitic acid and lysophospholipid (Allegrini et al., 1983) in which it was reported that, although quadrupole splittings approached the theoretical maximum in the gel state for chain segments C-2–C-13, at positions further toward the chain terminus orientational order still decreased progressively and substantially.

While relative proximity to the lipid phase transition appears to be the primary determinant of overall orientational order, secondary considerations related to chain structure do apparently affect relative molecular oder in both the liquid-crystalline and gel phase. The disordering effect of the various isomeric cis double bonds in the gel state does not contradict, but rather complements, the ordering effect of these and other substituents in the liquid-crystalline state. The properties of these substituents which tend to increase the orientational order in the liquid-crystalline state, i.e., an irregular, relatively inflexible structure, are those which in turn result in a perturbation and disordering of the gel state. Several experimentally verifiable predictions follow from this conclusion. The ^{19}F NMR order profiles of methyl iso- and methyl anteiso-branched fatty acid enriched membranes in the gel state should demonstrate a gradient of disorder analogous to that observed in the presence of 18:1cΔ14 and 18:1cΔ15. Similarly, the gel-state ^{19}F NMR order profiles in the presence of 18:1cΔ9 should be characteristically disordered if the liquid-crystalline/gel-ordered/disordered duality holds. Furthermore, one might predict that, if the temperature of the gel to liquid-crystalline phase transition is a reflection of the stability of the gel state, those acyl chain structures which most decrease the phase transition temperature should display the greatest gel phase disorder.

Finally, some points addressing the relevance of studies of the gel state of membrane lipids are as follows. Although many organisms are capable of "homeoviscous" or "homeophasic" adaptation, and adjust their membrane lipid composition under conditions of environmental stress so as to maintain a suitably "fluid" or liquid-crystalline state, other organisms, such as *A. laidlawii* B, are incapable of such compositional tailoring [for recent reviews, see Russell (1984) and McElhaney (1984)]. However, growth of *A. laidlawii* B remains unhindered until the proportion of gel-state lipid reaches 50% and ceases entirely only when the proportion of gel-state lipid exceeds approximately 90%. Thus, the gel state can become of direct biological relevance. Furthermore, it is through studies of the gel state of lipid bilayers that insights into the manner in which specific fatty acyl structural variants accomplish their role as "fluidizers" will be gained.

Registry No. 18:1cΔ4, 19308-06-6; 18:1cΔ5, 676-29-9; 18:1cΔ6, 593-39-5; 18:1cΔ7, 13126-31-3; 18:1cΔ8, 5684-71-9; 18:1cΔ9, 112-80-1; 18:1cΔ10, 2442-70-8; 18:1cΔ11, 506-17-2; 18:1cΔ12, 13126-37-9; 18:1cΔ13, 13126-39-1; 18:1cΔ14, 13126-41-5; 18:1cΔ15, 13126-43-7; 6F-16:0, 58763-54-5; 8F-16:0, 86569-21-3; 10F-16:0, 90866-62-9; 12F-16:0, 90866-63-0; 14F-16:0, 86569-22-4.

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Effects of a Surfactant-Associated Protein and Calcium Ions on the Structure and Surface Activity of Lung Surfactant Lipids†

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ABSTRACT: Previous studies have demonstrated that lung-specific proteins are associated with surfactant lipids, particularly the highly surface active subfraction known as tubular myelin. We have isolated a surfactant-associated protein complex with molecular weight components of 36 000, 32 000, and 28 000 and reassembled it with protein-free lung surfactant lipids prepared as small unilamellar liposomes. The effects of divalent cations on the structure and surface activity of this protein–lipid mixture were investigated by following (1) the state of lipid dispersion by changes in turbidity and by electron microscopy and (2) the ability of the surfactant lipids to form a surface film from an aqueous subphase at 37 °C. The protein complex markedly increased the rate of Ca^{2+}-induced surfactant–lipid aggregation. Electron microscopy demonstrated transformation of the small unilamellar liposomes (median diameter 440 Å) into large aggregates. The threshold Ca^{2+} concentration required for rapid lipid aggregation was reduced from 13 to 0.5 mM by the protein complex. This protein-facilitated lipid aggregation did not occur if Mg^{2+} was the only divalent cation present. Similarly, 5 mM Ca^{2+} but not 5 mM Mg^{2+} improved the ability of the protein–lipid mixture to form a surface film at 37 °C. Extensive aggregation of the surfactant lipids without protein by 20 mM Ca^{2+} or 20 mM Mg^{2+} did not promote rapid surface film formation. These results add to the growing evidence that specific Ca^{2+}–protein–lipid interactions are important in determining both the structure and function of extracellular lung surfactant fractions.

Pulmonary surfactant lipids are synthesized in the alveolar type II cell [reviewed in Van Golde (1976)]. Within the cell, these lipids are tightly packed into a membrane-bound organelle, the lamellar body (Gil & Reiss, 1973; Williams, 1982). After secretion of the lamellar body contents into the liquid layer lining the alveolar surface, at least some of the lamellae are transformed into tubular myelin, a characteristic latticelike structure found in fetal lung liquid (Williams, 1977), adult lungs (Williams, 1982), and lung washings (Gil & Reiss, 1973). This structure is thought to be the immediate precurosor of the monomolecular surface film which stabilizes the

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