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# Regulation and Phase Equilibria of Membrane Lipids from *Bacillus megaterium* and *Acholeplasma laidlawii* Strain A Containing Methyl-Branched Acyl Chains†

Leif Rilfors,* Jón B. Hauksson, and Göran Lindblom

*Department of Physical Chemistry, University of Umeå, S-901 87 Umeå, Sweden*

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ABSTRACT: Phosphatidylethanolamine (PE) was isolated from *Bacillus megaterium* grown at 20 and 55 °C (PE-20 and PE-55). Iso and anteiso methyl-branched, saturated acyl chains are predominant in *B. megaterium*, and the value of the molar ratio of iso/anteiso acyl chains is more than 20-fold higher in PE-55 than in PE-20. Moreover, about 21 mol % of the acyl chains of PE-20 are monounsaturated. The phase equilibria differ between the two PE preparations: (1) PE-20 is more prone to form reversed nonlamellar phases than PE-55; (2) PE-20 forms both reversed cubic ($I_2$) and reversed hexagonal ($H_{II}$) phases while PE-55 forms only an $H_{II}$ phase; and (3) the lamellar liquid-crystalline ($L_\alpha$) phase of PE-20 takes up about 70% more water than the $L_\alpha$ phase of PE-55. These differences can be explained by the differences in the acyl chain composition. When the growth temperature is raised, PE molecules with a reduced tendency to form nonlamellar phases are probably synthesized by *B. megaterium* in order to counteract the bilayer destabilizing effect of the temperature. The regulation of the acyl chain composition is not needed in order to regulate the temperature for the transition between gel/crystalline and $L_\alpha$ phases of the membrane lipids. *Acholeplasma laidlawii* strain A-EF22 was grown at 37 °C on 15-(1,1,1-$^2H_3$)methylhexadecanoic acid, 14-(1,1,1-$^2H_3$)methylhexadecanoic acid or 13-(1,1,1-$^2H_3$)methylhexadecanoic acid, and these acids constituted 84–89 mol % of the acyl chains in the membrane lipids. The molar ratio between the two dominating lipids, monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), decreased, and the molar fraction of the anionic lipids increased, when the methyl branch was moved from position 15 to position 13. Concomitantly, the order of the methyl branch increased in cells as well as in total lipid extracts. The phase equilibria of total lipid extracts (neutral lipids removed) were studied with 20 wt % of water, and $H_{II}$ and $I_2$ phases were formed above 63–67 °C. These results indicate that the regulation of the polar head-group composition compensates for the difference in acyl chain packing introduced into the bilayer by the three branched-chain fatty acids. The regulation of the polar head-group composition of the *A. laidlawii* lipids cannot regulate the temperature for the transition between gel/crystalline and $L_\alpha$ phases of the lipids, i.e. the transition to fluid acyl chains. Thus, both *B. megaterium* and *A. laidlawii* change the membrane lipid composition to adjust the balance between lipids forming lamellar and nonlamellar phases, but they appear to be largely insensitive to the transition between gel/crystalline and $L_\alpha$ phases. It is concluded that the physico-chemical properties of membrane lipids are influenced by the position of a methyl substituent on the acyl chains and that these differences in physico-chemical properties are sensed by living cells.

Iso and anteiso methyl-branched, saturated acyl chains occur in large amounts in the membrane lipids from several genera of Gram-positive as well as Gram-negative bacteria (Kaneda, 1977, 1991). The genus *Bacillus* has been the most extensively studied one with respect to these branched-chain fatty acids. Organisms having membrane lipids with a large fraction of iso and anteiso acyl chains often regulate the molar ratio between these chains upon changes in the growth temperature (Rilfors et al., 1978; Kaneda, 1991; Suutari & Laakso, 1992). It is consequently believed that iso and anteiso fatty acids are functional equivalents of the straight-chain saturated and cis-unsaturated fatty acids, respectively (Kaneda, 1977; Rilfors, 1985; Lewis & McElhaney, 1985), which occur in the majority of organisms. It has also been suggested that the branched-chain membrane lipid system represents an ancient system, which emerged before the appearance of the oxygenic atmosphere on Earth (Kaneda, 1977).

An increasing interest in the physico-chemical properties of synthetic membrane lipids containing acyl chains with various alkyl substituents in different positions can be noticed (Silvius & McElhaney, 1979, 1980; Kannenberg et al., 1983; Lewis & McElhaney, 1985; Mantsch et al., 1985, 1987; Silvius et al., 1985; Nuhn et al., 1986; Rice et al., 1987; Lewis et al., 1987, 1989; Menger et al., 1988a,b). Synthetic lipids with iso and anteiso methyl-branched acyl chains have different physico-chemical properties: the temperature, $T_m$,[1] for the transition between a lamellar gel ($L_\beta$) phase and a lamellar liquid-crystalline ($L_\alpha$) phase and the temperature, $T_{LH}$, for the transition between an $L_\alpha$ phase and a reversed hexagonal liquid-crystalline ($H_{II}$) phase are lower for lipids with anteiso

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[1] Abbreviations: $L_\beta$, lamellar gel phase; $L_\alpha$, lamellar liquid-crystalline phase; $T_m$, temperature for the $L_\beta$ to $L_\alpha$ phase transition; $I_2$, reversed cubic liquid-crystalline phase; $H_{II}$, reversed hexagonal liquid-crystalline phase; $T_{LH}$, temperature for the $L_\alpha$ to $H_{II}$ phase transition; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE-20 and PE-55, PE isolated from *Bacillus megaterium* grown at 20 and 55 °C; MGlcDAG, monoglucosyldiacylglycerol; DGlcDAG, diglucosyldiacylglycerol; 15-$C^2H_3$-16:0, 15-(1,1,1-$^2H_3$)methylhexadecanoic acid; 14-$C^2H_3$-16:0, 14-(1,1,1-$^2H_3$)methylhexadecanoic acid; 13-$C^2H_3$-16:0, 13-(1,1,1-$^2H_3$)-methylhexadecanoic acid.

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acyl chains; and lipids containing anteiso acyl chains occupy larger areas per molecule in monolayers in the condensed state (Silvius & McElhaney, 1979, 1980; Kannenberg et al., 1983; Lewis & McElhaney, 1985; Lewis et al., 1987, 1989). In this study, the hydration properties and the ability to form reversed nonlamellar phases have been investigated for two preparations of phosphatidylethanolamine (PE) which have been isolated from two strains of *Bacillus megaterium* grown at 20 and 55 °C. The dominating membrane lipid in these strains is PE, and the molar ratio of iso/anteiso acyl chains is regulated by changing the growth temperature (Rilfors et al., 1978).

Branched-chain fatty acids can support growth of bacterial species in which they do not naturally occur, e.g. *Escherichia coli* (Silbert et al., 1973), *Mycoplasma mycoides* (Rodwell & Peterson, 1971), *Acholeplasma laidlawii* strains A (Rilfors, 1985) and B (Silvius & McElhaney, 1978), and *Clostridium butyricum* (Goldfine et al., 1987b). It has been shown in a large number of investigations that the polar head-group composition of the *A. laidlawii* membrane lipids is adjusted in accordance with the chemical structure of the fatty acids incorporated into the lipids (see Rilfors et al. (1993) for a review). For example, the fraction of membrane lipids forming reversed nonlamellar phases increases concomitantly with the molar ratio of straight-chain saturated/cis-unsaturated acyl chains in the lipids (Wieslander et al., 1980; Rilfors et al., 1993; Lindblom et al., 1993). As a consequence of this adjustment, the tendency of total *A. laidlawii* lipid extracts, with various ratios of saturated/cis-unsaturated acyl chains, to form reversed nonlamellar phases is maintained within narrow limits (Lindblom et al., 1986; Österberg et al., 1994).

In this investigation we have grown *A. laidlawii* strain A on three different methyl-branched derivatives of palmitic acid; the methyl branch is deuterated and is located in the iso and anteiso positions and on the carbon atom above the anteiso position. We have determined the polar head-group composition of the membrane lipids and the molecular ordering of the methyl branch in cells and total lipid extracts and studied the phase equilibria of the total lipid extracts. The results obtained are discussed in terms of lipid regulation in cell membranes.

## MATERIALS AND METHODS

*Organisms and Growth Conditions.* A facultatively thermophilic strain (Ft R32) and an obligately thermophilic strain (Ot 32) of *B. megaterium* (Rilfors et al., 1978) were grown at 20 and 55 °C, respectively, in a tryptone-soya broth (30 g/L; Oxoid Ltd., Hampshire, England). The two strains originate from the same mesophilic wild-type strain, M 1, of *B. megaterium*, and their isolation and characteristics are described by Ståhl & Olsson (1977). The temperature ranges of the strains were tested before the culture vessel was inoculated and after the growth was completed. A culture volume of 100 L of each strain was obtained form a 15-L fermentor vessel by continuously pumping in fresh medium and pumping out the cell suspension. The cells were harvested at 5 °C by continuous-flow centrifugation with a Beckman JCF-Z rotor; the flow rate was 135–175 mL/min, and the relative centrifugal force was 17 000*g* at the rotor wall. The cells were suspended in 0.25 M NaCl and stored at −70 °C until the membranes were prepared.

*A. laidlawii*, strain A-EF22 (Wieslander & Rilfors, 1977), was grown statically in the medium described by Eriksson et al. (1991). The growth medium was supplemented with 100 μM of one of the following branched-chain fatty acids: 15-(1,1,1-$^2H_3$)methylhexadecanoic acid (15-$C^2H_3$-16:0), 14-(1,1,1-$^2H_3$)methylhexadecanoic acid (14-$C^2H_3$-16:0), or 13-(1,1,1-$^2H_3$)methylhexadecanoic acid (13-$C^2H_3$-16:0). The specifically deuterated fatty acids were synthesized by Bodo Dobner and Peter Nuhn, Martin-Luther-Universität Halle-Wittenberg, Germany (Dobner & Nuhn, 1991), and the purity of these acids was 97%, as determined by gas–liquid chromatography (GLC). The *A. laidlawii* cells were grown for 20–24 h at 37 °C. Portions (35 L) of each of the cultures containing 15-$C^2H_3$-16:0, 14-$C^2H_3$-16:0, or 13-$C^2H_3$-16:0 were harvested as described by Eriksson et al. (1991).

*Isolation and Purification of Lipids.* The preparation of cell membranes from *B. megaterium*, the extraction of the membrane lipids, and the purification of the lipid extracts were performed as described by Rilfors et al. (1982). PE obtained from strain Ft R32 grown at 20 °C (PE-20) was isolated by cellulose column chromatography (Comfurius & Zwaal, 1977). The lipid was eluted from the column with chloroform–methanol, 96:4 (v/v), and the purity of the preparation was >99%, as judged by thin-layer chromatography (TLC). PE obtained from strain Ot 32 grown at 55 °C (PE-55) was isolated by silicic acid chromatography (Eriksson et al., 1991). The other lipids occurring in *B. megaterium* were eluted from the gel with acetone and acetone–methanol, 80:20 (v/v), and PE-55 was eluted with acetone–methanol, 70:30 (v/v). The purity of the lipid preparation was >99%, as judged by TLC.

The extraction of the membrane lipids from the *A. laidlawii* cells grown on the deuterated branched-chain fatty acids, the purification of the lipid extracts, and the removal of pigments and neutral lipids (mostly diglycerides and free fatty acids) were performed as described by Eriksson et al. (1991). Divalent cations can affect the phase equilibria of anionic membrane lipids (see Lindblom et al. (1991) and references therein), and these ions were therefore removed by a modified version of the procedure described by Smaal et al. (1985). The lipids were dissolved in 1 unit of chloroform (100 mL of chloroform/300 mg of lipids), whereafter 2 units of methanol and 1 unit of 10 mM $CaCl_2$ in water were added. The mixture was shaken for 5 min, and a one-phase system was obtained. Half a unit each of chloroform and 10 mM $CaCl_2$ in methanol were added. The mixture was shaken for 1 min, and a one-phase system was still obtained. A two-phase system was induced by adding $^3/_2$ unit of 0.1 M EGTA, 0.1 M NaCl, and 0.05 M Tris-HCl (pH 8.2) and shaking the mixture for 5 min. Finally, 1 unit each of chloroform and the EGTA buffer were added and the mixture was shaken for 5 min. The chloroform phase was collected, and lipids dispersed in the water phase were extracted by washing twice with 1 unit of chloroform. The chloroform phases were pooled, and the solvent was evaporated. In order to assure that the sodium salt of the lipids was obtained, the lipids were finally dissolved in 1 unit of chloroform, 2 units of methanol, and 1 unit of 0.9% (w/v) NaCl in water. The mixture was shaken for 5 min, and a one-phase system was obtained. A two-phase system was generated by adding 1 unit each of chloroform and the NaCl solution and shaking the mixture for 5 min. The lipids were collected in the chloroform phase as described above.

*Determination of Lipid Composition.* The acyl chain composition of PE-20 and PE-55 from *B. megaterium* was determined by GLC and combined GLC–mass spectrometry (GLC-MS) after conversion of the acyl chains to their methyl esters (Rilfors et al., 1978). The GLC analyses were performed with a Carlo-Erba Instruments, Model HRGC 5300-HT, apparatus equipped with a polar SP-2380 capillary column (length 60 m; inner diameter 0.32 mm; film thickness 0.2 μm) from Supelco Inc. (Bellefonte, PA). The GLC apparatus was connected with a CE Instruments, Model DP700, electronic

integrator. The GLC-MS analyses were performed with a Hewlett-Packard, Model 5890, gas chromatograph connected with a Hewlett-Packard mass selective detector, Model 5970. The gas chromatograph was equipped with a nonpolar DB-5 capillary column (length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm) from J & W Scientific (Folsom, CA).

The polar head-group and acyl chain compositions of the total lipid extracts from *A. laidlawii* were determined as described by Rilfors (1985).

The large-scale cultivations of *B. megaterium* and *A. laidlawii* were performed in single batches. For a statistical treatment of the variation in the lipid composition between different batches, the reader is referred to the papers by Rilfors et al. (1978) and Rilfors (1985).

*Preparation of NMR Samples.* The preparation of the samples used for the NMR studies was performed as described by Eriksson et al. (1991) and Lindblom et al. (1993). The purity of the samples was checked by TLC after the NMR studies had been completed. About 1–2% of PE-20 and about 7–8% of PE-55 were found to be degraded. Trace amounts of free fatty acids were detected in the total lipids from *A. laidlawii*.

*NMR Spectroscopy.* Several previously published works have shown that $^{31}P$ and $^{2}H$ NMR can be conveniently used to study the phase behavior of membrane lipid–water systems (e.g. Ulmius et al., 1977; Brentel et al., 1985; Eriksson et al., 1985a,b; Lindblom et al., 1986).

$^{2}H$ NMR of $^{2}H_2O$ can often be used as a convenient method to study the phase equilibria of lipid–water mixtures (Ulmius et al., 1977; Lindblom et al., 1986; Lindblom & Rilfors, 1989). For a heterogeneous system consisting of two or more phases, one expects a superposition of the $^{2}H$ NMR spectra originating from the different phases in the sample, provided that the deuteron exchange between them is slow. $^{2}H$ NMR quadrupolar splittings of $^{2}H_2O$ are thus expected to be observed in lamellar and hexagonal phases, while the water in a cubic phase or an isotropic solution phase (e.g. a micellar phase) gives no quadrupolar splitting but only a single spectral peak (Ulmius et al., 1977). The $^{2}H$ NMR signal of a $C^{2}H_2$ or a $C^{2}H_3$ group on the acyl chains of a lipid can be used in an analogous fashion to study phase equilibria (Ulmius et al., 1982; Thurmond et al., 1993).

$^{2}H$ NMR spectra were recorded on a Bruker ACP-250 spectrometer operating in the quadrature detection mode at 38.4 MHz. The spectra were recorded using the quadrupole echo technique (Davis et al., 1976) with a $\pi/2$ pulse width of 25 μs, a spectral width of 100 kHz, an interpulse spacing of 80 μs, a preacquisition delay after the second $\pi/2$ pulse of 20 μs, and a relaxation delay between the pulse sequences of 1.0 s. The digital phase cycling was carefully adjusted until a pure absorption signal in the real quadrature channel was obtained. Before Fourier transformation the FID was left-shifted until the first data point represented the true maximum of the echo.

$^{31}P$ NMR can also be used to investigate the phase equilibria of lipid–water systems, since, for phospholipids, the appearance of the $^{31}P$ NMR signal depends on the symmetry of the aggregate structure(s) (McLaughlin et al., 1975; Cullis & De Kruijff, 1976; Lindblom et al., 1986; Brentel et al., 1987). A lamellar phase gives rise to a spectrum with a high-field peak and a low-field shoulder, while a hexagonal phase gives rise to a spectrum with a low-field peak and a high-field shoulder. For geometrical reasons, the magnitude of the chemical shift anisotropy for the hexagonal phase is roughly half of that obtained for the lamellar phase in the same system. Isotropic solution phases (e.g. micellar phases) and cubic liquid crystals usually give rise to a single, relatively narrow $^{31}P$ NMR signal. Multiphase systems give a superposition of the line shapes characteristic of each phase present.

$^{31}P$ NMR spectra were obtained with a Bruker ACP-250 Fourier transform spectrometer at 101.27 MHz. A phase-cycled Hahn echo sequence (Rance & Byrd, 1983) with high power proton decoupling was used, because it is not possible to record the first part of the free induction decay (FID) with a single pulse experiment. The $\pi/2$ pulse length was 9.6 μs, and the spectral width was 50 kHz. A relaxation delay of 0.75 s was used, and before Fourier transformation a line broadening of 20 Hz was applied. A variable-temperature unit was used to control the air flow around the sample in the spectrometer, and the temperature in the probe was measured with a calibrated thermistor.

In order to investigate the possible presence of a gel/crystalline phase in PE-55–water mixtures, high-power $^{1}H$ NMR spectra were recorded, and the second moment of the spectra were calculated, as described in Lindblom et al. (1993). $^{1}H$ NMR spectra were recorded from PE-20 dissolved in DMSO-$d_6$–$^{2}H_2O$, 98:2 (v/v), in order to examine if this lipid contains unsaturated acyl chains (Hauksson et al., 1994). The spectra were recorded at a $^{1}H$ frequency of 250.13 MHz with a spectral width of 2500 Hz. The $\pi/2$ pulse length was 10 μs, and a relaxation delay between successive scans of 10 s was used.

The pulsed magnetic field gradient technique was utilized in cases where $^{2}H$ NMR gave ambiguous results concerning the phase equilibria of total lipid extracts from *A. laidlawii*. $^{1}H$ NMR spectra were recorded as described in Lindblom et al. (1993), and the fact that a Hahn spin echo is easily observed in $^{1}H$ NMR for isotropic phases but not for anisotropic phases was utilized to check for the presence of isotropic phases.

*Computational Procedures.* All data processing was done on a Personal IRIS 4D/25 work station using Felix (Hare Research, Inc.).

## RESULTS

*Acyl Chain Composition of PE from B. megaterium.* PE, phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) are the dominating lipids in the membrane of *B. megaterium*, and PE constitutes about 55–65 mol % in the strains Ft R32 and Ot 32 within their entire growth temperature intervals (Rilfors et al., 1978). In contrast, the acyl chain composition of the membrane lipids is profoundly changed by the growth temperature (Table 1; Rilfors et al., 1978, 1982).

The identity of the fatty acids was established by GLC and GLC-MS analyses. The relative retention times of the methyl esters of the fatty acids in PE-20 and PE-55 were compared with those of known methyl esters of straight-chain saturated and unsaturated fatty acids and of saturated iso and anteiso fatty acids. Two capillary columns were used, one with a very polar stationary phase and one with a nonpolar stationary phase. Mass spectra were also recorded from methyl ester derivatives of the known as well the unknown fatty acids. An unequivocal identification was possible for the straight-chain $C_{16:1}$ fatty acid and for all the saturated straight-chain and branched-chain fatty acids given in Table 1. Thus, only 0.3 mol % of the acyl chains in PE-55 are unidentified.

The monounsaturated $C_{16}$ and $C_{17}$ acyl chains occurring in PE-20 are identified with sufficient certainty for this study (Table 1). The presence of one or more unsaturated carbon–carbon bonds was settled in three ways: (1) The relative retention times for these acyl chains were shorter on the nonpolar column than on the polar column, as compared to the saturated acyl chains; it is well documented that unsatur-

Table 1: Acyl Chain Composition (mol %) in PE Isolated from *B. megaterium* Strain Ft R32 Grown at 20 °C (PE-20) and Strain Ot 32 Grown at 55 °C (PE-55)

| acyl chain | PE-20 | PE-55 |
|---|---|---|
| iso-$C_{14}$ | 0.1 | 0.3 |
| normal-$C_{14}$ | 0.4 | 1.0 |
| iso-$C_{15}$ | 10.6 | 61.4 |
| anteiso-$C_{15}$ | 40.6 | 3.8 |
| normal-$C_{15}$ | | 0.1 |
| monounsaturated $C_{16}$ chain[a] | 4.7 | |
| iso-$C_{16}$ | 4.0 | 3.0 |
| normal-$C_{16:1}$ | 1.6 | |
| normal-$C_{16}$ | 1.4 | 5.6 |
| monounsaturated $C_{17}$ chains[b] | 14.8 | |
| iso-$C_{17}$ | 3.8 | 19.8 |
| anteiso-$C_{17}$ | 13.4 | 3.8 |
| normal-$C_{18}$ | 0.5 | 0.9 |
| Σbranched saturated acyl chains | 72.5 | 92.1 |
| Σnormal saturated acyl chains | 2.3 | 7.6 |
| Σmonounsaturated acyl chains | 21.1 | |
| Σunidentified acyl chains | 4.2 | 0.3 |
| Σiso acyl chains/Σanteiso acyl chains | 0.49[c] | 11.1 |

[a] Tentatively identified as a monounsaturated iso-$C_{16}$ acyl chain. [b] Tentatively identified as monounsaturated iso-$C_{17}$ (7.0 mol %) and anteiso-$C_{17}$ (7.8 mol %) acyl chains. [c] The tentatively identified monounsaturated branched acyl chains were included when calculating this value; the corresponding value with these acyl chains excluded is 0.35.

ated acyl chains are eluted before saturated ones of the same length on a nonpolar stationary phase, while the reverse relationship is valid when such acyl chains are chromatographed on a polar stationary phase (Christie, 1989); (2) the mass spectra of the methyl esters of these acyl chains all exhibit the ion fragments expected from unsaturated acyl chains (Christie, 1989); and (3) a $^1$H NMR spectrum recorded from PE-20 dissolved in DMSO-$d_6$–$^2H_2O$, 98:2 (v/v), shows signals with a chemical shift of approximately 5.3 ppm that is characteristic of olefinic protons (Hauksson et al., 1994). On the assumption that the unsaturated acyl chains in PE-20 are monounsaturated (see below), the fraction of unsaturated acyl chains was calculated from the $^1$H NMR spectrum to be 25 mol %. This value agrees very well with the value obtained from the GLC analyses, since some of the unidentified acyl chains in PE-20 probably are monounsaturated ones (Table 1). The relative retention times in the GLC chromatograms and the patterns of ion fragments in the mass spectra constitute the basis for the following tentative identification of the acyl chains: The $C_{16}$ chain is an unsaturated iso-$C_{16}$ chain, and the $C_{17}$ chains are unsaturated iso-$C_{17}$ and anteiso-$C_{17}$ chains. These acyl chains are most probably monounsaturated, since the occurrence of polyunsaturated chains has not been proven in *B. megaterium* (Quint & Fulco, 1973).

It is concluded that the molar ratio of iso/anteiso acyl chains in the present PE preparations increases more than 20-fold between 20 and 55 °C. Moreover, PE-20 contains about 21 mol % of monounsaturated acyl chains, of which 19.5 mol % are branched chains, while PE-55 does not contain any unsaturated acyl chains.

*Phase Equilibria and Maximum Hydration of PE from B. megaterium.* PE-20 and PE-55 were studied in mixtures with 3–17 mol of $^2H_2O$/mol of lipid and 3–11 mol of $^2H_2O$/mol of lipid, respectively. The phase equilibria were studied by $^{31}$P and $^2$H NMR.

The phase equilibria of PE-20 were investigated between 20 and 70 °C (Figure 1). An $L_\alpha$ phase is present up to about 50 °C with 3 mol of $^2H_2O$/mol of lipid, as inferred from $^{31}$P NMR spectra exhibiting a high-field peak and a low-field shoulder. The chemical shift anisotropy of a spectrum recorded at 40 °C is approximately −38 ppm. When the

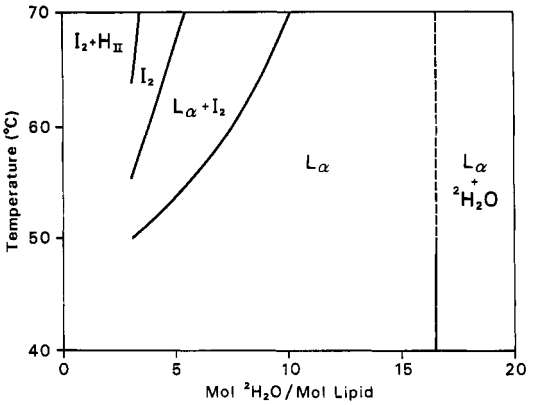


FIGURE 1: Tentative phase diagram for mixtures of $^2H_2O$ and PE isolated from *B. megaterium* strain Ft R32 grown at 20 °C (PE-20). The phase equilibria were determined by $^{31}$P and $^2$H NMR: $L_\alpha$, lamellar liquid–crystalline phase; $I_2$, reversed cubic liquid–crystalline phase; $H_{II}$, reversed hexagonal liquid–crystalline phase. The $L_\alpha$ phase extends down to at least 20 °C.

temperature is raised from 50 to 55 °C, the $^{31}$P NMR spectra show an increasing fraction of a narrow symmetrical signal in addition to the anisotropic spectral component. At such a low water content as 3 mol of $^2H_2O$/mol of lipid, the narrow signal represents a reversed cubic liquid-crystalline ($I_2$) phase (Rilfors et al., 1982; Eriksson et al., 1985a; Sjölund et al., 1987; Lindblom & Rilfors, 1989), and it was shown in earlier studies that this cubic phase is bicontinuous and belongs to the space group *Ia3d* (Rilfors et al., 1982; Lindblom & Rilfors, 1989). Thus, an $L_\alpha$ phase and an $I_2$ phase are in equilibrium between 50 and 55 °C. Between 55 and 64 °C the $^{31}$P NMR spectra exhibit just the narrow symmetrical signal and a pure $I_2$ phase is consequently formed. A spectral component consisting of a low-field peak and a high-field shoulder gradually appears above 64 °C, which indicates the formation of an $H_{II}$ phase (Eriksson et al., 1985a; Sjölund et al., 1987). The temperature for all the above-mentioned phase transitions is increased when the water content is increased above 3 mol of $^2H_2O$/mol of lipid (Figures 1 and 2a and b), and just an $L_\alpha$ phase is formed up to 70 °C with 10–16 mol of $^2H_2O$/mol of lipid. No traces of a gel/crystalline phase seem to be present at 20 °C in any of the PE-20–water samples, as judged from the line shape of the $^{31}$P NMR spectra.

$^2$H NMR spectra were recorded between 25 and 50 °C from the PE-20–water samples. A quadrupole splitting from the $^2H_2O$ was obtained when the water content was between 3 and 16 mol of $^2H_2O$/mol of lipid (Figure 2e); the splitting decreased from 2.3 kHz with 3 mol of $^2H_2O$/mol of lipid to 0.73 kHz with 15 mol of $^2H_2O$/mol of lipid. The water deuteron splitting as a function of the water content is shown in Figure 3. Except for the lowest water content, the splittings give a straight line passing through the origin. A sharp singlet superimposed on the quadrupole splitting was obtained in the $^2$H NMR spectra recorded from the sample with 17 mol of $^2H_2O$/mol of lipid (Figure 2f). This spectral feature indicates that the $L_\alpha$ phase is in equilibrium with free water (Figure 1).

The phase equilibria of PE-55 were investigated between 20 and 85 °C (Figure 4). The lipid forms an $L_\alpha$ phase up to 70 °C together with 3 mol of $^2H_2O$/mol of lipid (Figure 2c). The chemical shift anisotropy of a spectrum recorded at 40 °C is approximately −42 ppm. An $H_{II}$ phase is gradually formed between 70 and 82 °C (Figure 2d), and a pure $H_{II}$ phase is formed above 82 °C. The chemical shift anisotropy for the spectral component originating from the $H_{II}$ phase is 21 ppm, which is about half the value obtained from the $L_\alpha$ phase. During one of the heating cycles a fraction of the

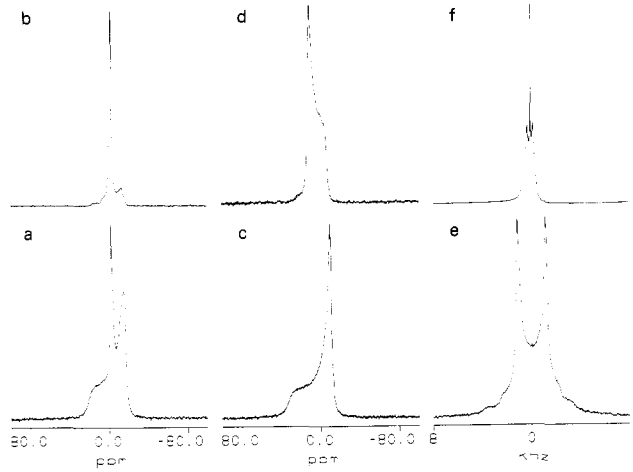


FIGURE 2: $^{31}P$ NMR spectra (a–d) and $^{2}H$ NMR spectra (e and f) recorded from mixtures of $^{2}H_2O$ and PE isolated from *B. megaterium* strain Ft R32 grown at 20 °C (PE-20) and strain Ot 32 grown at 55 °C (PE-55): (A) PE-20 with 5 mol of $^{2}H_2O$/mol of lipid at 55 °C; (B) same as panel A at 65 °C; (C) PE-55 with 3 mol of $^{2}H_2O$/mol of lipid at 40 °C; (D) same as panel C at 78 °C; (E) PE-20 with 3 mol of $^{2}H_2O$/mol of lipid at 25 °C; (F) PE-20 with 17 mol of $^{2}H_2O$/mol of lipid at 25 °C.

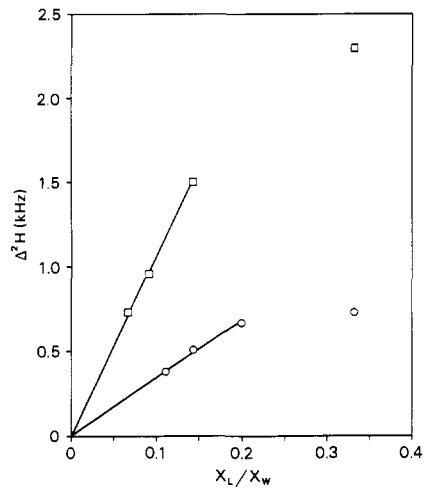


FIGURE 3: Water $^{2}H$ NMR quadrupole splitting as a function of the ratio between the mole fractions of lipid ($X_L$) and water ($X_W$) in the $L_\alpha$ phase formed by mixtures of $^{2}H_2O$ and PE isolated from *B. megaterium*. The spectra were recorded at 25 °C: (□) PE isolated from strain Ft R32 grown at 20 °C (PE-20); (○) PE isolated from strain Ot 32 grown at 55 °C (PE-55).

sample with 3 mol of $^{2}H_2O$/mol of lipid formed an $I_2$ phase, but this result could not be reproduced. However, it has been shown that dioleoyl-PE–water mixtures can form either an $H_{II}$ phase or an $I_2$ phase depending on the thermal history of a sample (Shyamsunder et al., 1988). A very small fraction of a PE-55 sample with 5 mol of $^{2}H_2O$/mol of lipid formed an $H_{II}$ phase between 75 and 85 °C, and samples with 7 and 9 mol of $^{2}H_2O$/mol of lipid formed just an $L_\alpha$ phase up to 85 °C (Figure 4). As judged from the line shape of the $^{31}P$ NMR spectra, no trace of a gel/crystalline phase was present in any of the PE-55–water samples at 20 °C. This conclusion was confirmed by high-power $^{1}H$ NMR studies, which showed that the line shape is super-Lorenzian (Wennerström, 1973), that the second moment is small and compatible with an $L_\alpha$ phase (Lindblom et al., 1993), and that it does not change significantly over the temperature range 17–38 °C.

The $^{2}H$ NMR spectra recorded at 25 °C from the PE-55–water samples exhibited a quadrupole splitting when the water content was between 3 and 9 mol of $^{2}H_2O$/mol of lipid. The splitting decreased from 0.73 kHz with 3 mol of $^{2}H_2O$/mol of lipid to 0.38 kHz with 9 mol of $^{2}H_2O$/mol of lipid. A

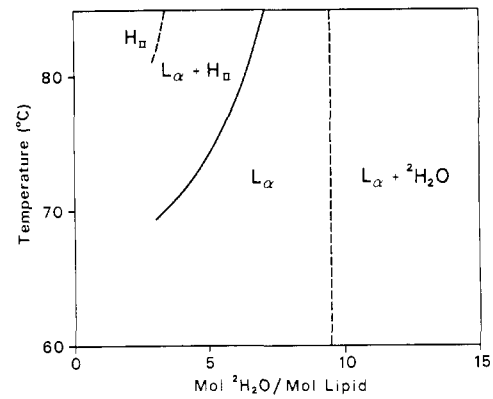


FIGURE 4: Tentative phase diagram for mixtures of $^{2}H_2O$ and PE isolated from *B. megaterium* strain Ot 32 grown at 55 °C (PE-55). The phase equilibria were determined by $^{31}P$ and $^{2}H$ NMR. Notations according to legends of Figure 1. The $L_\alpha$ phase extends down to at least 20 °C.

Table 2: Acyl Chain and Polar Head-Group Composition and Order Parameter in Total Lipid Extracts Isolated from *A. laidlawii* Strain A-EF22 Grown at 37 °C on Different Specifically Deuterated Branched-Chain Fatty Acids

| fatty acid supplemented to the growth medium | fraction of supplemented fatty acid in lipid extract (mol %) | ratio of MGlcDAG/ DGlcDAG | total fraction of anionic lipids[a] (mol %) | $S_{CD}$ of the $C^2H_3$ group[b] |
|---|---|---|---|---|
| 15-$C^2H_3$-16:0 | 89.0 | 1.32 | 33.2 | 0.004 |
| 14-$C^2H_3$-16:0 | 88.7 | 1.10 | 35.2 | 0.025 |
| 13-$C^2H_3$-16:0 | 84.1 | 0.68 | 41.1 | 0.028 |

[a] The anionic lipid fraction consists of PG and two different glycerophosphoryl derivatives of DGlcDAG (Rilfors et al., 1993; Hauksson et al., 1994). [b] The $S_{CD}$ values are calculated from the quadrupole splitting obtained at 35 °C from an $L_\alpha$ phase containing 20 wt % of water.

plot of the water deuteron splitting as a function of the water content exhibits a straight line passing through the origin when the splitting recorded at the lowest water content is excluded (Figure 3). With 10 and 11 mol of $^{2}H_2O$/mol of lipid the $^{2}H$ NMR spectra showed a sharp singlet superimposed on the quadrupole splitting, and these samples hence contained a free water phase in equilibrium with the $L_\alpha$ phase (Figure 4).

*Composition of A. laidlawii Total Lipids.* It has been shown previously that 13-methyltetradecanoic acid (iso-$C_{15}$), 12-methyltetradecanoic acid (anteiso-$C_{15}$), 15-methylhexadecanoic acid (iso-$C_{17}$), and 14-methylhexadecanoic acid (anteiso-$C_{17}$) support growth of *A. laidlawii* strain A-EF22 (Rilfors, 1985); however, iso-$C_{17}$ is less efficient in supporting growth than the other three acids. The organism is also able to grow on the branched-chain fatty acid precursors 3-methylbutanoic acid (iso-$C_5$) and 2-methylbutanoic acid (anteiso-$C_5$) (Rilfors, 1985). In the present investigation the *A. laidlawii* cells were grown on 15-$C^2H_3$-16:0, 14-$C^2H_3$-16:0, and 13-$C^2H_3$-16:0, although somewhat less efficiently on 15-$C^2H_3$-16:0 than on the other two acids. 13-$C^2H_3$-16:0 is not synthesized by living organisms but was included in the present study in order to ascertain the effects of moving the methyl branch one carbon atom above that of the anteiso position.

The three specifically deuterated branched-chain fatty acids were efficiently incorporated into the membrane lipids of *A. laidlawii* and constituted between 84 and 89 mol % of the acyl chains (Table 2). The remaining fraction of the acyl chains is dominated by myristoyl, palmitoyl, stearoyl, and oleoyl chains. As observed earlier in several investigations (see Rilfors et al. (1993) for a review), the polar head-group composition of the membrane lipids is changed in relation to

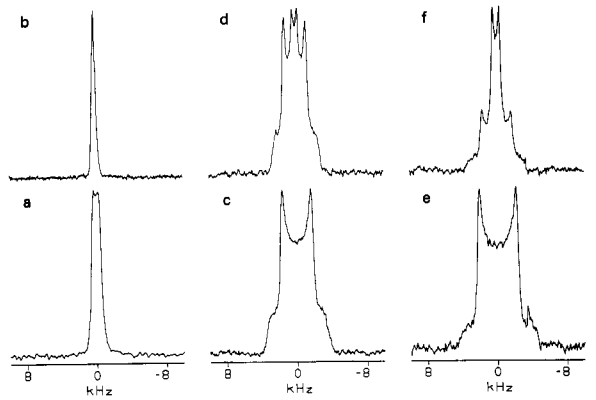


FIGURE 5: $^2H$ NMR spectra of total lipid extracts (neutral lipids removed) isolated from *A. laidlawii* strain A-EF22 grown at 37 °C on 15-$C^2H_3$-16:0 (a and b), 14-$C^2H_3$-16:0 (c and d), and 13-$C^2H_3$-16:0 (e and f). The water content was 10 wt % in all the samples. The $^2H$ NMR spectra were recorded at 35 °C (a, c, and e) and 60 °C (b, d, and f).

the structure of the acyl chains incorporated into the lipids. The molar ratio between the two dominating lipids, monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), decreases stepwise when the methyl branch is moved from position 15 to position 13 (Table 2). Concomitantly, the molar fraction of the three anionic lipids PG and two different glycerophosphoryl derivatives of DGlcDAG (Hauksson et al., 1994) increases.

*Phase Equilibria and Acyl Chain Ordering of A. laidlawii Total Lipids.* The phase equilibria of the total lipids (neutral lipids removed) isolated from *A. laidlawii* grown in the media supplemented with 15-$C^2H_3$-16:0, 14-$C^2H_3$-16:0, and 13-$C^2H_3$-16:0 were investigated with samples containing 10 and 20 wt % of deuterium-depleted water; these fractions correspond to 5 and 12 mol of water/mol of lipid.

$^2H$ NMR spectra were recorded from the 15-$C^2H_3$-16:0 lipid sample with 10 wt % of water between 25 and 74 °C. The spectra show a spectral component with a width of approximately 44 kHz up to 33 °C; this component originates from a gel/crystalline phase (Eriksson et al., 1991). Besides this broad component just a quadrupole splitting is present in the spectra up to 55 °C; the magnitude of the splitting is 513 and 342 Hz at 35 and 45 °C, respectively (Figure 5a). Thus, an $L_\alpha$ phase is in equilibrium with the gel/crystalline phase up to 33 °C, and just an $L_\alpha$ phase is present between 33 and 55 °C. Above 55 °C a new spectral component is seen in the center of the $^2H$ NMR spectra (Figure 5b). This component represents either an unresolved quadrupole splitting from an $H_{II}$ phase or a narrow $^2H$ signal from a cubic phase. The lipid–water sample was investigated with the pulsed magnetic field gradient technique above 55 °C, and no echo was observed. The presence of a cubic phase in the sample should have given rise to an echo (Lindblom et al., 1993), and the conclusion is therefore drawn that the narrow spectral component represents an $H_{II}$ phase. The intensity of this spectral component gradually increases when the temperature is raised above 55 °C.

$^2H$ NMR spectra were recorded between 25 and 76 °C from the 15-$C^2H_3$-16:0 lipid sample with 20 wt % of water. A gel/crystalline phase was present in the sample up to 29 °C, as seen from the occurrence of a component in the spectra with a width of approximately 48 kHz. Besides this component just a quadrupole splitting is seen in the spectra up to 63 °C; the magnitude of the splitting is 488 and 268 Hz at 35 and 45 °C, respectively. This spectral component represents an $L_\alpha$ phase. Above 63 °C a new spectral component is seen in the center of the $^2H$ NMR spectra also at this water content. By applying the pulsed magnetic field gradient technique it was concluded that the narrow spectral component represents an $H_{II}$ phase. The intensity of this component slowly increases when the temperature is raised, and the fraction of the 15-$C^2H_3$-16:0 lipids forming an $H_{II}$ phase at a certain temperature is smaller when they are mixed with 20 wt % water as compared with 10 wt % water. Finally, a $^2H$ NMR spectrum was recorded at 37 °C from a pellet of the *A. laidlawii* cells grown on 15-$C^2H_3$-16:0; a narrow spectrum with a small, unresolved quadrupole splitting was observed.

$^2H$ NMR spectra recorded from the 14-$C^2H_3$-16:0 lipid sample with 10 and 20 wt % of water do not exhibit any component representing a gel/crystalline phase at 25 °C. One quadrupole splitting is seen in the spectra from a sample with 10 wt % of water up to 49 °C; the magnitude of the splitting is 3.33 kHz at 35 °C (Figure 5c). Above 49 °C a new splitting of smaller magnitude appears, and at 52 °C the two splittings are 2.73 and 0.68 kHz, respectively (Figure 5d). The small splitting originates from an $H_{II}$ phase, and the fraction of this phase gradually increases when the temperature is raised. Note that if the geometry of the lipid aggregates alone is determining the difference in the quadrupole splittings, then the splitting of an $L_\alpha$ phase should be twice as large as that one observed for an $H_{II}$ phase (Wennerström et al., 1974; Thurmond et al., 1993). $^2H$ NMR spectra recorded from the sample with 20 wt % of water exhibit one quadrupole splitting up to 67 °C. The splitting decreases from 3.15 kHz at 35 °C to 2.03 kHz at 67 °C. At 69 °C an isotropic component and a quadrupole splitting of 0.59 kHz appear in the spectrum, representing an $I_2$ phase and an $H_{II}$ phase, respectively. The fraction of both these phases increases when the temperature is raised to 85 °C. A $^2H$ NMR spectrum recorded at 37 °C from a pellet of *A. laidlawii* cells grown in a medium supplemented with 14-$C^2H_3$-16:0 shows a quadrupole splitting of about 2.4 kHz.

The *A. laidlawii* lipids enriched in 13-$C^2H_3$-16:0 form an $L_\alpha$ phase both with 10 and 20 wt % of water at 25 °C. Just a quadrupole splitting is seen in the $^2H$ NMR spectra up to 42 °C in the sample with 10 wt % of water and up to 65 °C in the sample with 20 wt % of water. The magnitude of the splitting at 35 °C is 4.17 and 3.59 kHz with 10 and 20 wt % of water, respectively (Figure 5e). A second quadrupole splitting of smaller magnitude appears in the spectra above 42 and 65 °C in the samples containing 10 and 20 wt % of water, respectively (Figure 5f). The two splittings are 3.32 and 0.81 kHz at 55 °C with the lower water content and are 2.56 and 0.61 kHz at 69 °C with the higher water content. The smaller splitting originates from an $H_{II}$ phase. The intensity of the smaller splitting gradually increases, and the intensity of the larger splitting decreases, when the temperature is raised. Above 70 °C a very small fraction of the sample with 20 wt % of water forms an $I_2$ phase. A $^2H$ NMR spectrum was also recorded from a pellet of the *A. laidlawii* cells grown on 13-$C^2H_3$-16:0, and a quadrupole splitting of 3.0 kHz was obtained.

## DISCUSSION

*B. megaterium.* The acyl chain composition of PE from *B. megaterium* is markedly regulated in response to changes in the growth temperature of the organism (Table 1). The molar ratio of iso/anteiso acyl chains increases more than 20-fold when the growth temperature is raised from 20 to 55 °C. This result agrees with previous observations (Rilfors et al., 1978, 1982); however, in the present PE preparations, the increase in the acyl chain ratio is somewhat more pronounced. PE-55 does not contain any unsaturated acyl chains, while PE-20

contains about 21 mol % of monounsaturated chains (Table 1). The PE-20 preparation studied previously (Rilfors et al., 1982) contained about 11 mol % of unidentified acyl chains; monounsaturated iso and anteiso acyl chains most probably constituted the major fraction of these chains. The degree of unsaturation is thus approximately twice as high in the present PE-20 preparation.

The occurrence of monounsaturated iso and anteiso acyl chains in the membrane lipids of *B. megaterium* as well as in other species of the genus *Bacillus* has been reported previously. When *B. megaterium* is grown at 20 °C or lower temperatures, the synthesis of a $\Delta^5$-desaturase is induced (Fulco, 1972; Fujii & Fulco, 1977). Quint and Fulco (1973) showed that this organism is able to desaturate iso-$C_{16}$, iso-$C_{17}$, and anteiso-$C_{17}$ acyl chains but not iso-$C_{15}$ and anteiso-$C_{15}$ acyl chains. The tentative identification of the monounsaturated acyl chains present in PE-20 (see Results and Table 1) is consequently in complete agreement with the observations made by Quint and Fulco (1973). In a recent investigation Suutari and Laakso (1992) demonstrated that *B. megaterium* increases the fraction of $\Delta^5$-unsaturated acyl chains when the cells are grown below 35 °C; pentadecenoyl, hexadecenoyl, and isohexadecenoyl chains constituted about 21 mol % of the acyl chains at 20 °C.

To summarize, the *B. megaterium* strains studied in this work synthesize fatty acids with lower melting points at a lower growth temperature. Since low-melting acyl chains will decrease the $T_m$ value of the membrane lipids, it could be argued that this kind of regulation may be needed by the cells in order to avoid a large proportion of the lipids entering the gel phase; if that would occur, membrane-bound enzymes and transport proteins would be inactivated (McElhaney, 1984). However, this is presumably not the reason for the regulation of the acyl chain composition; both PE-20 and PE-55 have melted acyl chains and form an $L_\alpha$ phase at 20 °C, and PE has the highest $T_m$ value among the three dominating lipids in *B. megaterium* (Rilfors et al., 1993, and references therein).

The phase equilibria of lipid–water mixtures differ between PE-20 and PE-55 in three ways (Figures 1 and 4): PE-20 is more prone to form reversed nonlamellar phases than is PE-55; PE-20 forms both $I_2$ and $H_{II}$ phases while PE-55 forms only an $H_{II}$ phase; and the $L_\alpha$ phase of PE-20 takes up about 70% more water than the $L_\alpha$ phase of PE-55. These differences can to a large part be explained by the differences in the acyl chain composition. PE-20 contains about 21 mol % of monounsaturated acyl chains, and such chains generally shift the phase equilibria of membrane lipids toward reversed nonlamellar phases (Rilfors et al., 1984; Lewis et al., 1989; Israelachvili, 1991). The introduction of a cis double bond into a saturated hydrocarbon chain reduces the length of the chain (Israelachvili et al., 1977) and probably increases the average volume occupied by the chains in the lipid molecule. The phase equilibria of membrane lipids are likewise shifted toward reversed nonlamellar phases by moving a methyl branch from the iso to the anteiso position. It has been argued (Menger et al., 1988b) that a methyl branch can be viewed as a "kink-inducing unit" that, like a cis double bond, provides an inherent bend. When the methyl group is moved closer to the center of the hydrocarbon chain, a longer segment of the chain is skewed out of position by the torsional displacement introduced by a so-called 2g1 kink. Thus, the lower molar ratio of iso/anteiso acyl chains in PE-20 probably also increases the average volume of the hydrocarbon chains. The results obtained with the two native PE preparations can be compared to those obtained with synthetic PE compounds containing iso and anteiso acyl chains. The anteiso derivatives have a $T_{LH}$ that is 8–10 °C lower than that of the corresponding iso derivatives (Lewis et al., 1989).

A comparison can be made between the phase equilibria of the present PE preparations and the PE preparations studied previously (Rilfors et al., 1982). The older PE-20 and PE-55 were investigated between 26 and 65 °C. The phase diagrams for both these lipids exhibit exactly the same phases in this temperature interval as the diagrams for the new lipids: PE-20 formed an $L_\alpha$ phase, a mixture of $L_\alpha$ and $I_2$ phases, and a pure $I_2$ phase, while PE-55 formed only an $L_\alpha$ phase. However, a small difference is noted between the two PE-20 preparations; the temperature for the transition from the $L_\alpha$ phase to the $I_2$ phase increases more slowly with an increasing water content for the older preparation (see Figure 21 in Lindblom and Rilfors (1989)). This difference is difficult to explain due to the complex acyl chain composition of the lipids. The older PE-20 has a somewhat lower molar ratio of iso/anteiso acyl chains, which is expected to shift the phase equilibria toward the $I_2$ phase. On the other hand, the older PE-20 also contains a smaller fraction of monounsaturated acyl chains, which ought to shift the phase equilibria in the reverse direction.

The differences in phase behavior between PE-20 and PE-55 may be relevant for the ability of the *B. megaterium* cells to adapt to different growth temperatures. An increase in the temperature will increase the tendency of PE, with various acyl chain compositions, to form $I_2$ and $H_{II}$ phases (Figures 1 and 4; Cullis & De Kruijff, 1978; Lewis et al., 1989). In order to maintain an optimal packing of the membrane lipid molecules, giving for example suitable permeability properties to the bilayer and a suitable negative curvature of the lipid monolayers, it is probably necessary for *B. megaterium* to synthesize PE molecules with a reduced tendency to form reversed nonlamellar phases when the growth temperature is increased from 20 to 55 °C. In the present study PE was isolated from two different strains of *B. megaterium*. However, it should be pointed out that a shift in the growth temperature of strain Ft R32, when it is in the early logarithmic phase of growth, elicits a rapid and profound change in the acyl chain composition which is analogous to the one noticed between the two strains (Rilfors et al., 1982). The adaptation of the acyl chain composition therefore seems to be of vital importance for a proper membrane function. Several mesophilic as well as thermophilic *Bacillus* species exhibit a temperature-dependent alteration of the molar ratio of iso/anteiso acyl chains like the one found in the present *B. megaterium* strains (Shen et al., 1970; Chan et al., 1973; Kaneda, 1991; Suutari & Laakso, 1992), and the results presented in this work are therefore of general relevance.

The difference in acyl chain composition between the two PE preparations also influences their ability to take up water. For the PE-20 and PE-55 studied in the present work, a free water phase is obtained with 17 and 10 mol of $^2H_2O$/mol of lipid, respectively (Figures 1 and 4); with the older PE-20 and PE-55 preparations a free water phase was observed with 15 and 9 mol of $^2H_2O$/mol of lipid, respectively (Rilfors et al., 1982). From the deuterium NMR quadrupole splittings of $^2H_2O$ it can be concluded that when the bilayers in the $L_\alpha$ phase have a maximal amount of water associated to the bilayer surfaces (the polar head-groups have a filled "hydration shell" of "bound" water), then further addition of water to the $L_\alpha$ phase only results in an increase of "free" water molecules between the lipid lamellae. The decrease in the $^2H$ NMR quadrupole splitting of the water with increasing water concentration can be rationalized by applying a simple two-site model (see for example Finer and Darke (1974) and

Lindblom et al. (1976, 1991)), where "free" and "bound" water molecules are assumed to exchange rapidly between the sites. The observed quadrupole splitting within this model then follows

$$\Delta^2H = nX_L/X_W|\chi S| \quad (1)$$

where $X_L$ and $X_W$ are the mole fractions of lipid and water, $\chi$ is the quadrupole coupling constant, $n$ is the average number of water molecules "bound" to each head-group, and $S$ is the order parameter characterizing an average orientational order of the water molecules.

It can be inferred from Figure 3 that the observed $^2H$ NMR quadrupole splittings follow eq 1 and give a straight line, passing *through the origin*, over most of the water concentrations studied. A deviation is observed only at the lowest water content, where most probably two-dimensional swelling occurs (Lindblom et al., 1978). The slopes of the straight lines in Figure 3 are found to be equal to 10.5 kHz for PE-20 and 3.4 kHz for PE-55. The different slopes obtained for the two lipids can be due to either the hydration of the head-groups (the $n$-values) or the molecular ordering ($S$) of the water molecules in the "bound" site being different for PE-20 and PE-55. Note, that $\chi$ in eq 1 is an intramolecular constant, which for heavy water is equal to about 220 kHz (Glasel, 1972). It can be assumed that the $n$-values may be estimated from the deviation of the straight lines in Figure 3, i.e. a break or bend in the straight line is obtained at a point where a further increase in the water content begins to give a site of "free" water. From the limited experimental data, estimates of the $n$-values are found to be about 6 for PE-20 and about 4.5 for PE-55. Since the ratio between the slopes is equal to 3 and the ratio between the $n$-values is equal to 1.3, it can be concluded that also the molecular ordering of the water molecules around the polar head-group is affected by the difference in acyl chain composition between the two PE preparations. A more detailed description of the ordering of the water molecules in PE-20 and PE-55 is, however, not possible.

In crystals of PE with saturated, straight, acyl chains, the polar head-group forms a very compact, rigid network at the bilayer surface in which the ammonium groups interact directly with the unesterified phosphate oxygens by way of both hydrogen bonds and salt bridges (Hauser et al., 1981). There are indications that this compact molecular packing is maintained in the presence of water (Hauser et al., 1981). It has also been shown that the area occupied by the phosphorylethanolamine group is somewhat smaller than that occupied by two saturated, straight acyl chains (Pascher et al., 1981). If acyl chains occupying stepwise larger areas than such chains are introduced into the PE molecule, the phosphorylethanolamine groups will lose their close contact. In order to stabilize such an expanded lamellar phase, water molecules have to penetrate into the polar region and bridge the gaps between the head-groups (Hauser et al., 1981). PE-20 contains a larger fraction of anteiso and monounsaturated acyl chains than PE-55, and these chains occupy a larger area than iso and saturated acyl chains (Weitzel et al., 1951; Tourtellotte, 1972). The polar head-group lattice is thus probably more loosened up in PE-20 and is forced to bind more water molecules. This conclusion is supported by the fact that, at the same water content, the value of the quadrupole splitting observed for $^2H_2O$ in PE-20–$^2H_2O$ mixtures is 3 times larger than the value obtained from PE-55–$^2H_2O$ mixtures. Such results indicate that a larger fraction of the water molecules present in the PE-20–$^2H_2O$ mixtures is ordered by the interaction with the polar head-group. The fact that the value of the chemical shift anisotropy for PE-20 is about 4 ppm smaller than that for PE-55 also indicates that the area per lipid molecule is slightly larger for PE-20, since a larger head-group area will lead to a more extensive motional averaging, reducing the chemical shift anisotropy. When the acyl chain composition is changed from that characteristic for PE-55 to that characteristic for PE-20, a critical limit may be passed in order to maintain a tightly packed head-group lattice. This assertion is favored by the fact that the maximum hydration for dioleoyl-PE in the $L_\alpha$ phase is 18 mol of $H_2O$/mol of lipid (Gruner et al., 1988), i.e. just one water molecule more than that for PE-20. The hydration properties of PE can be compared to those of phosphatidylcholine (PC), MGlcDAG, and DGlcDAG. No ammonium links between the unesterified phosphate oxygens exist in crystals of PC with saturated, straight, acyl chains; these links are replaced by water molecules of hydration (Hauser et al., 1981). The relatively weak lateral interaction between the phosphorylcholine groups allows for a larger hydration of PC compared to PE, and the maximum hydration of dioleoyl-PC in the $L_\alpha$ phase is 30–34 mol of water/mol of lipid (Sjölund et al., 1987; Bergenståhl & Stenius, 1987; Gruner et al., 1988). On the other hand, MGlcDAG and DGlcDAG take up smaller amounts of water than PE and the maximum hydration of the glucolipids seems to be less sensitive to the acyl chain composition than is PE (Lindblom et al., 1986; Rilfors et al., 1993). This indicates that the hydrogen bonding is stronger between the head-groups of MGlcDAG and DGlcDAG, and it has been shown that, in crystals of glycolipids, the molecules are packed in such a way that intermolecular hydrogen bonding can occur (Moews & Knox, 1976; Pascher & Sundell, 1977).

*A. laidlawii*. When fatty acids supplied in the growth medium or synthesized by *A. laidlawii* are incorporated into the membrane lipids, the relative distribution of the polar head-groups is adapted to the chemical structure and thus the physico-chemical properties of the fatty acids. Three kinds of changes have been observed to occur (Rilfors et al., 1993): (1) the proportion between MGlcDAG and DGlcDAG; these lipids usually make up 55–75 mol % of the membrane lipids in strain A; (2) the proportion between the nonionic glucolipids and the anionic lipids; and (3) the proportion of a third glucolipid, monoacyl-MGlcDAG. In the present investigation the molar ratio of MGlcDAG/DGlcDAG decreases, and the molar fraction of anionic lipids increases, when the methyl branch is moved from position 15 to position 13 in the acyl chains incorporated into the membrane lipids (Table 2). The fraction of monoacyl-MGlcDAG was not quantified, but it is known from earlier experiments that this lipid constitutes merely 1–4 mol % of the membrane lipids when *A. laidlawii* strain A is grown on various branched-chain fatty acids (Rilfors, 1985). The regulation of the above-mentioned glucolipid ratio has generally been interpreted as a striving of the organism to maintain a certain balance between bilayer-forming and non-bilayer-forming lipids (Wieslander et al., 1980; Rilfors et al., 1993), giving a slightly negative curvature of the lipid monolayers in the membrane (Österberg et al., 1994). This model can be applied also to the present results. Anteiso fatty acids destabilize an $L_\alpha$ phase of PE more than iso acids (Figures 1 and 4; Lewis et al., 1989). It can be anticipated that a further destabilization is obtained by moving the methyl branch one carbon atom above the anteiso position, since such an acid occupies a larger area at the air–water interface than an anteiso acid (Weitzel et al., 1951). Moreover, Menger et al. (1988b) observed that the disruptive property of a methyl branch increases step by step when it is moved from the chain terminus to the center of the chain. *A. laidlawii*

reduces the fraction of MGlcDAG in the membrane (Table 2) when branched-chain fatty acids with an increasing tendency to destabilize a lipid bilayer are incorporated into the lipids; in these experiments MGlcDAG is the major lipid able to form reversed nonlamellar phases (Wieslander et al., 1978; Lindblom et al., 1986; Rilfors et al., 1993). Concomitantly, the fraction of anionic lipids increases and these lipids are likely to further stabilize a lipid bilayer. However, at least two phenomena can decrease the stabilizing ability of anionic lipids: counterion condensation (Lindblom et al., 1991) and specific interactions, like hydrogen bonding, between the polar head-groups (Nilsson et al., 1991).

The phase equilibria of total lipid extracts (neutral lipids removed) from *A. laidlawii* enriched in 15-$C^2H_3$-16:0, 14-$C^2H_3$-16:0, or 13-$C^2H_3$-16:0 were investigated. In the presence of 20 wt % of water, $H_{II}$ and $I_2$ phases formed above 63–67 °C in the lipid extracts. In the presence of 10 wt % of water just an $H_{II}$ phase was formed and the phase transition occurred at lower temperatures as compared with the cases of samples containing 20 wt % of water. The latter observation is in line with results obtained from several other lipid–water systems (Figures 1 and 4; Rilfors et al., 1984; Eriksson et al., 1985a; Lindblom et al., 1991). Moreover, with 10 wt % of water, the $H_{II}$ phase appeared at gradually lower temperatures when the methyl branch was moved up the hydrocarbon chain. At this low water content, however, there is probably no "free" water molecules between the lamellae (see above), and the phase transitions are more strongly influenced by changes in the interactions between water and the head-groups which are not relevant to the discussion of lipid regulation in cell membranes. Thus, these results indicate that the regulation of the polar head-group composition does compensate for the difference in acyl chain packing introduced into the bilayer by the three branched-chain fatty acids. The phase-transition temperatures obtained for the total lipid extracts in this study can be compared to those obtained from another recent investigation comprising a series of five total lipid extracts (neutral lipids removed) with different proportions of palmitoyl and oleoyl chains (Österberg et al., 1994). The water content in these samples was 17.5–18.5 wt % (10 mol of water/mol of lipid), and the fraction of oleoyl chains varied from 35 to 84 mol %. An $I_2$ phase and/or an $H_{II}$ phase appeared between 54 and 64 °C, but there is no correlation between the phase transition temperature and the degree of unsaturation in the membrane lipids. From both the above-mentioned series of total lipid extracts it can be concluded that the membrane lipid composition in *A. laidlawii* is regulated so that the ability to form nonbilayer structures is kept within a rather narrow range.

In one of the three lipid extracts from *A. laidlawii* studied in this work, a fraction of the lipids forms a gel/crystalline phase above 25 °C. This phase disappeared at 33 and 29 °C, in the lipid extract enriched in 15-$C^2H_3$-16:0, in the presence of 10 and 20 wt % of water, respectively. The result is in good agreement with the results obtained by Silvius et al. (1980) from *A. laidlawii* strain B-PG9. Differential thermal analysis of a total lipid extract from this strain enriched in iso-$C_{17}$ showed that the transition to an $L_\alpha$ phase had gone to 90% of completion at about 31 °C. However, the regulation of the polar head-group composition of the *A. laidlawii* lipids observed in the present study cannot regulate the $T_m$ value of the lipids. When branched-chain fatty acids with higher melting points are incorporated into the membrane lipids, the cells respond by increasing the molar ratio between MGlcDAG and DGlcDAG and decreasing the molar fraction of anionic lipids. Since MGlcDAG has a higher $T_m$ value than DGlcDAG with corresponding acyl chains and since the dominating anionic lipid, PG, has a lower $T_m$ value than both the glucolipids (Rilfors et al., 1993, and references therein), the result of the lipid regulation is to further raise the $T_m$ value.

The order parameter, $S_{CD}$, of the C–$^2$H bond of a methyl group can be obtained from the observed $^2$H NMR quadrupole splitting, $\Delta^2H$, by the equation (Lindblom et al., 1976)

$$\Delta^2H = 3/4|\chi S_{CD}| \quad (2)$$

The $S_{CD}$ values calculated for the different total lipid extracts containing acyl chains with a deuterated methyl branch are shown in Table 2. As expected, the $S_{CD}$ value increases as the methyl branch is moved upward from the chain terminus. This result is in agreement with a large number of investigations of so-called order parameter profiles in bilayers of different lipids (see e.g. Stockton et al. (1977), Bloom et al. (1991), Eriksson et al. (1991), and Thurmond et al. (1993)). Furthermore, by taking into account the rapid rotation about the symmetry axis of the methyl group, which results in an order parameter equal to $3S_{CD}$ (Lindblom et al., 1976), it is found that even the values of the order parameters at the different positions along the acyl chains are very similar to those obtained from straight acyl chains (Stockton et al., 1977; Eriksson et al., 1991). Thus, the perturbation of the molecular order caused by the introduction of a methyl branch in the acyl chain is small.

Macdonald et al. (1983) have studied the acyl chain order of membranes of *A. laidlawii* strain B-PG9 enriched with iso and anteiso acyl chains of the same length. Specifically monofluorinated palmitic acid probes were biosynthetically incorporated into the membrane lipids, and the orientational order profiles were determined by $^{19}$F NMR. At 37 °C the orientational order was higher in membranes enriched with iso acyl chains than in membranes enriched with anteiso chains. Moreover, the plateau region of the order parameter profiles of the branched acyl chains was extended when compared with that of a straight acyl chain and it was concluded that the presence of a methyl branch has a local ordering effect (Macdonald et al., 1983).

*Lipid Regulation in Other Bacteria.* The regulation of the membrane lipid composition occurring in three other genera of bacteria has also been interpreted to be an active adjustment of the balance between lipids forming lamellar phases and lipids forming nonlamellar phases. A very brief summary of these results is given below.

PE has been isolated from *Pseudomonas fluorescens* grown at 5 and 22 °C (Cullen et al., 1971). The fraction of unsaturated acyl chains is higher at the lower temperature. When studied at the same temperature, PE (5 °C) is more prone to form an $H_{II}$ phase than PE (22 °C). Therefore, the balance between the lamellar and nonlamellar phases is kept approximately constant at the growth temperatures. It is noteworthy that monolayer results obtained from the two PE preparations indicated that the $T_m$ value is below 5 °C for both of them (Cullen et al., 1971). It was recently reported that total lipid extracts from *P. fluorescens* are able to form cubic and $H_{II}$ phases (Mariani et al., 1990; Luzzati et al., 1992).

Goldfine and colleagues have performed a series of experiments with *Clostridium butyricum* (Goldfine et al., 1987a,b; MacDonald & Goldfine, 1991). The major membrane lipids are PE, plasmaenylethanolamine (PlaE), and the glycerol acetal of PlaE (GAPlaE). The PE plus PlaE fraction has a pronounced tendency to form an $H_{II}$ phase at physiological temperatures, while GAPlaE forms an $L_\alpha$ phase up to at least

50 °C even when it is highly enriched with oleoyl chains (Goldfine et al., 1987a). The ratio of (PE + PlaE)/GAPlaE is decreased when the degree of acyl chain unsaturation of the membrane lipids is increased. It has also been observed that this lipid ratio is lower when the lipids are enriched with anteiso-$C_{17}$ acyl chains as compared to the cases of lipids enriched with iso-$C_{14}$ and iso-$C_{16}$ acyl chains (Goldfine et al., 1987b). From experiments where *C. butyricum* was grown in the presence of cyclohexane or *n*-octanol, MacDonald and Goldfine (1991) drew the conclusion that the lipid ratio did not serve the function to regulate the $T_m$ value of the membrane lipids. Similar regulatory mechanisms thus seem to operate in *A. laidlawii* and *C. butyricum*, although the membrane lipids involved differ completely.

Johnston and Goldfine (1992) have studied the regulation of the phospholipid and glycolipid composition in another species of *Clostridium*, namely *Clostridium acetobutylicum*. The membrane of this organism contains substantial amounts of glycolipids with galactosyl and glucoyl–galactosyl headgroups besides the phospholipids present in *C. butyricum*. When the fraction of unsaturated and cyclopropane acyl chains in the lipids is varied, the polar head-group composition is regulated in such a way that the changes affect the equilibria between lipids forming lamellar and nonlamellar phases.

A mutant of *E. coli* has been used to investigate the polymorphic regulation of membrane lipid composition (Rietveld et al., 1993). The lack of PE in this mutant is compensated by a large increase in the fractions of PG and DPG. The cells have an absolute requirement for high concentrations of $Mg^{2+}$, $Ca^{2+}$, and $Sr^{2+}$, which are known to induce the formation of cubic and $H_{II}$ phases in DPG–water systems. It was shown that total lipid extracts (neutral lipids removed) from this *E. coli* strain began to form nonlamellar phases about 10 °C above the growth temperature in the presence of the growth-promoting cations at concentrations where there is maximal growth. In contrast, in the absence of divalent cations or at ion concentrations where no growth occurred, the lipid extracts formed a bilayer structure up to 75 °C. Finally, results from our laboratory show that also wild-type cells of *E. coli* regulate the balance between lipids forming lamellar and nonlamellar phases (S. Morein, A.-S. Andersson, L. Rilfors, & G. Lindblom, to be published). Cells were grown at 17, 27, and 37 °C, and the degree of acyl chain unsaturation decreases with increasing growth temperature. For total lipid extracts (neutral lipids removed) isolated from these cells, there is a direct relationship between the temperature at which nonlamellar phases begin to form and the growth temperature.

## CONCLUSIONS

It is evident from previous studies of synthetic membrane lipids, together with the present studies of native lipids, that the physico-chemical properties of the lipids are influenced by the position of a methyl substituent on the acyl chains. Moreover, these differences in physico-chemical properties are sensed by living cells: the bacterium *B. megaterium* regulates the molar ratio of iso/anteiso acyl chains in the membrane lipids upon changes in the growth temperature, and the bacterium *A. laidlawii* adjusts the polar head-group composition of the lipids when the methyl branch is moved up along the chain from the terminus. The present investigation extends and lends further support to two conclusions made earlier: (1) the perturbing effect of a methyl substituent on the packing of the acyl chains increases when it is moved upward from the chain terminus; and (2) iso and anteiso acyl chains are able to fulfill roles similar to those played by straight saturated and cis-monounsaturated acyl chains; however, the difference in physico-chemical properties is less between the two branched acyl chains than between the two straight acyl chains. The above-mentioned regulations of the acyl chain and polar head-group compositions do not seem to be needed in order to regulate the $T_m$ value of the membrane lipids (*B. megaterium*), or change the $T_m$ value of the lipids in the wrong direction (*A. laidlawii*). A more plausible explanation to the regulation of the lipid composition is a striving of the bacterial cells to keep the balance between bilayer-forming and nonbilayer-forming lipids, and thus the curvature of the lipid monolayers, within certain limits. The model for the regulation of the membrane lipid composition that is discussed in this work for *B. megaterium* and *A. laidlawii* has also been used to interpret the lipid regulation mechanisms observed in the bacteria *P. fluorescens* (Cullen et al., 1971), *C. butyricum* (Goldfine et al., 1987a,b; MacDonald & Goldfine, 1991), *C. acetobutylicum* (Johnston & Goldfine, 1992), and *E. coli* (Rietveld et al., 1993).

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