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ARTICLE *in* BIOCHEMISTRY · DECEMBER 1986

Impact Factor: 3.02 · DOI: 10.1021/bi00371a037 · Source: PubMed

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Phase Equilibria of Membrane Lipids from *Acholeplasma laidlawii*: Importance of a Single Lipid Forming Nonlamellar Phases†

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Received March 31, 1986; Revised Manuscript Received July 22, 1986

ABSTRACT: A basis for the reorganization of the bilayer structure in biological membranes is the different aggregate structures formed by lipids in water. The phase equilibria of *all* individual lipids and several in vivo polar lipid mixtures from acyl chain modified membranes of *Acholeplasma laidlawii* were investigated with different NMR techniques. All dioleoyl (DO) polar lipids, except monoglucosyldiglyceride (MGDG), form lamellar liquid crystalline (L_α) phases only. The phase diagram of DOMGDG reveals reversed cubic (I_{II}), reversed hexagonal (H_{II}), and L_α phases. In mixtures of DOMGDG and dioleoyldiglycosyldiglyceride (DODGDG), the formation of an I_{II} (or H_{II}) phase is enhanced by DOMGDG and low hydration or high temperatures. For in vivo mixtures of *all* polar DO lipids, a transition from an L_α to an I_{II} phase is promoted by low hydration or high temperatures (50 °C). The phospholipids are incorporated in this I_{II} phase. Likewise, I_{II} and H_{II} phases are formed at *similar* temperatures in a series of in vivo mixtures with different extents of acyl chain unsaturation. However, their melting temperatures (T_m) vary in an expected manner. All cubic and hexagonal phases, except the I_{II} phase with DOMGDG, exist in equilibrium with excess water. The maximum hydration of MGDG and DGDG is similar and increases with acyl chain unsaturation but is substantially lower than that for, e.g., phosphatidylcholine. The translational diffusion of the lipids in the cubic phases is rapid, implying *bicontinuous* structures. However, their appearances in freeze-fracture electron microscope pictures are different. The I_{II} phase of DOMGDG belongs to the *Ia3d* space group. It is concluded that the formation of nonlamellar phases by *A. laidlawii* lipids depends critically upon the MGDG concentration.

Biological membranes consist of a large number of different lipids which together with proteins constitute the bilayer structure. The physicochemical properties of the membrane lipids, in their role as structure builders and their participation in membrane-associated physiological processes, have been only partly studied. In this respect, the contributions from all the individual components of the in vivo lipid mixtures are very little known. For an understanding of the processes involving, e.g., the reorganization of the membrane structure, we need a better characterization of the various components and molecular interactions involved. A basic feature here is the different aggregate structures lipids can form in water and the different liquid crystalline phases they build up. In most studies hitherto performed, phase equilibria are studied only in lipid systems containing excess water. However, although the biological cell can experience excess water in its surroundings, the intracellular milieu or the conditions on membrane surfaces and between closely apposed membranes are not the same as in bulk water. Hence, the study of phase equilibria must embrace also these conditions.

In order to be able to investigate all lipid species in a biological membrane, it must have a rather simple composition or lend itself to manipulation. This holds for the cell wall less prokaryote (mycoplasma) *Acholeplasma laidlawii*. With certain lipid-exhausted growth media, this organism is completely dependent upon exogenous supply of fatty acids for synthesis of membrane polar lipids. Thereby, acyl chain homogenous lipids can be produced (within certain limits), substantially reducing the number of intramolecular lipid variants. Upon variations in the structure of fatty acids incorporated, as well as changes in growth temperature or the presence of foreign molecules like sterols, these cells in a characteristic manner change the proportions between the two major polar membrane (gluco) lipids and the relative fractions of ionic and and nonionic lipids synthesized (Rilfors et al., 1984; Wieslander et al., 1980).

Here we have studied the phase equilibria of all individual *A. laidlawii* lipids, as well as a number of different in vivo mixtures.[1] A transition from a lamellar to a nonlamellar phase occurs in *all* in vivo extracts and is strictly regulated. The means for this regulation is the lipid MGDG.

Materials and Methods

Cell Growth. *Acholeplasma laidlawii*, strain A EF22 (Wieslander & Rilfors, 1977), was grown statically in filled bottles (5% inoculum) in a lipid-depleted tryptose–bovine serum albumin medium (Christiansson et al., 1985). For large-scale preparation of individual oleoyl lipids, the growth medium was supplemented with 150 μM oleic acid, and the cells were grown for 24 h at 30 °C in 4-L bottles. A total of 200 L of culture was harvested by continuous-flow centrifugation, 140 mL/min at 29000*g* and 5 °C, and the cells were

† This work was supported by the Swedish Natural Science Research Council.

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[1] Abbreviations: L_α, lamellar liquid crystalline phase; H_{II}, reversed hexagonal phase; I_{II}, reversed cubic phase; T_m, gel to liquid crystalline phase transition (interval midpoint) temperature; DO, dioleoyl; PO, palmitoyloleoyl; DE, dielaidoyl; MGDG, monoglucosyldiglyceride; DGDG, diglucosyldiglyceride; PG, phosphatidylglycerol; GPMGDG, glycerophosphomonoglucosyldiglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; in vivo lipid mixture, mixture of all *A. laidlawii* polar membrane lipid species from a specific growth condition and at their natural percentages; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-7502$01.50/0

Table I: Polar Lipid Composition in Membranes from *A. laidlawii* Cells Grown with Different Fatty Acid Supplements

fatty acid in the growth medium	cell growth temp (°C)	acyl chain composition in polar lipids (mol %)[a]	ratio MGDG/DGDG (mol/mol)	fraction ionic lipids (mol %)[b]
oleic, 150 μM	30	97	0.59	40.3
palmitic/oleic (μM/μM)				
140/10	37	83/17	1.70	22.3
120/30	37	75/25	1.33	26.2
100/50	37	66/33	1.07	30.1
80/70	37	53/47	0.64	32.0
60/90	37	39/61	0.42	36.6
40/110	37	26/74	0.24	37.5
20/130	37	13/87	0.21	39.0
0/150	37	5/95	0.18	40.1

[a] From gas–liquid chromatography and liquid scintillation counting. [b] Ionic lipids: phosphatidylglycerol, glycerophospho derivatives of MGDG and DGDG, and diphosphatidylglycerol (minor amounts, ≤0.5%).

washed once in β buffer and freeze-dried. Preparation of membranes was not necessary since mycoplasmas have no internal membranes and the cellular lipids are localized in the cytoplasmic membrane only.

For the preparation of the total polar lipid extracts with different acyl chain composition, growth media were supplemented with palmitic and oleic acids in the following proportions (μM/μM): 140/10, 120/30, 100/50, 80/70, 60/90, 40/110, 20/130, and 0/150. Lipids were labeled by adding sterile [^{3}H]palmitic acid (30 μCi/L) and [^{14}C]oleic acid (10 μCi/L), from Amersham International; 4-L batches were grown for 20 h at 37 °C, harvested, washed, and freeze-dried.

Purification and Separation of Lipids. The dried cell batches were extracted 3 times with chloroform–methanol, 2:1 (v/v), followed by methanol, on a magnetic stirrer at 22 °C. Nonlipid contaminants were removed by chromatography on Sephadex G-25 fine (Wells & Dittmer, 1963). Lipids were then applied to a column with silicic acid (Bio-Sil HA from Bio-Rad). Neutral lipids and pigments in *all* samples were eluted with chloroform. Palmitoyl-containing total polar lipids were then eluted with methanol *or* oleoyl glucolipids with acetone followed by oleoyl phospholipids and phosphoglucolipids with methanol, respectively (Wieslander et al., 1978). The amounts of lipids in the chloroform fraction constituted at the most 5 mol % of the total lipids; cf. Christiansson and Wieslander (1980). A slight N_2 pressure was maintained over chromatography columns in order to prevent lipid oxidation and to secure constant flow rates. Individual polar lipids with oleoyl hydrocarbon chains were separated by thin-layer chromatography on 1-mm plates of silica gel, H 60 (Merck), developed with chloroform–methanol–water, 65:25:4 (v/v). Before activation, plates were washed by empty runs in the same solvent system. Individual lipids on wet, transparent plates were visualized by light and eluted from the gel at 5 °C by chloroform–methanol, 2:1 (v/v), followed by methanol. Oleoyl phospholipids and the palmitoyl-containing total extracts were extensively washed with Na_2EDTA in order to remove divalent cations. Lipids were analyzed for identity and constancy before and after NMR runs with thin-layer chromatography, liquid scintillation counting, and gas–liquid chromatography as described previously (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1980; Wieslander et al., 1978). Purified polar lipids contained >97% oleoyl acyl chains, and these lipids are considered as dioleoyl (DO) species. A total of 9.15 g of polar oleoyl lipids and 0.15 g each of the in vivo mixtures were obtained after all purification steps. The lipids were stored in solution under N_2 at −70 °C. Membrane lipid composition in the harvested cells is shown in Table I. For comparison, dielaidoyl-MGDG (DEMGDG) and palmitoyloleoyl-MGDG (POMGDG) from *A. laidlawii* were also prepared (Wieslander et al., 1978).

Sample Preparation. Approximately 100 mg of lipid was dried to a film in an NMR tube by N_2 gas followed by drying over night at a pressure less than 0.1 mmHg (13 Pa). Heavy water from Ciba-Geigy was added to the lipid followed by a flush of N_2 before sealing with silicon rubber stops. Lipids and heavy water were mixed by extended centrifugation, or vortexing (excess water), and the tubes were stored at −20 °C or colder; cf. the procedures in Wieslander et al. (1978, 1981) and Khan et al. (1981).

^{2}H and ^{31}P NMR Spectroscopy. ^{2}H NMR on lipid–^2H_2O systems were obtained at 38.4 MHz with a Bruker WM 250 FT spectrometer equipped with a superconducting magnet. For the systems containing phospholipids, also ^{31}P NMR spectra were recorded at 101.3 MHz on the same spectrometer. It has been shown in several previous works that ^{2}H NMR (Ulmius et al., 1977; Persson et al., 1975; Brentel et al., 1985; Khan et al., 1985) and ^{31}P NMR (Eriksson et al., 1985a; Arvidson et al., 1985) can be conveniently used to determine phase diagrams of lipid systems. A homogeneous anisotropic liquid crystalline phase, like the lamellar and hexagonal phases, produces a quadrupole splitting in the ^{2}H NMR spectra; i.e., two equally intense peaks are observed (see Figure 1). An isotropic phase like a micellar solution or a cubic liquid crystalline phase yields only a rather sharp singlet peak, provided the static quadrupole interaction is averaged out to zero as a result of rapid and isotropic molecular motions. Such a situation is usually at hand, although it should be noted that recently static interactions were observed for a cubic phase of lysolecithin (Eriksson et al., 1985b). For a system with two or more phases, the ^{2}H NMR spectrum consists of a superposition of spectra from the various phases in the samples. Thus, for a sample containing hexagonal and cubic phases, a quadrupole splitting together with a single peak will be observed. Likewise, for a three-phase system with two anisotropic phases and one isotropic phase, the ^{2}H NMR spectrum contains two doublets and a central peak. For a detailed theoretical treatment of ^{2}H NMR on lipid systems, see Wennerström et al. (1974), Mantsch et al. (1977), and Davis (1983). The splittings, designated Δ, were obtained from the distances between the spectral peaks and are given in frequency units.

Analogously, the ^{31}P NMR spectrum also shows static interactions in anisotropic liquid crystals. A powder sample of a lamellar or hexagonal phase gives a ^{31}P NMR spectrum having a line shape that is characteristic for an axially symmetric chemical shift tensor (McLaughlin et al., 1975; Cullis & de Kruijff, 1976). Generally, a lamellar phase gives rise

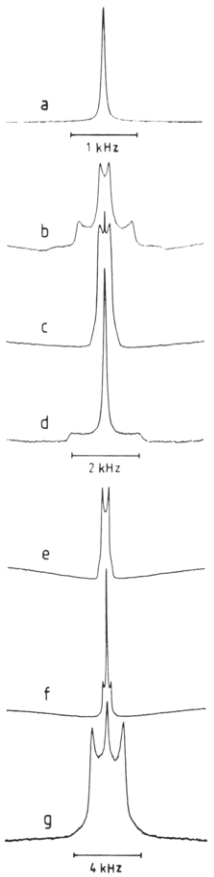

FIGURE 1: Typical ^2H NMR spectra showing quadrupole splittings in different samples composed of ^2H_2O and *A. laidlawii* lipids. Spectra a–f are from samples of DOMGDG with different amounts of heavy water and at different temperatures: (a) isotropic signal from a cubic phase with 5 mol of water at 20 °C; (b) two-phase sample with 9 mol of water at 0 °C, containing lamellar and hexagonal phases; (c) two-phase sample with 11 mol of water at 40 °C, containing a hexagonal phase and excess free water; (d) two-phase sample with 5 mol of water at 10 °C, containing lamellar and cubic phases; (e) hexagonal phase with 9 mol of water at 30 °C; (f) two-phase sample with 7 mol of water at 30 °C, containing hexagonal and cubic phases. Spectrum g is from a sample of in vivo polar lipids with oleoyl chains (cells grown at 30 °C) with 5 mol of water at 35 °C, containing lamellar and cubic phases. Compare spectrum g with the ^{31}P NMR spectrum of the same sample, shown in Figure 2c.

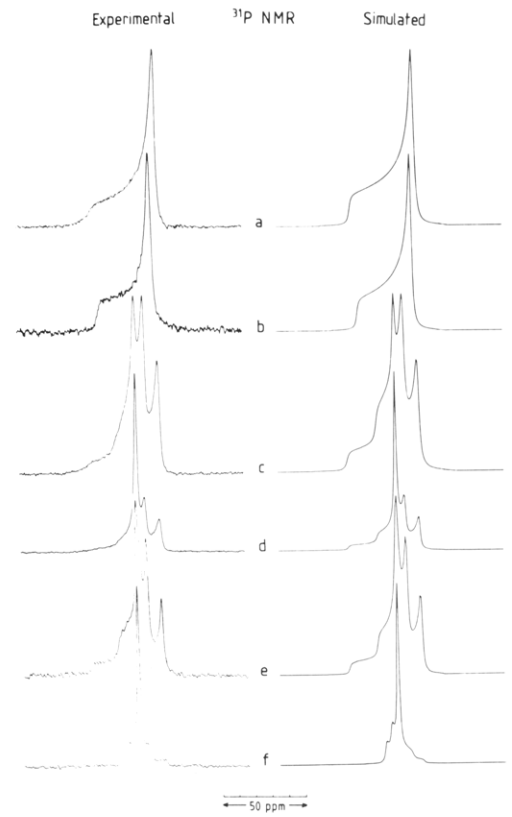

FIGURE 2: Experimental and simulated ^{31}P NMR spectra from samples composed of heavy water and different *A. laidlawii* phospholipids or in vivo lipid mixtures: (a) DOPG with 12 mol of water at 25 °C, containing a lamellar phase; (b) DOGPMGDG with 10 mol of water at 25 °C, containing a lamellar phase; (c) in vivo polar lipids with oleoyl chains (cells grown at 30 °C) with 5 mol of water at 35 °C, containing lamellar and cubic phases; (d) sample c at 45 °C; (e) in vivo polar lipids with oleoyl chains (cells grown at 37 °C) with 10 mol of water at 50 °C, containing lamellar and cubic phases; (f) in vivo polar lipids with 75% (mol/mol) palmitoyl and 25% oleoyl chains (cells grown at 37 °C) with 10 mol of water at 65 °C, containing hexagonal and cubic phases. Acyl chain and polar lipid compositions in total lipid mixtures are shown in Table I. The following parameters were used in the simulations from which the relative fractions of the different superimposed spectra were calculated [sample, $\Delta\sigma$ (ppm), $\nu_{1/2}$ (Hz), relative composition (%)]: a, −38.5, 175, 100; b, −32.6, 150, 100; c, $\Delta\sigma_1$ = −42.4, 200, 53, $\Delta\sigma_2$ = −16.8, 200, 40, $\Delta\sigma_3$ = 0, 200, 7; d, $\Delta\sigma_1$ = −42.4, 150, 39, $\Delta\sigma_2$ = −16.8, 150, 31, $\Delta\sigma_3$ = 0, 150, 30; e, $\Delta\sigma_1$ = −41.9, 50, 43.5, $\Delta\sigma_2$ = −17.3, 50, 36.5, $\Delta\sigma_3$ = 0, 100, 19.5; f, $\Delta\sigma_1$ = 0, 40, 44, $\Delta\sigma_2$ = 12.3, 60, 29.5, $\Delta\sigma_3$ = 22.2, 50, 26.5. Spectra for samples e and f were obtained at 40.5 MHz.

to a spectrum with a high-field peak and a low-field shoulder, while a hexagonal phase generates a spectrum with a low-field peak and a high-field shoulder. Often, the magnitude of the chemical shift anisotropy, $\Delta\sigma = \sigma_\parallel - \sigma_\perp$, for the hexagonal phase is half of that obtained for the lamellar phase in the same system. Furthermore, the sign of the chemical shift anisotropy is different for the two phases in question. A narrow symmetrical NMR signal is generally obtained from an isotropic liquid crystalline phase. As for ^2H NMR, ^{31}P NMR gives a superposition of spectra for a sample consisting of several different phases (cf. Figure 2). Spectra simulations were performed as described by Eriksson et al. (1985a).

By a systematic study of ^2H and ^{31}P NMR spectra as a function of sample composition and temperature, the phase diagrams of most of the *A. laidlawii* membrane lipids in water were established. The reproducibility of the equilibrium boundary lines between a one-phase liquid crystalline phase and heterogeneous phases is within 3–5%, the lower accuracy existing at high water contents.

NMR Diffusion Measurements. The translational diffusion coefficients of MGDG and DGDG in the lamellar and cubic phases were determined with the pulsed magnetic field gradient technique as described previously (Lindblom & Wennerström, 1977; Lindblom et al., 1979, 1981; Rilfors et al., 1986). These measurements were performed with a Bruker 322s pulsed NMR spectrometer equipped with a home-built digitized pulsed field gradient unit.

ESR Spectroscopy. The nitroxide spin-label Tempo (2,2,6,6-tetramethylpiperidine-1-oxyl) was utilized to determine phase transition temperatures according to a method developed by Shimshick and McConnell (1973). Spectra were registered with a Varian E-109 ESR spectrometer using 100-kHz modulation and an operating frequency of 9.0 GHz. The gel to liquid crystalline phase transition (interval midpoint) temperature (T_m) was thus determined for extracts containing in vivo mixtures of all the polar membrane lipids. The concentration of the probe molecule was 0.5 mol % of the lipid.

Polarizing Light Microscope, Freeze-Fracture Electron Microscopy, and Low-Angle X-ray Diffraction. The typical textures of the different lipid phases were studied with a polarizing microscope according to Rosevear (1954, 1968). Electron microscopy pictures were obtained for the cubic phase

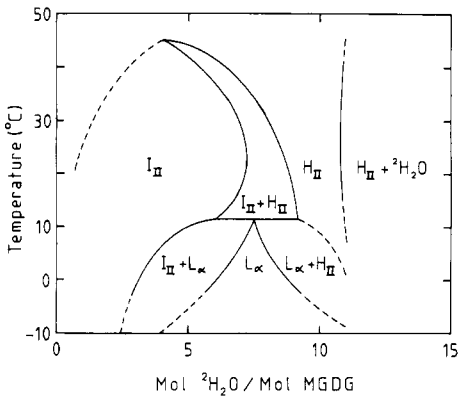

FIGURE 3: Phase diagram of the system dioleoylmonoglucosyldiglyceride–^2H_2O. The phase equilibria were deduced by NMR spectroscopy and polarized light microscopy. H_{II}, reversed hexagonal liquid crystalline phase; I_{II}, reversed cubic liquid crystalline phase; L_α, lamellar liquid crystalline phase. Hatched lines denote a slight uncertainty of phase boundaries.

with DOMGDG according to Gulik-Krzywicki et al. (1984), by use of a combination of freeze-fracture electron microscopy and low-angle X-ray diffraction before and after freezing.

Results and Discussion

^2H and ^{31}P NMR spectra were recorded at different compositions and temperatures for a large number of samples of practically all the individual oleoyl lipids from the membrane of *A. laidlawii*. Samples containing various mixtures of the two dominant lipids DOMGDG and DODGDG were also examined as well as mixtures of all the lipids present in the natural membrane. A study of the effect of variation in unsaturation in the acyl chains on the phase equilibria of in vivo mixtures was also performed. The NMR data obtained were then used to achieve the phase diagrams of the various lipid–^2H_2O systems as delineated under Materials and Methods. It should be noted that the NMR method for determination of phase diagrams is very convenient and rapid, and the practical problems often met with classical methods in separating the individual phases from each other do not exist here. Furthermore, the method is nondestructive.

In the study of phase diagrams of membrane lipids there is sometimes a problem of representation of data as described in a previous work by us (Brentel et al., 1985). This is mainly due to the heterogeneity in the composition of the acyl chains and the vast number of samples that has to be investigated in order to get a "true" phase diagram. This problem can often be circumvented by considering the membrane lipid mixture as "one component", which in some cases may lead to Gibbs' phase rule not being followed in these kinds of "binary" phase diagrams. However, if one is aware of this, it usually does not create any problem in the interpretation and conclusions drawn (Brentel et al., 1985).

DOMGDG–^2H_2O Phase Equilibria. From an investigation of about 25 samples with DOMGDG–^2H_2O content in the composition range of 0.5–15 mol of water/mol of lipid at temperatures between −20 and 50 °C, the phase diagram shown in Figure 3 could be drawn. The most notable features of this diagram are as follows. Above about 10 °C, only nonlamellar phases exist—reversed hexagonal (H_{II}) and cubic (I_{II}) phases. Below 10 °C there is a narrow region with a lamellar phase (L_α). The reversed hexagonal phase stands in equilibrium with excess water, and the maximum hydration of this phase is about 11 mol of water/mol of lipid. The cubic phase area extends between about 1 and 7 mol of water/mol of lipid and between 10 and 45 °C. (For DEMGDG and POMGDG, no cubic phases have been found under similar conditions.) A gel phase is formed at about −15 °C (not shown in Figure 3). No deuteron quadrupole splitting was obtained below this temperature in any MGDG sample studied. It has been observed previously that for *A. laidlawii* lipids with substantially higher gel to liquid crystalline phase transition temperatures (T_m) the splittings also vanished below the T_m (Wieslander et et al., 1978). By use of differential thermal analysis calorimetry, a T_m of −13 °C was obtained for total oleoyl lipids from *A. laidlawii* (McElhaney, 1984). For synthetic dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylethanolamine (DOPE), this T_m transition takes place at −18, −16, −22, and −16 °C, respectively [see review by Silvius (1982)]. The difference in T_m for these dioleoyl lipids (DOPG, DOPC, DOMGDG, and DOPE) thus lies within less than 10 degrees. It can therefore be concluded that for lipids having oleoyl acyl chains the polar head group has a very small effect on the gel to liquid crystalline phase transition. However, with other acyl chains the situation is different; e.g., DEMGDG and POMGDG have a T_m at 30–35 °C and 20–25 °C, respectively (Wieslander et al., 1978). This is very close to T_m's of the corresponding PE's (Van Dijck et al., 1976) but approximately 25 °C higher than for the corresponding PC's (Seelig & Waespe-Sarcevic, 1978). The T_m's of the monosugar glucosphingolipids and ether glucolipids are also substantially higher than the T_m's for PC having corresponding saturated chains (Maggio et al., 1985; Hinz et al., 1985). Obviously, for lipids containing saturated (or transunsaturated) acyl chains there is a strong dependence of the polar head group on T_m. This can most probably be explained by the fact that the packing and hydration properties of the lipid molecules are more critically affected by the head group for saturated chains.

Finally, it is interesting to note that transition temperatures for DOPE and DOMGDG follow each other remarkably closely, not only for the gel to liquid crystalline transition but also for the L_α to H_{II} transitions (Figure 3; Cullis et al., 1985).

DODGDG–^2H_2O Phase Equilibria. Deuteron NMR on heavy water was investigated for a few samples in this system as a function of composition and temperature, together with water penetration experiments with a polarizing microscope according to a method described by Leigh et al. (1981). Below a water content of about 3–4 mol of ^2H_2O/mol of lipid and the temperature range 10–50 °C, no splitting was observed in this crystalline phase. In the lamellar liquid crystalline phase that existed at water contents between 4 and 10 mol of ^2H_2O/mol of lipid a typical quadrupole splitting observed was about 2 kHz (30 °C; 4.5 mol of ^2H_2O). The splitting decreased with increasing water content to about 1.4 kHz at 10 mol of water/mol of lipid. Above 10 mol of ^2H_2O/mol of lipid, the lamellar phase was in equilibrium with excess water. It was found that T_m was about −20 °C, i.e., similar to the value obtained for DOMGDG (see above). Thus, the phase diagram for the system DODGDG–^2H_2O consists only of a lamellar liquid crystalline phase between 4 and 10 mol of ^2H_2O/mol of lipid above −20 °C (the upper limit of the temperature has not been determined, since the lipid decomposes at high temperatures). Below that temperature a crystalline "gel" phase occurs. The lamellar phase structure has been characterized previously with X-ray diffraction (Wieslander et al., 1978).

DOPG–^2H_2O Phase Equilibria. The dominating phase in the DOPG–water system was a lamellar liquid crystalline phase with an extension between 4 and 12 mol of ^2H_2O/mol of lipid as revealed by polarizing microscope, ^2H quadrupole

Table II: Phase Equilibria in Mixtures of Monoglucosyldiglyceride and Diglucosyldiglyceride Obtained by ^2H_2O NMR

DOMGDG:DODGDG (mol/mol)	^2H_2O content (mol/mol of lipids)	phase	
		25 °C	45 °C
1.0:0	5.0	I_{II}[a]	H_{II}
	8.0	I_{II}[a] + H_{II}	H_{II}
	>10.0	H_{II}	H_{II}
3.0:1.0	2.5	I_{II}	I_{II}
	5.0	I_{II}	I_{II}
	10.0	L_α + H_{II} + I_{II}[c]	H_{II} + I_{II} (+L_α)[d]
2.5:1.0	5.0	I_{II}	I_{II}
1.2:1.0	5.0	L_α (+I_{II})[b]	I_{II} (+L_α)
0.8:1.0	3.0	I_{II}	I_{II}
	5.0	L_α	L_α (+I_{II})
0:1.0	>4.0	L_α	L_α

[a] *Ia3d*, the reversed bicontinuous cubic phase of DOMGDG. In the DOMGDG–DODGDG mixtures, I_{II} is the reversed bicontinuous cubic phase observed previously (Wieslander et al., 1981). [b] Symbols within parentheses denote minor amounts. [c] These three phases in this lipid mixture can exist in equilibrium with excess water. [d] This L_α phase is absent at 55 °C.

splittings, and ^{31}P chemical shift anistropies. At a water content below 4 mol of water/mol of lipid, a crystalline phase was present, and at water contents above 12 mol of ^2H_2O/mol of lipid the sample was homogeneously milky, and isotropic signals together with splittings or chemical shift anisotropies appeared in the ^2H and ^{31}P NMR spectra, respectively. This latter behavior of the samples continued up to the highest water content studied, 98% ^2H_2O (w/w). A probable explanation of these findings is that another lamellar phase is formed. This second lamellar phase seems to be able to swell infinitely in water at least up to 98% water. Furthermore, since isotropic peaks are observed in the ^{31}P NMR spectra, lipid vesicles are most probably formed spontaneously. The water deuteron splitting was found to increase with increasing temperature in the lamellar phase at low water content, indicating that the water deuterons are exchanging with the glycerol hydroxylic deuterons. Such an exchange has been observed previously with alcohols in other amphiphile systems (Persson et al., 1975). The T_m was found to be about −20 to −25 °C.

Dioleoylglycerophospho-MGDG (DOGPMGDG)–^2H_2O Phase Equilibria. In the temperature range studied, 25–45 °C, also for this system only lamellar liquid crystalline phases were observed, one at low water contents between 5 and 15 mol of water/mol of lipid and one at higher water contents as with DOPG. While the quadrupole splittings were similar to those observed for the other membrane lipids studied, it is interesting to note that the chemical shift anisotropy for DOGPMGDG was significantly smaller than that for DOPG, 34.9 and 42.0 ppm, respectively, at corresponding temperature and composition (cf. Figure 2). Recently, it was observed that the chemical shift anisotropy differed for DOPE and DOPC (Eriksson et al., 1985a) due to a difference in the molecular ordering of the polar head groups. Such an explanation is the most probable also here, since the charged phosphate group on the glucose residue of DOGPMGDG is located much further out in the water layer than that of DOPG. Therefore, the molecular ordering of the head group of DOGPMGDG as studied by ^{31}P NMR is lower than that of DOPG.

DOMGDG–DODGDG–^2H_2O Phase Equilibria. For this three-component system some selected samples have been studied as summarized in Table II. As can be inferred from this table, the phase equilibria of mixtures of DOMGDG and DODGDG exhibit an increasing disposition for formation of a lamellar phase with (i) decreasing ratio between DOMGDG/DODGDG, (ii) decreasing temperature, and (iii) increasing hydration. If the lipid ratio is above 1 at a water content of 5 mol/mol of lipid, almost exclusively nonlamellar phases are formed in the temperature range studied (0–50 °C). An exception is the sample in which this ratio is 1.2 and the temperature is 25 °C, but again, at 45 °C this sample contains a cubic phase with only a trace amount of a lamellar phase. A further remarkable feature of the phase behavior found for these mixtures is that if DODGDG is added to DOMGDG at 45 °C, then the reversed hexagonal phase is displaced by a cubic phase. This experimental finding can be explained in terms of a change in the molecular shape according to the self-assembly theory for lipids developed by Israelachvili et al. (1976), which will be discussed in more detail below. [See Translational Diffusion in Lamellar and Cubic Phases and the accompanying paper (Wieslander et al., 1986)]. Recently, a similar study of the phase equilibria of chloroplast galactolipids was reported by us (Brentel et al., 1985), and it was found that the phase behavior for the two different pairs of sugar lipids at various ratios between the mono- and the disugar species shows the same trends. The most interesting observation here is that the presence of MGDG in the lipid mixture in most cases leads to the formation of a nonlamellar phase, particularly at high temperatures; i.e., MGDG seems to play a dominant role for the phase structure.

Table III: Phase Equilibria in in Vivo Polar Lipid Mixtures with Oleoyl Acyl Chains from Membranes of *A. laidlawii* Cells Grown at 30 °C[a]

^2H_2O content (mol/mol of lipid)	temp (°C)	phase
5.0	25	L_α (+I_{II})[b]
	35	L_α (+I_{II})
	45	I_{II} + L_α
	55	I_{II} (+L_α)
10.0 and 15.0	25	L_α
	35	L_α
	45	L_α
	55	L_α (+I_{II})

[a] Phase equilibria obtained with ^{31}P and ^2H_2O NMR; cf. Figures 1 and 2. Membrane polar lipid composition is as shown in Table I. [b] Symbols within parentheses denote minor amounts.

Phase Equilibria of Polar Lipid Mixtures from A. laidlawii Membranes. An investigation was performed of an in vivo polar lipid mixture (including 0.5% diphosphatidylglycerol, cf. Table I) with oleoyl acyl chains and a MGDG/DGDG ratio of 0.59 and 40 mol % phospholipids from cells grown at 30 °C (Table I). The results obtained are summarized in Table III. In this study, both ^{31}P and ^2H NMR were utilized (cf. Figures 1g and 2c), and it was found that at low temperatures (between about 25 and 45 °C) a lamellar phase was the most stable. At about 12 mol of water/mol of lipid, a low water containing lamellar phase was in equilibrium with another lamellar phase as found for the ionic lipids, DOPG and DOGPMGDG. The effects of hydration and temperature

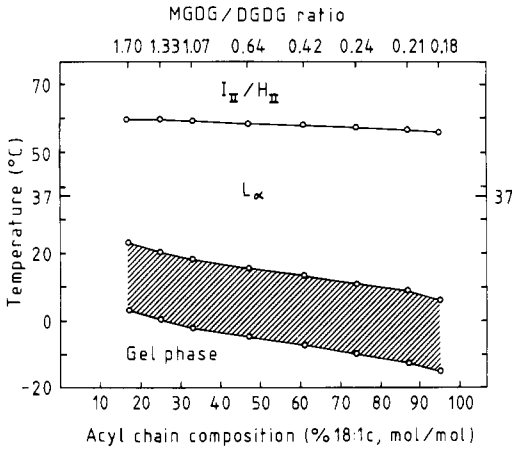

FIGURE 4: Phase equilibria of in vivo lipid mixtures, containing different amounts of palmitoyl and oleoyl acyl chains, from membranes of *A. laidlawii* grown at 37 °C. Water contents were 10 mol/mol of lipid, i.e., close to the maximum hydration for the glucolipids. The lower x axis shows the contents (mol %) of palmitoyl and oleoyl chains in the polar lipids; cf. Table I. The upper x axis shows the metabolically obtained MGDG:DGDG ratios. The ionic lipids vary from 22 to 40% (mol/mol) over the series (Table I). The hatched area denotes the gel to liquid crystalline phase transition interval as determined by ESR. Above the upper border of the L_α phase, *no* lamellar phases were found. Two- and three-phase regions (L_α, H_{II}, and I_{II}) occur just below this border (within less than 10 °C).

were found to be similar to those of the MGDG/DGDG mixtures. The most striking observation from this study of an in vivo lipid mixture is that the effect of addition of ionic phospholipids to an MGDG/DGDG mixture is small (cf. Tables I–III). This is indeed unexpected since the phospholipids form only lamellar phases, but in spite of that the membrane lipid mixtures can form a cubic liquid crystalline phase. For example, a sample with 5 mol of ^2H_2O at 55 °C and a sample with 10–15 mol of ^2H_2O at about 60 °C formed a cubic liquid crystalline phase.

Concerning the water ordering in the membrane lipid extracts, it can be concluded from the observed water quadrupole splittings that the ordering decreases with increasing water content. Thus, for the dioleoyl-containing mixtures the splittings for samples with 5, 10, and 15 mol of water (Table III) were 1620, 1020, and 660 Hz, respectively. The ^{31}P NMR chemical shift anisotropy, however, varied only slightly over the whole range of concentrations. As can be seen in Figure 2, the chemical shift anisotropies of DOGPMGDG and DOPG were well resolved in the lipid mixtures (28.9 and 36.5 ppm at 25 °C and 5 mol of water, respectively, and 35.9 and 40.2 ppm at 25 °C and 15 mol of water, respectively), having values found in lamellar phases of DOGPMGDG–water and DOPG–water. The temperature dependence of the shift anisotropy was also similar to that observed in the binary lamellar phases.

An investigation of the effect on the phase equilibria of alterations in the saturation of the acyl chains in vivo was also performed. The degree of unsaturation affects the amounts of MGDG, DGDG, and ionic lipids in the membrane (cf. Table I). It was found for all samples studied (Figure 4) that a phase transition from a lamellar phase to either a hexagonal or a cubic phase occurred at about 45–55 °C (cf. the phase transitions in Table III). However, the T_m for the samples varied over a wide range from 20 to −12 °C (Figure 4). Hence, the area of existence of the lamellar phase varied markedly for these in vivo lipid extracts, but the upper temperature limit was almost constant, independent of the unsaturation in the acyl chains.

For the two-phase samples, the fractions of the individual phospholipids in the different phases could be calculated from the simulated ^{31}P spectra [Figure 2; cf. Eriksson et al. (1985a)]. It is found that, close to the maximum hydration of the glucolipids (Figure 2e,f), PG was slightly enriched in the cubic phase compared to GPMGDG.

Lipid Hydration. At present, there is a large interest in the surface-induced solvent structure of amphiphile aggregates, proteins, and DNA [see e.g., Jönsson (1985)]. In particular the existence and quantification of the repulsive hydration force are a matter of intensive study (Israelachvili, 1985; Jönsson, 1985; Marra & Israelachvili, 1985; Marra, 1985, 1986; Evans & Ninham, 1986). Both direct (Israelachvili & Adams, 1978) and indirect (Le Neveu et al., 1977; Parsegian et al., 1979) methods have been used to measure forces between lipid bilayers. A third approach has been taken by Wennerström and co-workers, who base their theoretical considerations upon phase diagrams [Khan et al. (1985) and references cited therein). Obviously, the hydration properties of lipids are an important subject, and here we will discuss the maximum hydration of all the lipids present in the membrane of *A. laidlawii*, roughly estimated from the phase diagrams obtained. It should be remembered that the maximal uptake of water in lamellar and hexagonal phases is very conveniently determined from an investigation of the ^2H NMR of heavy water (Ulmius et al., 1977; Brentel et al., 1985). A system containing an anisotropic liquid crystalline phase in equilibrium with excess water will give rise to a spectrum with a quadrupole splitting superimposed on a narrow central peak. The maximum hydration at 35 °C for the nonionic glucolipids forming lamellar phases was approximately 11, 9, and 7 mol of water/mol of lipid for DODGDG, PODGDG, and DEDGDG, respectively.

For the monoglucosyldiglyceride lipids forming a hexagonal phase, the maximum hydration at 35 °C was found to be 11, 8, and 7 mol of water/mol of lipid for DOMGDG, POMGDG, and DEMGDG, respectively. Here some striking results are noted: (i) The maximal uptake of water is approximately the same for MGDG and DGDG, in spite of the fact that they form reversed hexagonal and lamellar phases, respectively. A similar result was recently also found for the galactolipids from chloroplasts (Brentel et al., 1985). (ii) An increase in the number of acyl chains with cis unsaturation leads to an increase in the water binding capacity. Notice that mono- and digalactosyldiglycerides from chloroplasts with mostly linolenoyl acyl chains (three double bonds) have even higher hydration capacities, taking up 12–13 mol and 14–15 mol of water, respectively (Brentel et al., 1985). Furthermore, PE enriched in anteiso acyl chains takes up more water than PE enriched in iso acyl chains (Rilfors et al., 1982). A most plausible explanation to this effect of cis unsaturation and anteiso branching, is that the surface area per lipid in the aggregate increases, allowing for an increase in the water binding. However, it should also be noticed that glucose has a slightly lesser water binding capacity than galactose (Iwamoto et al., 1982), but this cannot explain the whole difference observed between the lipids in question. (iii) Compared to the nonionic lipids, the hydration properties of the ionic ones are quite different. Both DOPG and DOGPMGDG form a lamellar phase with a maximal hydration of about 12 mol of water/mol of lipid. At higher water contents, most probably another lamellar phase is formed. This behavior is also in contrast to the behavior of the zwitterionic lipid DOPC that binds about 31–33 mol of water/mol of lipid and where the lamellar phase at higher water contents is in

equilibrium with excess water (Gutman et al., 1984; Hauser, 1984). However, ordinary ionic surfactants, generally, can take up large amounts of water in the lamellar phase (Ekwall, 1975). At present it is not possible to give an unambiguous explanation in terms of electrostatics for these observations.

Let us now discuss in more detail the results obtained in (i) and compare the maximum hydration of PC and that of the sugar lipids. For zwitterionic phospholipids it is well established that a strong hydration force (Lis et al., 1978) leads to large separations (10–30 Å) between adjacent bilayers. This repulsive hydration force has been suggested to arise from an ordering of the water molecules around the lipid head groups (hydration) (Marra & Israelachvili, 1985). If, however, PC is replaced by digalactosyldiglyceride, the repulsive forces are very different (Marra, 1986), and it is found that the interaction between the sugar lipid bilayers across the water is 6 times stronger than that between PC bilayers. This difference in hydration force might be due to the fact that the hydroxyl-containing sugar head groups associate with water so that there is almost no difference between the bulk and the vicinal surface water. This is supported by thermodynamic studies (Taylor & Rowlinson, 1955) of solutions of glucose and sucrose. They found evidence for stronger hydrogen bonding between these sugars and water compared to that between water molecules. Recent investigations of micelles (Drummond et al., 1985) and membranes (Crowe et al., 1984) also indicate that carbohydrate head groups can be considered as "aqueous-like" in nature. This means that if the gluco head groups of MGDG and DGDG behave like "water molecules", there will be no hydration force and the water uptake will be strongly limited by the number of hydrogen bonds that can be formed between glucose and water.

Water quadrupole splittings contain information on the hydration in terms of the fraction of water molecules oriented at the surface of the aggregate and the degree of orientation characterized by an order parameter S, through

$$\Delta(^2\mathrm{H}) = (nX_\mathrm{L}/X_\mathrm{W})\nu_\mathrm{Q}S$$

where ν_Q is the quadrupole coupling constant and n is the average hydration number of the lipid. X_L and X_W are the mole fractions of lipid and water, respectively. This equation is derived (Wennerström et al., 1975) under the assumption that only two sites, free or bound, exist for water molecules; i.e., either they reside in the bulk water, or they participate in the hydration of the lipids. Then a plot of $\Delta(^2\mathrm{H})$ against the molar ratio of the lipids to water should be linear. This is, however, not found for MGDG and DGDG, indicating that several sites exist. Such a situation should be expected if water molecules are hydrogen-bonded to different hydroxylic groups and if the amount of "free" water is very low.

Translational Diffusion in Lamellar and Cubic Phases. The translational diffusion coefficients have been determined for DOMGDG, DODGDG, and mixtures of these lipids. The results are summarized in Table IV. For comparison, the lateral diffusion coefficient of DOPC is included. It can be inferred from this table that (i) the lateral diffusion of DODGDG is much larger than that for DOPC, (ii) the diffusion coefficient decreases upon addition of DOMGDG to a lamellar phase of DODGDG, (iii) the diffusion coefficient of the cubic phases is much smaller than it is in the lamellar phases, and (iv) addition of DODGDG to a cubic phase of DOMGDG decreases the lipid diffusion coefficient.

Previously it has been shown that the polar head group is one important factor as concerns the value of the lateral diffusion coefficient (Lindblom & Wennerström, 1977; Eriksson et al., 1984). Thus, it is not surprising that DODGDG and DOPC have very different lateral diffusion coefficients, although it is not possible at present to give an explanation to the observed difference in terms of molecular interactions. One possibility might be that the aqueous-like behavior of the sugar groups is involved. The effect observed in (ii) is more difficult to understand. It is, however, likely that DOMGDG has the property of changing the molecular packing of the lipids in the bilayers in such a way that they become undulated, since DOMGDG forms nonbilayer aggregates and therefore prefers to be located at nonplanar surfaces. A wavy lamellar aggregate will affect the measured diffusion coefficient in two ways; first, an undulation increases the distance of travel between two points at the lamellar surface, thus leading to a decrease in the diffusion coefficient obtained; second, DOMGDG, preferably located in "valleys" of the bilayers, will cause a restriction in the lipid translational motion. An increase in the concentration of DOMGDG eventually leads to a phase transition to a cubic structure, which then most probably has an even stronger discrete distribution of DOMGDG at regions of high curvature due to packing constraints. This should lead to a substantial decrease in the measured diffusion coefficient, in accordance with our observations. The same explanation can be applied to the faster diffusional motion in a cubic phase of only DOMGDG and water compared to that in a cubic phase containing a mixture of DOMGDG and DODGDG.

Table IV: Lipid Translational Diffusion Coefficients Determined by the NMR-Pulsed Field Gradient Method.

lipid	^2H_2O content (mol/mol of lipid)	temp (°C)	phase[a]	D ($\times 10^{12}$ m^2 s^{-1})
DOMGDG	4.0	30	cubic	1.7
DODGDG	5.8	45	lamellar	37
DOMGDG: DODGDG				
2.5:1.0[b]	5.1	45	cubic	1.0
1.2:1.0	5.0	25	lamellar	15
1.2:1.0	5.0	45	cubic	1.0
DOPC[c]	10	35	lamellar	5.0

[a] According to NMR and X-ray diffraction. [b] Data from Wieslander et al. (1981). [c] DOPC, dioleoylphosphatidylcholine. Diffusion coefficient from Lindblom et al. (1981).

The diffusion NMR technique (Charvolin & Rigny, 1971; Bull & Lindman, 1974; Lindblom & Wennerström, 1977; Rilfors et al., 1986) has been shown to be a powerful method for distinguishing between the two fundamentally different structures building up cubic liquid crystalline phases: (i) phases having continuous regions of both water and hydrocarbon and (ii) phases with discontinuous hydrocarbon regions and continuous water domains ("oil in water") or with continuous hydrocarbon regions and discontinuous water regions ("water in oil") [see, e.g., Luzzati (1968), Eriksson et al. (1982, 1985a), and Rilfors et al. (1984)]. In structures of the first type, lipids can diffuse over macroscopic distances. For structures of the second type, diffusional motion is restricted to occur within the aggregates. The measured diffusion coefficient, as performed with the NMR method, will be substantially lower in the phase composed of closed aggregates than in the bicontinuous phase [see Lindblom and Wennerström (1977)]. It can be concluded that since the measured lipid diffusion coefficients in the cubic phases are in the order of 10^{-12} m^2/s, as has been found for most bicontinuous cubic phases containing membrane lipids hitherto studied (Rilfors et al., 1986), the phases in the present investigation are bicontinuous too, having one of the structures proposed previously (Luzzati & Spegt, 1967; Scriven, 1976;

FIGURE 5: Freeze-facture electron micrograph of cubic phase of dioleoylmonoglucosyldiglyceride with 4 mol of water quenched from 25 °C. The cubic structure was verified by low-angle X-ray diffraction before and after freezing; see Gulik-Krzywicki et al. (1984) for technical details. Horizontal bar equals 200 nm.

Lindblom et al., 1979). Due to the low water content, they must be of the reversed type.

The cubic phase of DOMGDG was visualized by freeze-fracture electron microscopy, verifying the cubic structure before and after cryofixation with low-angle X-ray diffraction (Gulik-Krzywicki et al., 1984). Figure 5 shows a freeze-fracture picture of DOMGDG with 4 mol of water/lipid at 25 °C. Note the absence of globular structures (cf. below), so-called "lipidic particles", frequently occurring in other cubic and isotropic lipid systems (Gulik-Krzywicki et al., 1984; Verkleij, 1984). The striated pattern is similar to that obtained from the cubic phase in the low-water and high-temperature part of the phase diagram of egg yolk phosphatidylcholine (Gulik-Krzywicki et al., 1984). According to the X-ray diffraction results, the cubic phase of DOMGDG is of the body-centered space group *Ia3d*. A freeze-fracture electron microscopy photo of the reversed cubic phase composed of a mixture of DOMGDG and DODGDG at low-water content and high temperature is shown by Verkleij (1984, Figure 5J,K). This picture shows stacked layers of typical lipidic particles in a body-centered packing, quite different from the striation pattern of the *Ia3d* structure of the cubic phase of DOMGDG (Figure 5). However, the diffusion data show that also this phase is bicontinuous [see also Rilfors et al. (1986), where a more detailed discussion of this matter has been given].

Conclusions

In this work, phase diagrams of nearly all the different lipids in the membrane of *A. laidlawii* have been determined. It was found that all the membrane lipids except MGDG form a lamellar phase at all water concentrations at the growth temperature of the bacterium. Thus, only *one* lipid, MGDG, forms nonlamellar phases (cubic and hexagonal), which has been shown to have great consequences for the phase equilibria of a system containing a mixture of all the lipids present in the natural membrane. The most important result is that for in vivo mixtures of membrane lipids the transition temperature for the formation of a nonlamellar phase from a lamellar one is almost independent of the degree of unsaturation of the acyl chains. Note, however, that the gel to liquid crystalline phase transition temperature depends in an expected way on the acyl chain unsaturation (cf. Figure 4). These observations might have important biological implications for the functioning of the natural membrane. Thus, it can be speculated that the lipid composition in the membrane always is such that the bilayer structure easily (with low-energy consumption) may be transformed to open structures with, e.g., water channels. These structures may appear in the membrane for a short period of time and are then quickly closed to form an intact bilayer structure again. It is believed that such transformations only occur at certain regions of the membrane, where the nonlamellar lipid is concentrated. Such a concentrated distribution of a lipid can be brought about by, for example, an increase in the curvature of the membrane (locally or at larger parts of the membrane); cf. the simple calculations by Maggio (1985). The rapid diffusional motion of the lipids will facilitate a fast rearrangement of the lipid composition in different domains of the cell membrane. The biological role played by such a formation of holes in the membrane for fusion, exo- and endocytosis, and transmembrane traffic of macromolecules and ions has been discussed before [cf. Rilfors et al. (1984) and Cullis et al. (1985)].

The phase diagrams also constitute an important base for further investigations of the physicochemical properties of the lipids at the molecular level. Previously, it has been shown (Israelachvili et al., 1976, 1980; Israelachvili, 1985) that most of the difficult problems of describing the intermolecular forces, lipid self-assembly, or the mechanism behind the organization in different aggregate shapes can be encompassed by a very simple approach considering the geometry or shape of the lipid molecules. The rules provided by this simple theory have been shown to be very powerful in predicting the behavior of the lipids in the membrane of *A. laidlawii* (Wieslander et al., 1980, 1981; Rilfors et al., 1984). A prerequisite for successful predictions to be made, however, is a detailed knowledge of the phase equilibria and phase structures of the lipids involved. This is also clearly demonstrated in the investigation reported in the accompanying paper, where the experimental planning and the conclusions drawn heavily lean on access to the phase diagrams.

Acknowledgments

We thank Dr. T. Gulik-Krzywicki, Centre de Gênêtique Moléculaire, CNRS, Gif-sur-Yvette, France, for performing the combined X-ray and freeze-fracture electron microscopy analyses and Dr. A. Khan for recording NMR spectra in the initial state of this investigation.

Registry No. DOMGDG, 78279-19-3; DODGDG, 104486-15-9; DOPG, 62700-69-0; DOGPMGDG, 104422-21-1; oleic acid, 112-80-1; palmitic acid, 57-10-3.

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