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Influence of Lipid Composition on the Orientational Order in *Acholeplasma laidlawii* Strain B Membranes: A Deuterium NMR Study†

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ABSTRACT: ^{2}H NMR techniques have recently been developed to determine the complete orientational order profile of lipid bilayers employing lipids containing perdeuteriated palmitic acid [Lafleur, M., Fine, B., Sternin, E., Cullis, P. R., & Bloom, M. (1989) *Biophys. J. 56*, 1037–1041]. In this work, these techniques have been applied to study order profiles in intact membranes derived from *Acholeplasma laidlawii* strain B. It is shown that complete orientational order profiles can be readily obtained from the intact membranes of *A. laidlawii* B grown on equimolar amounts of perdeuteriated palmitic acid and a nondeuteriated fatty acid of varying length and unsaturation. By variation of the fatty acid composition employing mixtures of perdeuteriated palmitic acid with myristic, elaidic, oleic, or linoleic acid, a range of hydrocarbon order compatible with high rates and extents of cell growth has been obtained where the average order parameter, $\langle S \rangle$, varies over the range 0.140–0.176. This same variation in order is seen for liposomes derived from total lipids extracted from these intact membranes. ^{2}H NMR studies on liposomes composed of individual species of the extracted lipids indicate that modulation of the membrane lipid headgroup composition has the potential to play an important role in maintaining the membrane order within this range.

Deuterium nuclear magnetic resonance spectroscopy (^{2}H NMR)[1] is an important technique for characterizing order and dynamics in the hydrocarbon region of model and biological membranes (Davis et al., 1980; Lafleur et al., 1989; Rance et al., 1982; Jarrell et al., 1982). Orientational order profiles of the hydrocarbon chains have usually been constructed using a series of membranes each containing a specifically CD_2-labeled fatty acyl chain. Recent reports have detailed the development of convenient ^{2}H NMR procedures to obtain complete hydrocarbon order profiles for lipid systems containing perdeuteriated long-chain saturated hydrocarbons. This includes systems containing perdeuteriated tetradecanol (Sternin et al., 1988) as well as systems containing perdeuteriated palmitic acid (Lafleur et al., 1989, 1990a). Here we extend this type of measurement to intact biological membranes in which perdeuteriated palmitic acid has been biosynthetically incorporated into the membrane lipids. The membranes of the mycoplasma *Acholeplasma laidlawii* strain B are ideally suited for such a study since the fatty acid composition of membrane lipids can be widely manipulated by the incorporation of exogenously supplied fatty acids [see, for example, Silvius and McElhaney (1978)], and because these membranes have been extensively studied by many physical techniques (McElhaney, 1984, 1989). The use of one preparation containing perdeuteriated palmitic acid, as opposed to a series of preparations each containing a different specifically CD_2-labeled palmitic acid, offers significant advantages with respect to cost and time and minimizes the biological variability inherent in the use of different preparations. Here we show that biosynthetically incorporated perdeuteriated palmitic acid in *A. laidlawii* allows the straightforward generation of the complete hydrocarbon order profile for a saturated chain. We have employed this technique to investigate the range of order profiles consistent with normal growth characteristics of this organism. Further, we have examined the hydrocarbon order in liposomal dispersions derived from total membrane lipid extracts and from the major *A. laidlawii* membrane lipids. Order profiles measured for these systems indicate that the nature of the lipid polar headgroups in the intact *A. laidlawii* membrane can strongly modulate the order profile.

MATERIALS AND METHODS

The fatty acids used in this study were obtained from Nuchek Prep Inc. (Elysian, MN). Palmitic acid was perdeuteriated using the methodology of Hsiao et al. (1974), purified by column chromatography, and recrystallized from ethanol.

Isolation of A. laidlawii Membranes. The organism *A. laidlawii* B was cultured in the presence of the desired exogenously supplied fatty acids under conditions where endogenous fatty acid biosynthesis and exogenous fatty acid chain elongation have been inhibited by the inclusion of avidin in the growth medium [see Silvius and McElhaney (1978)]. The membranes were isolated by differential centrifugation after cell lysis by osmotic shock [see Silvius and McElhaney (1978)].

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[1] Abbreviations: MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; PG, phosphatidylglycerol; GPDGDG, glycerophosphoryldiglucosyldiacylglycerol; O-APG, *O*-aminoacylphosphatidylglycerol; GLX, glycolipid-X; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; FID, free induction decay; TLC, thin-layer chromatography.

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Preparation of Samples for ²H NMR Spectroscopy. Intact *A. laidlawii* membranes were prepared for ^{2}H NMR spectroscopy by a method that is essentially similar to that described by de Kruijff et al. (1976). Briefly, a fresh sample was centrifuged for 20 min at 45 000 rpm (Beckman Ti75 rotor) and the pellet resuspended in deuterium-depleted buffered water (20 mM Hepes/100 mM NaCl, pH 7.5), recentrifuged as above, and resuspended in the same buffer to a final volume of 0.7 mL. Lipid dispersions for ^{2}H NMR spectroscopy were prepared by the gentle vortexing of the freeze-dried lipid with either the deuterium-depleted buffer or deuterium-depleted water at temperatures above that of the gel/liquid-crystalline-phase transition temperature of the lipid sample. Under comparable conditions, ^{2}H NMR spectra of a sample dispersed in deuterium-depleted water were indistinguishable from those of dispersions of the same material in deuterium-depleted buffer.

Extraction of A. laidlawii Lipids and Preparation of the Polar Lipid Fraction. Total lipid extracts were obtained by a modified method of Bligh and Dyer (1959). The aqueous dispersion of intact *A. laidlawii* membranes was diluted with an equal volume of methanol and the mixture heated at 70 °C for 45 min. After the mixture had cooled, it was diluted with an equal volume of water and 4 times its volume of chloroform. After vigorous shaking, the chloroform layer was separated and washed twice with ice-cold 2 M hydrochloric acid[2] and twice with water. The chloroform layer was dried by filtration through chloroform-wetted filter paper and concentrated by rotary evaporation. Next, the lipid concentrate was redissolved in benzene/acetone (1:1 by volume), and the final traces of water were removed by azeotropic evaporation of the solvent. The dried lipid extract was redissolved in chloroform and applied to a column of silicic acid (Biosil A, Bio-Rad) in chloroform. The column was washed with at least 3 volumes of chloroform, and the polar lipids were subsequently eluted with methanol. The methanol fraction was subsequently concentrated to dryness by rotary evaporation, and the polar lipids were freeze-dried from benzene, flushed with nitrogen, and stored at 20 °C until required.

Thin-Layer Chromatography. Analytical TLC was used to determine if there was significant lipid degradation during the NMR experiments, and preparative TLC was used primarily in the determination of the lipid polar headgroup composition of the *A. laidlawii* membrane preparations. Samples were analyzed on 0.5-mm silica gel G plates which were developed with a solvent system consisting of chloroform/methanol/water (75:25:3, by volume). The lipid components were visualized either by charring (analytical studies) or by staining with iodine (preparative studies).

Column Chromatographic Separation of A. laidlawii Membrane Lipids. The chromatographic separation of the MGDG, DGDG, and PG present in the total polar lipid extract of *A. laidlawii* membranes was as follows. The total polar lipid fraction was redissolved in chloroform and applied to a column of silica gel (Davisil grade 634, Aldrich) in chloroform. The column was washed with chloroform and then developed sequentially with 10 column volumes each of chloroform/acetonitrile (75:25 by volume) and of chloroform/acetonitrile (1:1 by volume), 4 column volumes of acetonitrile, 10 column volumes of chloroform/methanol (95:5 by volume), 4 column volumes of chloroform/methanol (90:10 by volume), and 10 column volumes each of chloroform/methanol (85:15 by volume) and then chloroform/methanol (80:20 by volume) and finally chloroform/methanol (7:3 by volume). Under these conditions, the MGDG elutes in the chloroform/acetonitrile 1:1 fractions, the DGDG in the chloroform/methanol 95:5 fractions, and the PG mainly in the chloroform/methanol 90:10 and 80:20 fractions. The fractions containing each of the purified lipid components were separately concentrated by rotary evaporation, lyophilized from benzene, and stored at −20 °C until required.

Determination of Polar Headgroup Composition. After separation of the polar lipid components by preparative TLC, the individual components were visualized and the various lipid fractions scraped quantitatively into clean screw-capped tubes. At this stage, a known amount of an appropriate phosphatidylcholine was added as an internal standard, and the entire mixture was transesterified with 5% H_2SO_4 in methanol. The lipid components were each quantified by gas chromatographic analysis of the methyl esters formed, and from this, the polar lipid composition of the given lipid mixture was determined.

²H NMR Measurements. ^{2}H NMR measurements were performed at 46.175 MHz on a home-built spectrometer (Sternin, 1985). The quadrupolar echo pulse sequence (Davis, 1983) was used with a 300-ms recycle delay time, a 50-μs interpulse spacing, and a 30.5-μs ring-down delay. FIDs were acquired with an eight-step phase cycle sequence. Spectral widths for all spectra acquired were either 200 or 500 kHz. Experiments were repeated for all systems studied with the exception of the myristic acid-containing samples.

Derivation of Order Profiles. The spectra of *A. laidlawii* membranes and derived liposomes recorded at 37 °C are typical of lipids bearing perdeuteriated chains in the L_α phase. Since the lipid system shows an axial symmetry, it is possible to apply a method previously discussed (Sternin et al., 1988; Lafleur, et al., 1989) to derive the general shape of the order profile. First, dePakeing was performed by the method of Sternin et al. (1983). The dePaked spectrum represents the continuous probability distribution of order for the deuteriated acyl chain.[3] Assuming that CD_2 groups contribute equal intensity to the dePaked spectrum and that there is a monotonic decrease of order from the interface toward the middle of the bilayer, an average value of the quadrupolar splitting, $\Delta\nu_q$, was assigned to each methylene group denoted by its acyl chain position, n = 2, 3, ..., 16. The order parameters, $S(n)$, were then calculated using eq 1 where e^2qQ/h = 167 kHz is

$$\Delta\nu_q = \frac{3}{4}\frac{e^2qQ}{h}S(n) \qquad (1)$$

the quadrupolar coupling constant (Davis, 1983). Average order values, mentioned in the text, were determined by calculating an arithmetic mean, $\langle S \rangle$, for the $S(n)$, for $2 \le n \le 16$. Corrections were made for $S(16)$ by a linear extrapolation of $S(14)$ and $S(15)$ (Lafleur et al., 1989).

RESULTS

The first set of experiments was aimed at demonstrating that the ^{2}H NMR dePakeing approach for obtaining mem-

[2] The acid washing step converts PG (and all other negatively charged lipids) to the protonated form. This step is essential to ensure a predictable and consistent elution of PG during the column chromatographic separation of MGDG, DGDG, and PG.

[3] As is often found in studies of this type, a small departure from the Pake doublet shape manifests itself in a small negative-going base line just outside the main dePaked spectrum (see Figure 1). This flat negative spectral region was used to define the base line. Changes in the order profile arising from different plausible choices for the base line are of the order of the random changes resulting from repetition of the experiment.

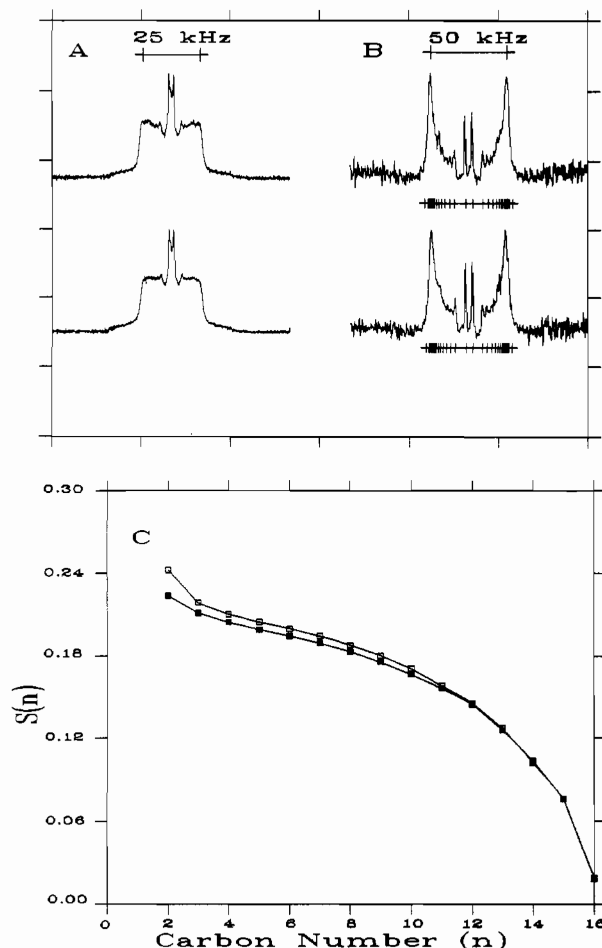

FIGURE 1: Sample ^{2}H NMR spectra (A) and corresponding dePaked spectra (B) of *A. laidlawii* B intact membranes and derived liposomes with a fatty acyl chain composition of $16{:}0d_{31}/18{:}1c\Delta9$, 47:53 (mol %). (c) Order profiles derived from (B) as described under Materials and Methods. The ticks beneath the dePaked spectra give the frequency assigned to a carbon position of unit area (Lafleur et al., 1989). Spectra were recorded at 37 °C, using the quadrupolar echo pulse technique (Davis 1983). A total of 60 000 transients were recorded for each spectrum.

brane hydrocarbon order profiles could be applied to an intact biological membrane system. Here, perdeuteriated palmitic acid was biosynthetically incorporated into the membrane lipids of *A. laidlawii* B and not simply intercalated into the membrane bilayers. This was achieved by culturing the microorganism in avidin-containing media supplemented with perdeuteriated palmitic acid and another fatty acid. Under these conditions, endogenous fatty acid synthesis is suppressed, and the growth of the microorganism is totally dependent on the exogenous supply of fatty acids (Silvius & McElhaney, 1978). It should also be noted that under these conditions the exogenous $16{:}0d_{31}$ is esterified almost exclusively to the *sn*-1 position of the glycerol backbone (McElhaney & Tourtellotte, 1970; Saito et al., 1977). The ^{2}H NMR spectra obtained from the purified *A. laidlawii* membrane, as well as liposomes prepared from the total extracted lipids, is shown in Figure 1A. The "dePaked" spectra derived from these line shapes are shown in Figure 1B. Few of the quadrupolar splittings arising from deuterons at different positions on the chain can be resolved; however, a smoothed orientational order profile can be achieved by assuming a monotonic decrease in order along the acyl chain (Sternin et al., 1988; Lafleur et al., 1989). The resulting order profiles derived from the dePaked spectra are shown in Figure 1C. Two points may be noted: first, as expected, both profiles exhibit the plateau region characteristic of lipid bilayer systems (Seelig & Seelig, 1980); second, the order profile in the intact membrane is essentially the same, within experimental error, as that observed for the liposomes composed of the extracted lipids.

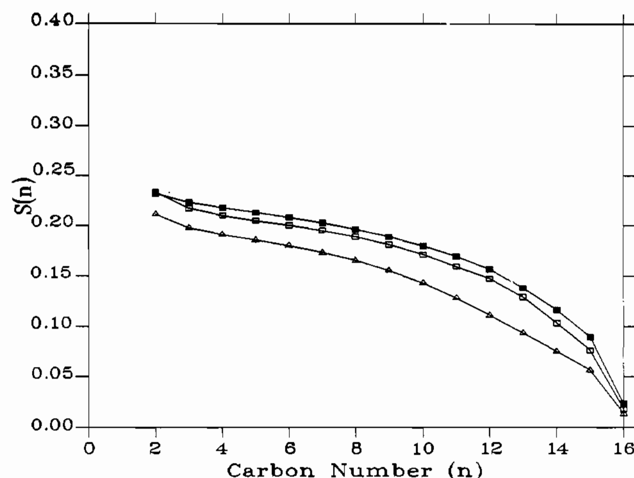

FIGURE 2: Order profiles derived from the dePaked spectra of intact *A. laidlawii* membranes. All spectra were the sum of 60 000 transients, recorded at 37 °C. Fatty acid compositions corresponding to individual profiles are as follows: $16{:}0d_{31}/18{:}1t\Delta9$, 46:54 mol % (closed rectangles); $16{:}0d_{31}/18{:}1c\Delta9$, 47:53 mol % (open rectangles); $16{:}0d_{31}/18{:}2c\Delta9,\Delta12$, 41:59 mol % (open triangles).

A major objective of this study was to determine the range of order profiles compatible with growth of the microorganism *A. laidlawii* B. Previous studies of fatty acid-enriched and fatty acid-homogeneous cultures of *A. laidlawii* B have shown that the growth of the microorganism is inhibited when the growth temperature is more than 50 °C above the gel/liquid-crystalline-phase transition temperature of the membrane lipids, or when more than 50% of the membrane lipids are in the gel state at any given growth temperature (McElhaney, 1974; Silvius & McElhaney, 1978; Silvius et al., 1980). Thus, the organism will grow poorly or not at all on pure palmitic or pure oleic acid, for example (vide infra). Given these limitations, there may be a range of membrane lipid order within which normal growth and function can occur. It is possible to examine the membrane hydrocarbon order at the limits of growth by varying the effective unsaturation of fatty acids supplied to the *A. laidlawii* growth medium. We have done this in two ways: (1) by varying the number and type (cis or trans) of double bonds in the fatty acids, keeping the saturated:unsaturated ratios constant; (2) by varying the ratios of saturated and cis-monounsaturated fatty acids.

In order to compare the variation in $\langle S \rangle$ derived from our *A. laidlawii* membrane preparations with those in fluid model membranes, we have measured $\langle S \rangle$ for $DPPC_{d62}$ and for $DOPC/POPC_{d31}$ (90:10) liposomes. These values correspond to $\langle S \rangle = 0.177$ and $\langle S \rangle = 0.133$ for the $DPPC_{d62}$ and $DOPC/POPC_{d31}$ liposomes, respectively, and are used to define the maximum and minimum observable values of hydrocarbon order for a fluid membrane lacking cholesterol. Since $DPPC_{d62}$ is only fluid at temperatures above 40 °C, we have measured the order parameters at 42 °C for both of the above liposome samples. On the basis of differences in order profile measurements of the $POPC_{d31}$ dispersions taken at different temperatures, we estimate that the values of $\langle S \rangle$ given above for both the $DPPC_{d62}$ and $DOPC/POPC_{d31}$ (90:10) are approximately 5% lower than expected at 37 °C.

The order profiles shown in Figure 2 are those of *A. laidlawii* B membranes prepared from fatty acid auxotrophic cultures grown at 37 °C in the presence of predeuteriated palmitic

Table I: ALB Membrane Lipid Composition

fatty acid compositions	MGDG	DGDG	PG	OAPG + GPDGDG	GLX
$16{:}0d_{31}/14{:}0$, 45:54 (mol %)	4	7	19	9	60
$16{:}0d_{31}/18{:}1t\Delta9$, 46:54 (mol %)	34	34	26	5	tr
$16{:}0d_{31}/18{:}1c\Delta9$, 78:22 (mol %)	55	5	18	1	21
$16{:}0d_{31}/18{:}1c\Delta9$, 47:53 (mol %)	33[a]	25[a]	27[a]	15[a]	tr
$16{:}0d_{31}/18{:}1c\Delta9$, 21:79 (mol %)	34	28	28	9	tr
$16{:}0d_{31}/18{:}2c\Delta9,\Delta12$, 41:59 (mol %)	32	21	23	24	tr

[a] Average from analyses of several *A. laidlawii* ($16{:}0d_{31}/18{:}1c\Delta9$) preparations.

acid and an equimolar quantity of either elaidic ($18{:}1t\Delta9$), oleic ($18{:}1c\Delta9$), or linoleic ($18{:}2c\Delta9,\Delta12$) acids. As may be expected, the order decreases for more unsaturated acyl chain substituents in the sequence $16{:}0d_{31}/18{:}1t\Delta9 > 16{:}0d_{31}/18{:}1c\Delta9 > 16{:}0d_{31}/18{:}2c\Delta9,\Delta12$. This trend was not unexpected and is essentially consistent with what one would expect from an examination of the melting points of the three unsaturated fatty acids concerned as well as the results of previous ^{19}F NMR studies of MacDonald et al. (1984, 1985a,b). The average order $\langle S\rangle$ (calculated as described under Materials and Methods) decreased by approximately 20% over the range where $\langle S\rangle = 0.173$ for the $16{:}0d_{31}/18{:}1t\Delta9$ system and 0.140 for the $16{:}0d_{31}/18{:}2c\Delta9,\Delta12$ preparation. It should be noted that, in particular, the linoleate content of the linoleate-containing membranes (59 mol %; see Table I) is close to the maximum which can be incorporated into *A. laidlawii* B membranes while supporting normal or near-normal growth of the microorganism (Silvius, 1979). Thus, the $\langle S\rangle = 0.140$ may well be approaching the lower limits of hydrocarbon order which can support normal growth and membrane function in *A. laidlawii* B at 37 °C.

In the next series of experiments, the order profiles were determined for *A. laidlawii* B membranes in which the ratios of $16{:}0d_{31}$ and $18{:}1c\Delta9$ in the membrane were varied. It has been demonstrated previously that neither highly saturated (e.g., palmitic acid) nor highly unsaturated (e.g., oleic acid) fatty acids alone can support the growth of *A. laidlawii* B when made fatty acid auxotrophic by the inclusion of avidin in the growth medium. However, the organism will grow normally when the growth medium is supplemented with mixtures of a high-melting and a low-melting fatty acid (Silvius & McElhaney, 1978). These limitations can be rationalized by the suggestion that *A. laidlawii* membrane lipids derived from appropriate fatty acid mixtures will have an order ($\langle S\rangle$) within the range that can support normal growth and membrane function. Membrane lipids derived from either of the pure fatty acids, however, will result in either membranes that are too disordered or membranes containing too much gel-phase lipid for the microorganism to function normally at the growth temperature (vide infra).

In recent experiments, it was found that mixtures of palmitic and oleic acids can support good cell growth provided that the mixture contains no more than 80 mol % of any single component (Lewis et al., unpublished results). Assuming that these are close to the limits of saturation consistent with the normal growth and membrane function of the microorganism, we measured the hydrocarbon order in intact *A. laidlawii* membranes grown in media supplemented with $16{:}0d_{31}/18{:}1c\Delta9$ (80:20) and $16{:}0d_{31}/18{:}1c\Delta9$ (20:80). The observed

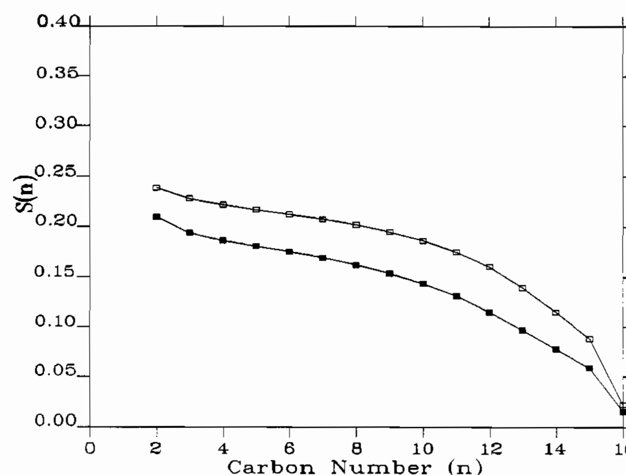

FIGURE 3: Order profiles of *A. laidlawii* intact membranes from spectra recorded at 37 °C. The top and bottom profiles correspond to membranes containing the fatty acid compositions $16{:}0d_{31}/18{:}1c\Delta9$ 78:22 mol % and $16{:}0d_{31}/18{:}1c\Delta9$ 21:79 mol %, respectively. Methods for deriving the order profiles are described in the text.

order profiles are illustrated in Figure 3. The systems containing larger amounts of oleic acid are significantly more disordered, as expected. This is expressed by the average order $\langle S\rangle$ which decreases from 0.176 for the $16{:}0d_{31}/18{:}1c\Delta9$ (80:20) membranes to 0.140 for the $16{:}0d_{31}/18{:}1c\Delta9$ (20:80)-containing membranes. Since DSC studies (data not presented) have shown that the upper boundary of the gel/liquid-crystalline-phase transition of the palmitate (80)/oleate (20) membranes falls above the growth temperature of 37 °C, it seems likely that the order parameters observed with these membranes may be close to the upper limits that are compatible with the normal functioning of *A. laidlawii* membranes (at 37 °C). It should be noted that the $16{:}0d_{31}/18{:}1c\Delta9$ (80:20) membranes gave rise to a spectrum characteristic of a mixture of gel (approximately 50% based on enthalpy estimates from DSC measurements, data not shown) and liquid-crystalline phases at 37 °C. Thus, it was necessary to perform a spectral subtraction method outlined previously to obtain an order profile for those lipids in the liquid-crystalline state (Vist & Davis, 1990). Spectra obtained at 37 and 42 °C were used in this case. We estimate that $\langle S\rangle = 0.176$ is probably 5% lower than what would be obtained from a pure liquid-crystalline membrane preparation based on the difference between order profile determinations of POPC dispersions at different temperatures.

In an effort to further probe these limits, we have also examined the order profile of membrane preparations isolated from cultures supplemented with the fatty acids $16{:}0d_{31}$ and 14:0. Studies on *A. laidlawii* membranes have previously shown that good growth occurs when *A. laidlawii* is cultured in an avidin-free medium on single fatty acid species with chain lengths in the interval 13–19 carbons (Silvius et al., 1980; McElhaney, 1984). The order profiles obtained from intact membranes and liposomes composed of the total extracted lipids of *A. laidlawii* grown on an equimolar mixture of perdeuteriated palmitic acid and myristic acid are shown in Figure 4. It may be observed that order in the intact membrane is within the range given above, where $\langle S\rangle = 0.176$. DSC studies of this system (not shown) indicate that a small proportion (approximately 10–15%) of the membrane lipids are in the gel phase. A gel-phase component was not detected in the ^{2}H NMR spectrum probably due to a combination of the signal-to-noise ratio obtained and the breadth of frequencies over which such a gel-phase component would

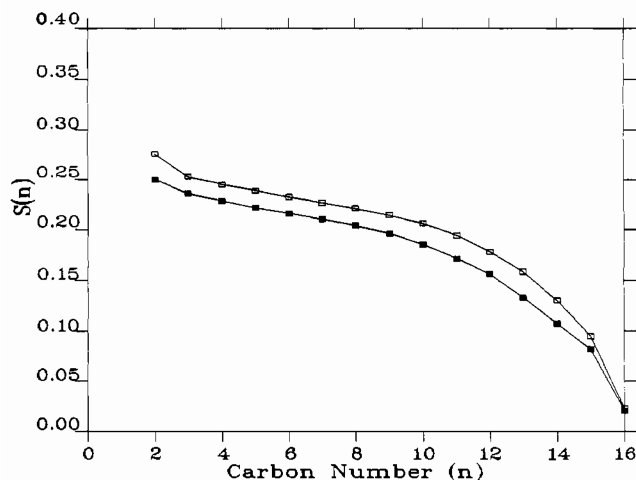

FIGURE 4: Order profiles of intact *A. laidlawii* membranes (closed rectangles) and derived liposomes (open rectangles) with the following fatty acid composition: 16:0d_{31}/14:0, 46:54 mol %. Spectra were recorded at 37 °C. A total of 60 000 transients were recorded for each spectrum. Methods for deriving the order profiles are described in the text.

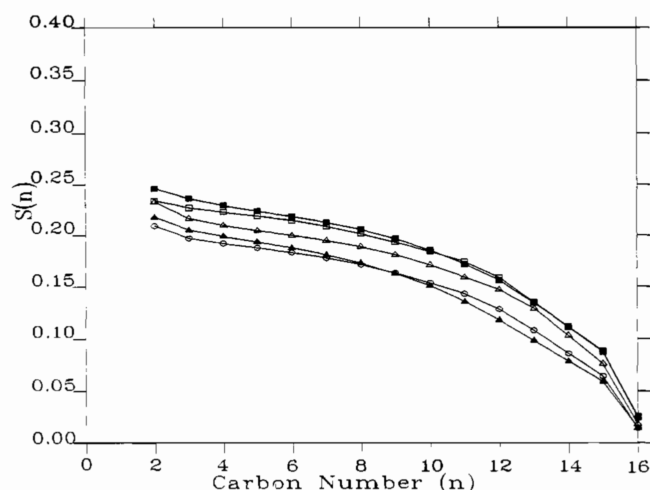

FIGURE 5: Order profiles obtained from the dePaked spectra of derived *A. laidlawii* liposomes. All spectra were the sum of 60 000 transients, recorded at 37 °C. Symbols used in profiles corresponding to specific fatty acid compositions are as follows: 16:0d_{31}/18:1cΔ9, 78:22 mol % (closed rectangles); 16:0d_{31}/18:1tΔ9, 46:54 mol % (open rectangles); 16:0d_{31}/18:1cΔ9, 47:53 mol % (open triangles); 16:0d_{31}/18:2cΔ9,Δ12, 41:59 mol % (closed triangles); 16:0d_{31}/18:1cΔ9, 21:79 mol % (open circles). Methods for deriving the order profiles are described in the text.

normally be observed. The order in the derived liposomes was found to be significantly higher than in the intact membrane (⟨S⟩ = 0.195), in contrast to the system grown on 16:0d_{31}/18:1cΔ9 (see Figure 1). In an attempt to understand the basis of this difference, the lipid composition of these *A. laidlawii* membranes was analyzed and compared to the compositions of the other preparations employed in this study. As shown in Table I, the membrane grown on the 16:0d_{31}/14:0 mixture contained large amounts of a glycolipid (referred to as GLX) which was synthesized at the expense of MGDG and DGDG. The structure of this lipid has only recently been characterized in *A. laidlawii* membranes (Bhakoo et al., 1987) and exhibits a hydrocarbon region comprised of an exogenously supplied fatty acid and a 20-carbon polyprenyl chain. The function of GLX in these membranes is presently unknown, but it has been surmised that its flexibility is similar to that of the phytanyl lipids. Further study would be necessary for a complete understanding of the function of GLX in these membranes.

It is of interest to compare the range of order observed for the intact *A. laidlawii* membranes with that observed for liposomes composed of the total extracted lipids as well as individual lipid species. As shown in Figure 5, the range of order profiles for liposomes composed of total lipids is very similar to that observed in the intact membranes (Figures 2 and 3). The ⟨S⟩ values range from 0.176 to 0.140 for the 16:0d_{31}/18:1cΔ9 (80:20) and 16:0d_{31}/18:1cΔ9 (20:80) systems. Further, it may be noted that, with the exception of the 16:0d_{31}/14:0 preparation noted above, the order profiles of the intact membranes and the derived liposomes are practically identical, indicating that membrane protein does not significantly influence either acyl chain motion or packing in the hydrocarbon region.

From the above results and from previous studies (Seelig & Waespe-Sarcevic, 1978), it is evident that the order parameters measured are dependent upon the degree of saturation of the fatty acid composition. However, with *A. laidlawii* B, it is also known that changes in the fatty acid composition tend to be accompanied by changes in the polar headgroup composition of the membrane lipids (McElhaney, 1984, 1989; Wieslander et al., 1980). The lipids usually found in *A. laidlawii* B membranes are predominantly the glycolipids MGDG and DGDG, which tend to form inverted hexagonal (H_{II}) and bilayer phases, respectively (Wieslander et al., 1980), and PG, a bilayer-forming phosphatide present in quantities of up to 30 mol % of polar lipid content. Since changes in the polar headgroup composition can also influence the magnitude of the order parameters measured, we have examined the order profiles of these polar lipids. Multilamellar vesicle (MLV) dispersions of MGDG, DGDG, and PG extracted from 16:0d_{31}/18:1cΔ9 *A. laidlawii* membranes were therefore prepared and order profiles determined by ^{2}H NMR methods. The results are presented in Figure 6.

The glucolipids MGDG and DGDG exhibit vastly different ^{2}H NMR spectra at 37 °C. By analogy with ^{2}H NMR results obtained for bilayer- and H_{II}-preferring phospholipids (Perly et al., 1985; Lafleur et al., 1990a,b), the DGDG spectrum is characteristic of bilayer structure whereas the MGDG dispersions give rise to a spectrum characteristic of a mixture of (predominantly) H_{II} and bilayer phases at 37 °C. Due to the small contribution of the bilayer component (less than 10%), these spectra are not easily separable, which would be necessary to apply the dePakeing and integration techniques (Lafleur et al., 1989; Sternin et al., 1988). Thus, an order profile for MGDG liposomes is not presented. As shown in Figure 6, both PG and DGDG form bilayers with the characteristic order profile shape although the magnitude of the order in PG bilayers is significantly higher than in DGDG bilayers.

MGDG/DGDG/PG (40:35:25) lipid dispersions, mimicking the lipid composition of the parent membrane, were investigated by the above methods. The hydrocarbon order profile obtained (Figure 6B) is essentially the same as that given by the parent, intact membrane (16:0d_{31}/18:1cΔ9 in Figure 2), showing that the proportions of the component lipid species are important determinants of the magnitude of the order profile in *A. laidlawii*. Further, whereas the proportion of PG is relatively unchanged over the range of fatty acids employed, the proportion of DGDG can change considerably along with GPDGDG. As noted previously (Lafleur et al., 1990a; Eriksson et al., 1991), H_{II}-preferring lipids in bilayers can exert an ordering effect. This is also exhibited by liposomes of MGDG/DGDG (1:1). The mixture

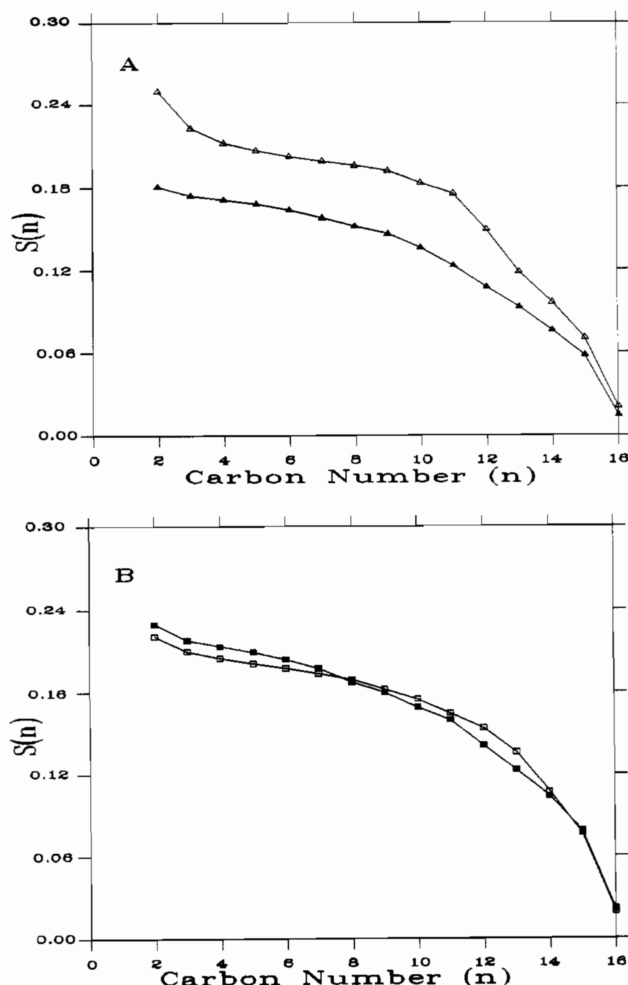

FIGURE 6: Bilayer order profiles of "model membranes". (A) PG liposomes (open triangles) and DGDG liposomes (closed triangles). (B) MGDG/DGDG/PG dispersions in 40:35:25 molar ratios (open rectangles); MGDG/DGDG dispersions in 50:50 molar ratios (closed rectangles). All spectra were recorded at 37 °C as for the above figures. Each spectrum giving rise to the order profiles was a sum of 24 000 transients. Fatty acyl chain composition was 16:0d_{31}/18:1cΔ9, 47:53 mol %. Methods for deriving the order profiles are described in the text.

MGDG/DGDG (1:1) exhibits a 20% increase in $\langle S \rangle$ over that of the DGDG alone where $\langle S \rangle = 0.164$ and $\langle S \rangle = 0.140$, respectively.

DISCUSSION

The major results of this study concern the measurement of hydrocarbon order profiles in intact biological membranes, the implications of the range of order profiles which are compatible with growth, and the roles of individual lipid components in modulating membrane order. With regard to the ability to measure complete order profiles for saturated chains in intact membranes, the ^{2}H NMR dePakeing technique clearly offers significant advantages in comparison to previous procedures (Lafleur et al., 1989; Jarrell et al., 1982) which require growing *A. laidlawii* on a series of specifically ^{2}H-labeled fatty acids. As pointed out elsewhere, for model systems (Sternin et al., 1988; Lafleur et al., 1989) the procedure describes the general features of the order gradient without the need for synthesis and, in the present use, biological incorporation of specifically labeled acyl chains. It would clearly be useful to extend these procedures to other biological membranes, such as those of eukaryotes, which are not fatty acid auxotrophs. In this regard, it has been shown that free fatty acids as well as certain long-chain alcohols, such as tetradecanol, induce little or no change in membrane order in concentrations up to 20 mol % (Pauls et al., 1983; Lafleur et al., 1990c) and that there is a strong correlation between the magnitude of the order parameters and the shape of the order profile. This suggests that perdeuteriated alcohols could be used as probes of order profiles in other biological membranes, a possibility which is currently under investigation.

Table II: Hydrophobic Thickness Calculated

fatty acids and compositions	intact membranes (Å)	derived liposomes (Å)
16:0d_{31}/14:0, 46:54 (mol %)	26.7	27.4
16:0d_{31}/18:1cΔ9, 78:22 (mol %)	26.7	26.7
16:0d_{31}/18:1tΔ9, 46:54 (mol %)	26.5	26.6
16:0d_{31}/18:1cΔ9, 47:53 (mol %)	26.2	26.2
16:0d_{31}/18:2cΔ9,Δ12, 41:59 (mol %)	25.2	25.5
16:0d_{31}/18:1cΔ9, 21:79 (mol %)	25.2	25.5

The range of order profiles compatible with high rates and extents of cell growth was determined to correspond to $\langle S \rangle = 0.140$ to $\langle S \rangle = 0.176$. These values appear to fall very close to the maximum and minimum values of $\langle S \rangle = 0.177 \pm 5\%$ and $\langle S \rangle = 0.133 \pm 5\%$ found for DPPC$_{d62}$ and DOPC/POPC$_{d31}$ (90:10), respectively, and probably reflect the limits of achievable hydrocarbon order in a fluid membrane lacking cholesterol. Since the incorporation of 30 mol % cholesterol in a model membrane would result in $\langle S \rangle \simeq 0.3$ [see, for example, Lafleur et al. (1990c)], one could argue that the maximum possible value of $\langle S \rangle$ for a "fluid" membrane is actually much higher than that obtained here and therefore the range $0.140 \leq \langle S \rangle \leq 0.176$ is narrow in comparison. However, cell growth is greatly inhibited for *A. laidlawii* preparations falling outside this range, suggesting a requirement for the hydrocarbon order to fall within this range in order to maintain normal membrane function. The intact membrane preparation of 16:0d_{31}/14:0 is an example of an *A. laidlawii* system which meets these conditions. High rates and extents of growth were observed for the preparation, and $\langle S \rangle = 0.176$ falls within the above limits. Since the myristate-containing membranes have a component in the gel phase, the order in this system may well be close to the maximum which can be obtained in an *A. laidlawii* B membrane.

The observation of a restricted range in hydrocarbon order may, in turn, reflect a requirement by the organism that other membrane-related parameters are maintained within fixed boundaries. For example, it has been postulated (Ipsen et al., 1990) [see also Seelig and Seelig (1974) and DeYoung and Dill (1988)] that membrane thickness, $2d$, is directly related to the average order parameter by the relation:

$$d = d_1[\alpha\langle S \rangle + \beta]$$

where d is the average projection of the acyl chain along the bilayer normal, $d_1 = 19.7$ Å is the length of an *all-trans* palmitoyl chain (Marcelja, 1974), and α and β are numerical parameters satisfying $0.5\alpha + \beta = 1$; values of $\alpha = 1$ and $\beta = 0.5$ have been used. According to this analysis, the variation in order profiles determined in this study would correspond to a change in hydrophobic thickness of 1.5 Å (Table II). Other groups have postulated a relationship between parameters such as polymorphism and hydrocarbon order (Epand, 1990). Since the interior of a membrane may be approximated as an incompressible fluid, the choice of lipid cross-sectional area as a membrane coordinate, which is used by others (Thurmond et al., 1991), is equivalent to that of bilayer thickness. However, the details of such quantitative relationships remain, as yet, unestablished.

The role of particular membrane constituents in establishing the order profile observed is of interest. In the exceptional case of the $16{:}0d_{31}/14{:}0$ preparation, the very different value of $\langle S \rangle$ obtained for the lipid dispersion relative to the intact membrane suggests that significant protein–lipid interactions may influence the observed order profile. Alternatively, since the DSC studies indicated that more gel phase was present in the derived liposomes than in the intact preparation, the proteins may interact with the lipids to reduce the gel-phase component in the membrane to allow continued viability of the *A. laidlawii* cells during growth. The interpretation of these results is complicated by the presence of the GLX and will require further examination to elucidate.

With the exception of the $16{:}0d_{31}/14{:}0$ preparation, the order profile observed for the intact membrane is effectively identical within experimental error to that for liposomes composed of the total extracted lipids. This indicates that membrane proteins do not significantly perturb acyl chain order and packing, at least for acyl chain lengths of 16 and 18 carbons. This is consistent with previous observations (Seelig & Seelig, 1980; Bloom & Smith, 1985; Bloom, 1979) and, in the spirit of the mattress model of Mouritsen and Bloom (1984), suggests that the hydrophobic regions of integral proteins are matched in a manner such that the presence of the protein does not perturb the motional freedom of the lipids on the NMR timescale.

The predominant lipid species in *A. laidlawii*, MGDG, DGDG, and PG differ significantly from one another with respect to their polymorphic phase preferences and hydrocarbon order. In addition, from the results obtained, MGDG and DGDG in combination appear to potentially play an important role in establishing the order profile in the intact membrane. It is clear that the presence of MGDG, which adopts the H_{II} phase in isolation, increases the order in bilayers formed with MGDG/DGDG mixtures. The increase has been attributed to an increase in lateral pressure induced by the nonbilayer-forming lipid on the acyl chains of the lipid mixture (Lafleur et al., 1990a). This effect has also been observed for POPE in mixtures with POPC (Lafleur et al., 1990a).

Wieslander et al. (1980) have shown for the *A. laidlawii* strain A that the MGDG to DGDG ratio varies for different fatty acid compositions in a manner which can be interpreted as conserving the overall polymorphic preference of the lipid bilayer. Such regulation has not been consistently observed in *A. laidlawii* strain B (McElhaney et al., 1984, 1989). As shown in Table I, the only consistent effect observed in *A. laidlawii* strain B is a variation in the DGDG to OAPG + GPDGDG ratio as a function of acyl chain unsaturation. Although OAPG and GPDGDG have not received detailed attention in this regard, they also may play a role in maintaining membrane order within defined limits.

In summary, this work demonstrates that ^2H NMR dePakeing methods may be used to determine the order gradient in the hydrocarbon region of *A. laidlawii* membranes grown on perdeuteriated palmitic acid. A relationship apparently exists between hydrocarbon order and high rates and extents of *A. laidlawii* cell growth. Under natural conditions, the organism may modulate this order via acyl chain and lipid headgroup composition. By restricting the fatty acyl chain composition while allowing for good growth of the organism, the lipid headgroup composition is varied in a manner which can be related to the need to achieve hydrocarbon order profiles lying within a fairly well-defined range. The contribution of lipids present in lesser amounts in the *A. laidlawii* membrane (OAPG and GPDGDG) appears to be important in this regard.

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Registry No. 16:0, 57-10-3; 14:0, 544-63-8; 18:1tΔ9, 112-79-8; 18:1cΔ9, 112-80-1; 18:2cΔ9,Δ12, 60-33-3.