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ARTICLE in *BIOCHIMICA ET BIOPHYSICA ACTA* · OCTOBER 2000

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# $\alpha$ -Methylene ordering of acyl chains differs in glucolipids and phosphatidylglycerol from *Acholeplasma laidlawii* membranes: $^2\text{H}$ -NMR quadrupole splittings from individual lipids in mixed bilayers

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Received 22 December 1999; received in revised form 4 July 2000; accepted 6 July 2000

## Abstract

A *Acholeplasma laidlawii* strain A-EF22 was grown in a medium supplemented with  $\alpha$ -deuterated oleic acid. Phosphatidylglycerol (PG), the glucolipids monoglucosyldiacylglycerol (MGlcDAG), diglucosyldiacylglycerol (DGlcDAG) and monoacyldiglucosyldiacylglycerol, and the phosphoglucolipid glycerophosphoryldiglucosyldiacylglycerol (GPDGlcDAG) were purified, and the phase behaviour and molecular ordering for the individual lipids, as well as for mixtures of the lipids, were studied by  $^2\text{H}$ -,  $^{31}\text{P}$ -NMR and X-ray scattering methods. The chemical structure of all the *A. laidlawii* lipids, except PG, has been determined and verified previously; here also the chemical structure of PG was verified, utilising mass spectrometry and  $^1\text{H}$  and  $^{13}\text{C}$  high resolution NMR spectroscopy. For the first time, lipid dimers were found in the mass spectrometry measurements. The major findings in this work are: (1) addition of 50 mol% of PG to the non-lamellar-forming lipid MGlcDAG does not significantly alter the transition temperature between lamellar and non-lamellar phases; (2) the  $^2\text{H}$ -NMR quadrupole splitting patterns obtained from the lamellar liquid crystalline phase are markedly different for PG on one hand, and DGlcDAG and GPDGlcDAG on the other hand; and (3) mixtures of PG and DGlcDAG or MGlcDAG give rise to  $^2\text{H}$ -NMR spectra consisting of a superposition of splitting patterns of the individual lipids. These remarkable features show that the local ordering of the  $\alpha$ -carbon of the acyl chains is different for PG than for MGlcDAG and DGlcDAG, and that this difference is preserved when PG is mixed with the glucolipids. The results obtained are interpreted in terms of differences in molecular shape and hydrophilicity of the different polar headgroups. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:**  $^2\text{H}$ -NMR; X-ray diffraction; Quadrupole splitting; Lamellar–non-lamellar phase transition;  $^1\text{H}$  high resolution NMR;  $^{13}\text{C}$  high resolution NMR; Mass spectrometry

## 1. Introduction

The single cell membrane of the bacterium *Acholeplasma laidlawii* A-EF22 contains several phospho-, phosphogluco- and glucolipids each possessing unique physico-chemical properties [1,2]. One of the advantages with this organism for physico-chemical

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studies of membranes and membrane lipids is that it can be grown under conditions where fatty acids cannot be endogenously synthesised, and the cells are therefore forced to incorporate the exogenously supplied fatty acids into its membrane lipids. Consequently, if fatty acids specifically deuterated in the  $\alpha$ -position are supplied, labeled lipids can be prepared and the phase equilibria and structural organisation can be conveniently studied by  $^2\text{H}$ -NMR [3,4].

In previous studies of *A. laidlawii* lipid mixtures, we observed that the anionic lipid fraction did not significantly increase the transition temperature ( $T_{\text{NL}}$ ) from a lamellar liquid-crystalline ( $L_\alpha$ ) phase to a non-lamellar phase [5,6]. This is a surprising result since ionic lipids are expected to shift the phase equilibria to an  $L_\alpha$  phase [7–10]. In a monolayer study of total lipid extracts and individual lipids prepared from *A. laidlawii* membranes, it was observed that the average molecular areas for the individual lipids are larger than the areas of the total lipid extracts [11]. We therefore considered the possibility that intermolecular interactions are present between the polar headgroups of 1,2-diacyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG) and the glucolipids, enabling tight lipid packing in total lipid extracts. One aim of the present study is to examine this possibility by determining the phase equilibria in three different binary mixtures of *A. laidlawii* lipids: (1) mixtures of the two lamellar-forming lipids PG and 1,2-diacyl-3-*O*-[ $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -D-glucopyranosyl]-*sn*-glycerol (DGlcDAG) were studied to see if such mixtures are able to transform into a non-lamellar arrangement; (2) mixtures of PG and the non-lamellar-forming lipid 1,2-diacyl-3-*O*-( $\alpha$ -D-glucopyranosyl)-*sn*-glycerol (MGlcDAG) were studied to examine how the phase equilibria of MGlcDAG are shifted by the presence of PG; and (3) mixtures of the phosphoglucolipid 1,2-diacyl-3-*O*-[glycerophosphoryl-6-*O*-( $\alpha$ -D-glucopyranosyl)-(1 $\rightarrow$ 2)-*O*- $\alpha$ -D-glucopyranosyl]-*sn*-glycerol (GPDGlcDAG) and the non-lamellar-forming lipid 1,2-diacyl-3-*O*-[ $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*-(6-*O*-acyl- $\alpha$ -D-glucopyranosyl)]-*sn*-glycerol (MADGlcDAG) were studied to examine if the ability of GPDGlcDAG to stabilise an  $L_\alpha$  phase in mixtures with the non-ionic lipid MADGlcDAG is of the same magnitude as the ability of GPDGlcDAG to stabilise an  $L_\alpha$  phase in

mixtures with the zwitterionic, non-lamellar-forming lipid dielaidoylphosphatidylethanolamine [12].

Previous  $^2\text{H}$ -NMR studies on *A. laidlawii* membranes and total lipid extracts containing  $\alpha$ -deuterated acyl chains have revealed a complex quadrupole splitting pattern, consisting of many overlapping splittings [2,6,13]. The second aim of the present study is to assign the splittings to separate lipids and to determine the splitting patterns generated by the three previously described binary lipid mixtures.

## 2. Materials and methods

### 2.1. Cell growth and lipid extraction

Strain A-EF22 of *A. laidlawii* was grown in a lipid-depleted bovine serum albumin/tryptose medium [14,15], where the tryptose had been prepared with an improved lipid-depletion procedure [16]. The medium was supplemented with 124–150  $\mu\text{M}$   $\alpha$ -deuterated oleic acid (18:1c- $d_2$ ). 18:1c- $d_2$  was synthesised according to Tulloch [17]. The cells were grown at 30°C and adapted to growth with 18:1c- $d_2$  by five consecutive daily inoculations. The volume of each culture was 5–7.5 l, and the growth time was 22–24 h. The cell cultures were harvested as described in Andersson et al. [1]. The cell pellets were pooled to 5 batches, each corresponding to 30 L of cell culture. The membrane lipids were extracted and non-lipid contaminants were removed as described in Andersson et al. [1].

### 2.2. Purification of MGlcDAG, DGlcDAG, PG and GPDGlcDAG

The total lipid extracts were applied to a silica gel (Bio-Sil HA minus 325 mesh, Bio-Rad Laboratories, Richmond, CA; silica gel 60, 230–400 mesh, Merck, Darmstadt, Germany; or silica gel S, 230–400 mesh, Riedel-de Haën, Seelze, Germany) column. Pigments and neutral lipids were eluted with chloroform, the glucolipids were eluted with acetone, and the anionic lipids were eluted with methanol.

The acetone fractions were further purified on a silica gel column (TLC-Silica gel 60H, particle size 15  $\mu\text{m}$ , Merck, Darmstadt, Germany). A slight  $\text{N}_2$

pressure was maintained over the column to prevent oxidation of the lipids. The column was eluted with chloroform to remove remnants of the neutral lipid fraction. The solvent chloroform/methanol/ammonia (91:35:10, v/v) (solvent system A) was applied to the column and small fractions were collected. The fractions enriched in MGlcDAG or DGlcDAG were selected in order to purify each lipid further. The fractions were applied on preparative thin-layer chromatography (TLC) plates pre-coated with a 0.5 mm layer of Silica gel 60 (Merck, Darmstadt, Germany). The chromatograms were developed with solvent system A, and the lipid spots were visualised by spraying the plates with deionised water; the gel was scraped off the plates and the lipids were eluted from the gel with chloroform/methanol (2:1, v/v).

PG was purified from the methanol fractions by preparative TLC using the solvent system chloroform/methanol/water (55:35:5, v/v) (solvent system B). It was eluted from the gel with chloroform/methanol (2:1, v/v) and methanol and was further purified with preparative TLC, using the solvent system chloroform/methanol/water/acetic acid (65:25:4:1, v/v) (solvent system C), and eluted from the gel as described previously.

GPDGlcDAG was purified from the methanol fractions by the use of a Chromatotron model 7924T (Harrison Research) where the circular plate was coated with Silica gel 60 PF<sub>254</sub> containing gypsum (Merck, Darmstadt, Germany) and saturated with solvent system B. The methanol fractions were applied, the lipids eluted and small fractions col-

lected. The fractions containing GPDGlcDAG only were pooled.

The MGlcDAG, DGlcDAG and PG purified from the different batches were pooled. GPDGlcDAG was only purified from one of the batches. Divalent cations were removed from the purified lipids and exchanged for sodium ions by a modified version [18] of the procedure described by Smaal and colleagues [19].

The MADGlcDAG preparation used in this study was purified according to the method previously described by us [1].

### 2.3. Determination of lipid composition

The acyl chain distribution in the purified lipids was determined by gas-liquid chromatography after converting the acyl chains to their methyl esters [20]. The analyses of the methyl esters and the calculations of the molar percentages were performed as in Andersson et al. [1]. The acyl chain composition of the purified lipids are presented in Table 1. It is obvious that the organism prefers to incorporate the fatty acids in different proportions into the different lipids [21].

The purity of the MGlcDAG, DGlcDAG and PG preparations were determined by TLC. The lipid spots were visualised by the use of a charring agent [22] and identified by proper references. The chromatograms were developed with solvent systems A and chloroform/methanol/water (65:25:4, v/v) (solvent system D) to determine the purity of DGlcDAG. The purity of MGlcDAG was determined by migra-

Table 1  
Acyl chain composition of purified membrane lipids from *A. laidlawii* A-EF22

Lipid	Acyl chain composition <sup>a</sup> (mol%)									UAC <sup>c</sup>
	12:0	13:0	14:0	15:0	16:0	17:0	18:0	18:1c-d <sub>2</sub>	C <sub>n</sub> <sup>b</sup>	
MGlcDAG	–	0.4	6.2	6.6	27.2	2.5	0.4	56.9	17.0	56.9
DGlcDAG	–	0.8	4.5	3.8	13.1	0.9	0.5	76.4	17.4	76.4
MADGlcDAG <sup>d</sup>	10.0	5.7	12.2	3.5	5.1	0	0	61.7	16.3	61.7
PG	–	–	2.4	2.0	8.0	–	–	87.6	17.7	87.6
GPDGlcDAG	2.2	1.3	4.7	2.0	7.5	–	1.1	81.2	17.4	81.2

<sup>a</sup>Acyl chains are denoted as *n:k*, where *n* is the number of carbons and *k* is the number of *cis* double bonds.

<sup>b</sup>Average acyl chain length.

<sup>c</sup>Unsaturated acyl chains (mol%).

<sup>d</sup>Values taken from Andersson et al. [1].

tion in solvent system A and the purity of PG was determined using solvent systems A, B and C. Finally the purity of GPDGlcDAG was determined using solvent systems B and D. Some of the TLC plates were sprayed with reagents specific for the detection of phospholipids or glycolipids [23].

The purity of the lipid preparations was determined to be  $\geq 98\%$  for MGlcDAG,  $\geq 99\%$  for DGlcDAG,  $\geq 95\%$  for PG and  $\geq 98\%$  for GPDGlcDAG. The contaminant in the PG preparation is not any of the other anionic lipids in *A. laidlawii*. The contaminant is neither a phospholipid nor a glycolipid, since it does not react with the spraying reagents specific for these lipid classes. Since it strongly absorbs and emits UV light, it is most probably a pigment.

#### 2.4. Determination of lipid structure

It was judged necessary to verify the chemical structure of PG isolated from *A. laidlawii*. This was done by two techniques, mass spectrometry and high resolution  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopy. Synthetic dioleoyl-PG (DOPG) (Avanti Polar Lipids, Alabaster, AL) was used as a reference lipid.

##### 2.4.1. Mass spectrometry

Electrospray ionisation (ESI) was performed on a Finnigan TSQ7000 triple quadrupole mass spectrometer (San Jose, CA) that had been fitted with a micro-ESI source [24,25]. The mass spectrometer was scanned from 200–2000 mass-to-charge ( $m/z$ ) units in 1 s and all data were acquired in the profile mode.

In order to reduce the volume of liquid flowing into the ESI source, a small inner diameter fused capillary needle was used. A 20-cm spray emitter/needle was constructed from fused silica capillary (Polymicro Technologies, Phoenix, AZ) (220  $\mu\text{m}$  o.d., 50  $\mu\text{m}$  i.d.). The polyimide coating was burned away from the tip (approximately 0.5 cm) and the tip was then lowered into a 49% solution of hydrofluoric acid (HF) while distilled  $\text{H}_2\text{O}$  was pumped through the capillary at 500 nl/min. The progress of the etching was monitored with a microscope until a tapered needle tip was obtained. The tip was then carefully ground by hand to a flat end using the flat side of a ceramic cutting stone. The opposite end of the needle

was attached to a stainless-steel zero dead volume fitting with a 0.02 in. through-hole. The other end of the zero dead volume union was connected to a syringe via a 30-cm fused silica transfer line. The high voltage was applied to the spray needle through the solvent at this union. Samples were analysed by using both positive and negative electrospray ionisation mode. Specific source conditions were: needle voltage,  $\pm 2.0$ – $3.0$  kV; heated capillary,  $\pm 10$  V and  $200^\circ\text{C}$ .

Samples were dissolved in 20  $\mu\text{l}$  of 50% methanol/0.25% acetic acid and analysed by continuous infusion at a flow rate of 400–500 nl/min. Infusions were performed by loading the sample into a 50- $\mu\text{l}$  gas tight Hamilton syringe. The sample was presented to the micro-ESI source via the 30-cm fused silica transfer line (220  $\mu\text{m}$  o.d., 50  $\mu\text{m}$  i.d.) connected to the syringe and the flow was driven by a Harvard Apparatus Model 2274 syringe pump.

Tandem MS (MS/MS) was performed to provide structure verification of *A. laidlawii*-PG. The first quadrupole of the tandem mass spectrometer was set to transmit the parent ion with an acceptance window of about  $\pm 1$   $m/z$  units. The parent ion was dissociated by collision with Ar gas (1.0–1.5 mTorr) in the second quadrupole region, and the third quadrupole was scanned over a mass range from 200 to 2000  $m/z$  units for the appropriate product ions. Mass measurement accuracy for the product ions was typically  $\pm 0.2$   $m/z$  units.

##### 2.4.2. NMR

The lipids were dissolved in  $\text{DMSO-}d_6/\text{H}_2\text{O}$  98:2 (v/v), a solvent that is known to give high-resolution spectra of lipids [26]. About 2 mg of lipid was dissolved in 700  $\mu\text{l}$  solvent. NMR measurements were performed at a temperature of 310 K on a Bruker AMX2-500 NMR spectrometer operating with a  $^1\text{H}$  frequency of 500.13 MHz and a  $^{13}\text{C}$  frequency of 125.76 MHz.

$^1\text{H}$  one-dimensional experiments were performed using a one-pulse sequence with water presaturation during the relaxation delay. A 5000 Hz spectral width was used and the relaxation delay was at least 3 s. The  $90^\circ$  pulse length was 7.4  $\mu\text{s}$ . 8192 data points were recorded, and zero-filling to 16384 points was performed before Fourier transformation. No window function was used. Data was processed using

the software Felix, version 2.30 from Biosym Technologies (San Diego, CA).

$^1\text{H}$  Double quantum-filtered correlated spectroscopy (DQF-COSY) experiments [27] were performed with water presaturation during the relaxation delay, using the states-TPPI method to get phase sensitivity, and with a modified phase-cycle according to Derome and Williamsson [28]. In all, 4096 data points were recorded in the F2 dimension with a spectral width of 5000 Hz and a relaxation delay of 3 s. A total of 1100 experiments were performed with the incremental delays set to correspond to a spectral width of 5000 Hz with averaging over 16 scans for each experiment. A squared sinebell window function was applied in both dimensions prior to Fourier transformation. Zero-filling to 1024 points was performed in the F1 dimension giving a  $4096 \times 1024$  2D data set. Data processing was done using X-Winnmr software on the spectrometer.

$^1\text{H}$ – $^{13}\text{C}$ -heteronuclear single-quantum correlation (HSQC) experiments were performed using a pulse program with magnetic gradients for coherence selection and water suppression [29–32]. In all, 1024 data points were recorded in the F2 dimension with a spectral width of 5000 Hz and a relaxation delay of 2 s. A total of 800 experiments were performed with the incremental delays set to correspond to a  $^{13}\text{C}$  spectral width of 8800 Hz (70 ppm) with averaging over eight scans for each experiment. A squared sinebell window function was applied in both dimensions prior to Fourier transformation. Zero-filling to 512 points was performed in the F1 dimension giving a  $1024 \times 512$  2D data set. Data processing was done using X-Winnmr software on the spectrometer.

## 2.5. Preparation of lipid samples for $^2\text{H}$ - and $^{31}\text{P}$ -NMR studies

Each lipid was dried to a film in an 8-mm o.d. glass tube with  $\text{N}_2$  and then dried to constant weight in vacuum. Samples aimed for lipid mixtures were prepared by dissolving each lipid in chloroform/methanol (2:1, v/v). The lipids were then pooled, thoroughly mixed and dried again. After the addition of 20 or 40 wt% deuterium-depleted water ( $^1\text{H}_2\text{O}$ ) (Fluka, Buchs, Switzerland), the tubes were centri-

fuged and flame-sealed. The samples were mixed by extended centrifugation and freeze-thawed for ten cycles to ensure complete equilibration. No decomposition of the lipids occurred during the NMR measurements as judged by TLC analyses performed on the samples after the measurements.

## 2.6. $^2\text{H}$ - and $^{31}\text{P}$ -NMR measurements and data processing

$^2\text{H}$ -NMR spectra were obtained for the lipid samples using a phase-cycled quadrupole echo pulse sequence [33]. The measurements were made at 61.42 MHz on a Varian CMX Infinity-400 spectrometer or at 76.77 MHz on a Bruker AMX2-500 spectrometer. The  $\pi/2$  pulse length was 4.5 (6.4)  $\mu\text{s}$  and the pulse separation 50 (40)  $\mu\text{s}$  for the two instruments, respectively. Temperature was controlled by a thermocouple placed close to the sample. The data were Fourier transformed from the echo maximum. The deuterium quadrupole splittings of the lipids ( $\Delta\nu_{\text{q}}$ ), defined as the frequency separation between the two  $90^\circ$  peaks in a powder spectrum, were determined from de-Paked spectra [34].

$^{31}\text{P}$ -NMR spectra were obtained for the phospholipid samples at a frequency of 162.1 MHz on a Varian CMX Infinity-400 spectrometer using a Hahn echo sequence [35] with a  $90^\circ$  pulse width of 23  $\mu\text{s}$  and an interpulse spacing of 50  $\mu\text{s}$ . Data was collected with WALTZ decoupling of the protons [36].

The deuterium quadrupole splitting, can be used to obtain information about the phase equilibria of lipid mixtures. Provided that the local ordering of the  $\text{C}$ – $^2\text{H}_2$  chain segment does not change upon phase transitions, one can expect the quadrupole splittings in the hexagonal phase to be reduced by a factor of two compared to the  $\text{L}_\alpha$  phase. An isotropic phase will give rise to a single line due to the complete averaging of the quadrupole interaction. Thus, the phase transitions between lamellar, hexagonal and isotropic phases can be followed by  $^2\text{H}$ -NMR [3]. As a complementary method  $^{31}\text{P}$ -NMR has been used on some samples. Again, spectra recorded from the lamellar, hexagonal and isotropic phases have characteristic lineshapes due to the chemical shift anisotropy of  $^{31}\text{P}$  [3,37].

## 2.7. X-ray diffraction

Some of the samples were investigated by X-ray diffraction in order to further strengthen the conclusions drawn from the NMR data. X-ray measurements of PG, GPDGlcDAG, DGlcDAG/PG (75:25, mol/mol) and MGlcDAG/PG (75:25, mol/mol) were performed at Station 8.2 at the Daresbury Laboratory (Cheshire, UK) as described in [2].

Typically, the temperature was changed at a rate of 3°C/min. At certain intervals, the temperature was held at a constant value for several minutes in order to ensure sample equilibration. No change in the diffractograms was observed during these constant temperature periods and it was concluded that the sample was close to thermal equilibrium at all times.

The SAXS reflections were used to distinguish the liquid crystalline phases [38]. After the measurements, the lipids were removed from the mica sheets and checked with TLC to make sure that no decomposition of the lipids had occurred.

## 3. Results

### 3.1. Verification of the structure of PG from *A. laidlawii*

Several reasons exist to make a careful investigation of the structure of the membrane phospholipid in *A. laidlawii* strain A-EF22 that, based on chemical analyses, was suggested by Wieslander and Rilfors

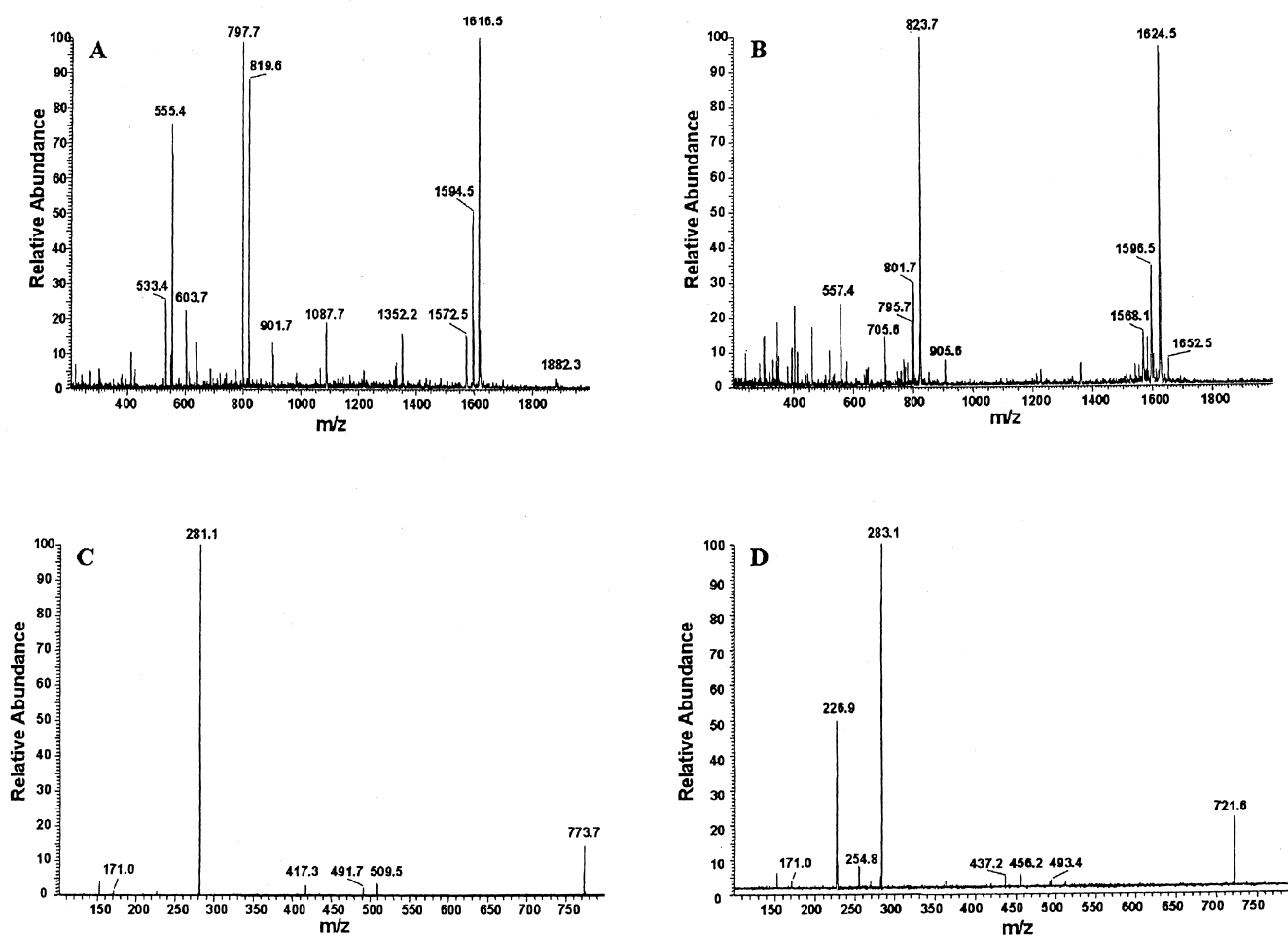


Fig. 1. Mass spectra of synthetic DOPG (A,C) and *A. laidlawii*-PG (B,D). The spectra in A and B were generated by positive electrospray ionisation, and the spectra in C and D are tandem mass spectra that were generated by negative electrospray ionisation.

[39] to be PG: (1) NMR studies indicated that *A. laidlawii*-PG may form two  $L_\alpha$  phases being in equilibrium with each other [5], while synthetic DOPG forms a single  $L_\alpha$  phase [40]; (2)  $^2\text{H}$ -NMR spectra recorded from *A. laidlawii*-PG containing two 18:1c- $d_2$  chains exhibit four quadrupole splittings (see Section 3.2) instead of the expected three splittings exhibited by several natural phospholipids containing two  $\alpha$ -deuterated acyl chains [41]; (3) small amounts of *O*-amino acid esters of PG have been found in membranes of *A. laidlawii* strain B-PG9 [42], and the possible occurrence of such a lipid in strain A-EF22 should be elucidated; and (4) PG is the only membrane lipid synthesised by *A. laidlawii* A-EF22 whose structure remains to be examined carefully [26,43,44].

In this study, the structure of *A. laidlawii*-PG containing 88 mol% 18:1c- $d_2$  (Table 1) was determined with mass spectrometry and NMR spectroscopy. Synthetic DOPG was used as a comparison.

### 3.1.1. Mass spectrometry

A mass spectrum of DOPG generated by the positive electrospray technique is shown in Fig. 1A. The mass-to-charge ( $m/z$ ) 797.7 corresponds to the sodium salt of DOPG, and  $m/z$  819.6 probably corresponds to a  $\text{Na}^+$  adduct of this molecule. The formation of dimers is also observed in the spectrum, and  $m/z$  1616.5, 1594.5, and 1572.5 correspond to dimer +21 ( $\text{Na}^+$  adduct), dimer –1, and dimer –23 (loss of  $\text{Na}^+$ ), respectively. Fig. 1B shows the mass spectrum of *A. laidlawii*-PG generated by the same technique.  $m/z$  801.7 corresponds to the sodium salt of DOPG- $d_4$ , and  $m/z$  823.7 corresponds to the  $\text{Na}^+$  adduct of the molecule. In this case, dimers are formed between the different molecular species present in the *A. laidlawii*-PG preparation. Besides DOPG- $d_4$  the species palmitoyl-oleoyl-PG (POPG- $d_2$ ) and myristoyl-oleoyl-PG (MOPG- $d_2$ ) exist (Table 1).  $M/z$  1624.5, 1596.5, and 1568.1 correspond to the  $\text{Na}^+$  adducts of the dimers DOPG- $d_4$ /DOPG- $d_4$ , DOPG- $d_4$ /POPG- $d_2$ , and DOPG- $d_4$ /MOPG- $d_2$ , respectively.

Both lipid molecules were also investigated with tandem mass spectrometry. Spectra generated with the negative electrospray technique are shown for DOPG and *A. laidlawii*-PG in Fig. 1C and D, respectively. The important fragments formed from

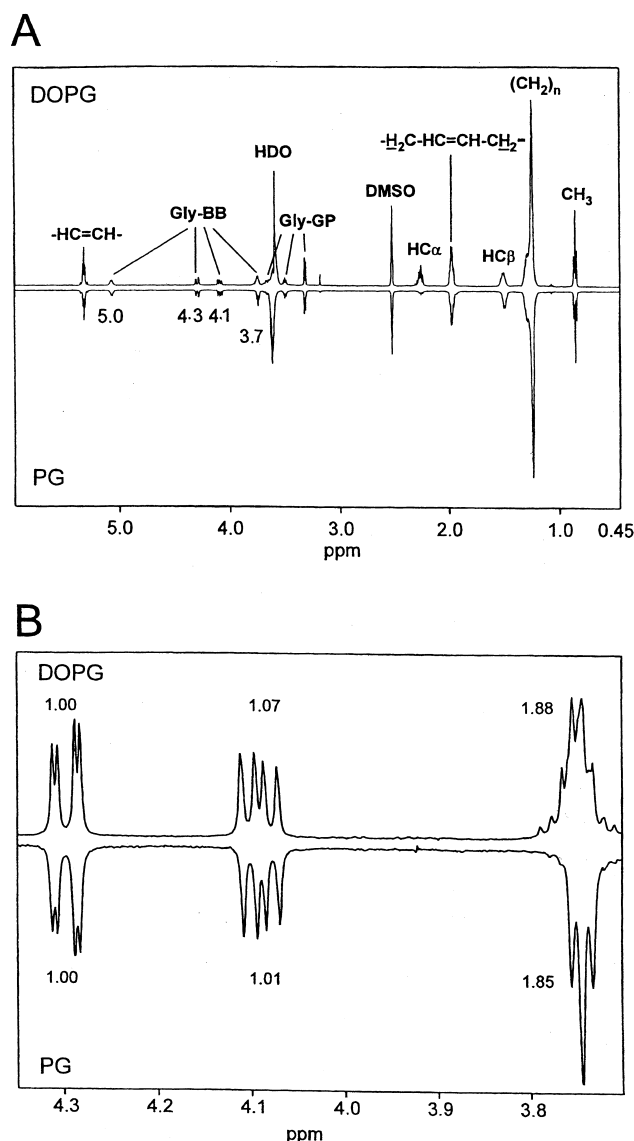


Fig. 2. One-dimensional  $^1\text{H}$ -NMR spectra of synthetic DOPG and *A. laidlawii*-PG. The upper spectrum in each panel corresponds to synthetic DOPG and the lower spectrum corresponds to *A. laidlawii*-PG. (A) The full spectral width with assignments indicated. Gly-BB is the backbone glycerol moiety, and Gly-GP is the glycerophosphoryl headgroup. Note the weaker signal at 2.26 ppm in *A. laidlawii*-PG due to  $\alpha$ -deuteration. (B) The backbone glycerol region. There is a difference in splitting pattern of the peak at 3.74 ppm from the two lipids. The relative integrals are shown for each multiplet (the integral of the signal at 4.30 ppm is set to 1.00) and are equal in the two lipids.

DOPG without  $\text{Na}^+$  ( $m/z$  773.7) are the ones having  $m/z$  281.1 and 171.0; the former fragment is the anion of 18:1c, and the latter fragment is the anion of phosphoglycerol, i.e. the polar headgroup of PG.



The spectrum recorded from the MOPG- $d_2$  species from *A. laidlawii* exhibits fragments with  $m/z$  283.1, 226.9, and 171.0, which corresponds to the anions of 18:1c- $d_2$ , myristic acid (14:0), and phosphoglycerol, respectively. Since the 18:1c fragment is more abundant than the 14:0 fragment, it is concluded that 18:1c is located in the *sn*-2 position and 14:0 in the *sn*-1 position [45]. Tandem mass spectra recorded from the other *A. laidlawii*-PG species show that generally 18:1c is located in the *sn*-2 position and that the saturated acyl chains are located in the *sn*-1 position.

### 3.1.2. NMR

There are only minor differences between the 1D  $^1\text{H}$ -NMR spectra of synthetic DOPG and the corresponding spectra of *A. laidlawii*-PG (Fig. 2A). The chemical shift of the methyl group was used as an internal reference and was set to 0.85 ppm in correspondence to Hauksson et al. [26]. The peak at 2.26 ppm represents the protons on the  $\alpha$ -carbon. This carbon atom is deuterated in the lipids from *A. laidlawii*, and therefore only the small fraction of non-

deuterated oleic acid gives a signal. The only other difference is found in the backbone glycerol region (Fig. 2B). The peak at 3.74 ppm is a triplet in *A. laidlawii*-PG but a more complex multiplet in synthetic DOPG, indicating a different coupling pattern between the spins. However, the integral of all peaks from the backbone glycerol protons is the same in both molecules, so the number of protons in this moiety is the same. Generally, exactly the same signals appear in the spectra from both DOPG and *A. laidlawii*-PG.

All the signals in the 1D  $^1\text{H}$ -spectrum were assigned with the additional information from the DQF-COSY and HSQC spectra. The assignments were consistent with the chemical shift values found earlier in other lipids from *A. laidlawii* strain A-EF22 [26,43,44]. Fig. 3 shows the polar headgroup region in the HSQC spectra of the two lipids. It is seen that there is only negligible differences between the two spectra. The HSQC and DQF-COSY spectra clearly show  $^1\text{H}$ - and  $^{13}\text{C}$ -signals at the same positions, and with the same relative intensities, in the spectra from both molecules. Moreover, there is no difference in

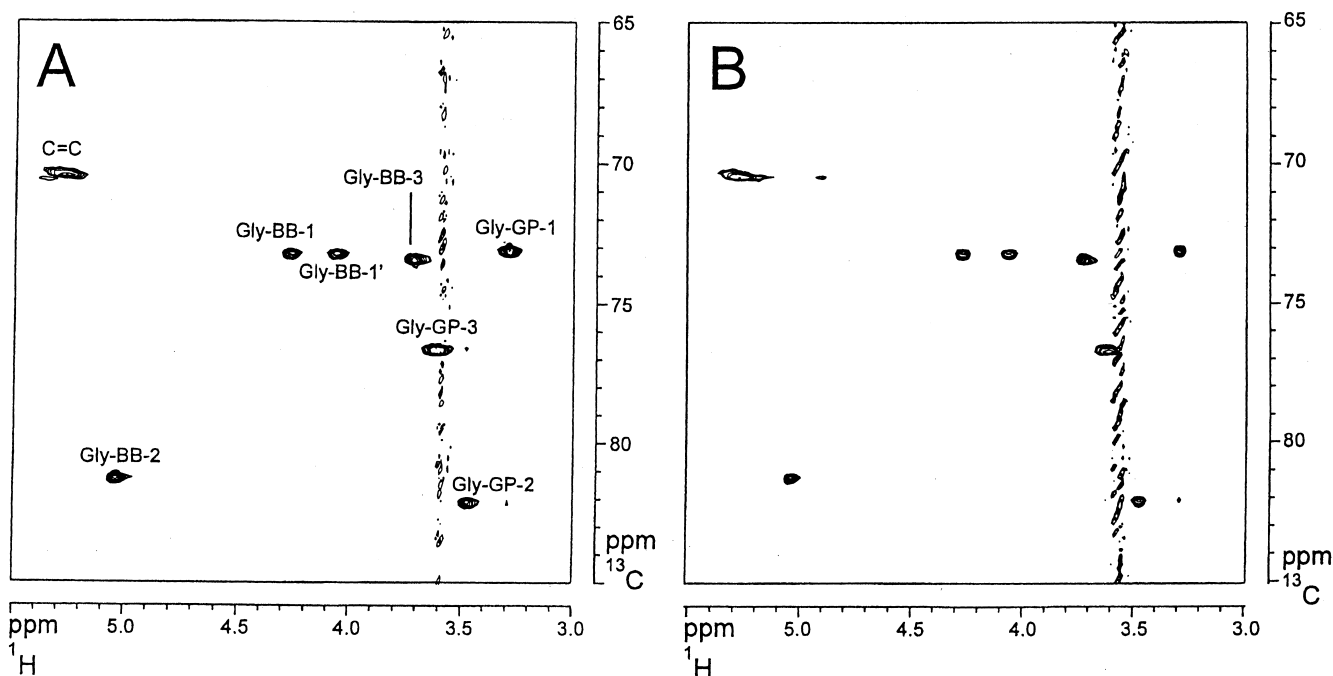


Fig. 3. A comparison of the HSQC spectra of the backbone glycerol region of *A. laidlawii*-PG (A) and synthetic DOPG (B). The assignments were obtained using HSQC data in combination with data from the DQF-COSY experiments (not shown). The numbering of the positions of the glycerols are chosen according to Hauksson et al. [26] and is not related to the *sn*-configuration of these moieties.

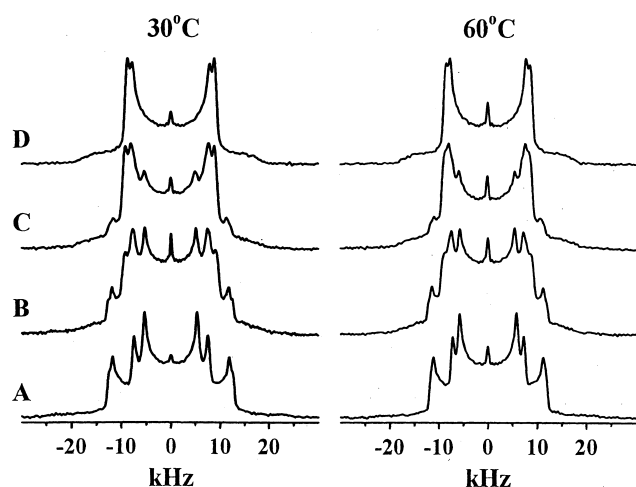


Fig. 4.  $^2\text{H}$ -NMR spectra of lipids isolated from *A. laidlawii* A-EF22 cells supplemented with  $18:1\text{-}c\text{-}d_2$ . The water content was 20 wt%. (A) PG. (B) DGlcDAG/PG (50:50, mol/mol). (C) DGlcDAG/PG (75:25, mol/mol). (D) DGlcDAG.

the coupling patterns as determined from the cross-peaks.

It is concluded from both the mass spectrometry and the NMR spectroscopy investigations that the phospholipid occurring in *A. laidlawii* strain A-EF22 in fact is PG.

### 3.2. Quadrupole splittings of the lipids in the $L_\alpha$ phase

$^2\text{H}$ -NMR spectra recorded from *A. laidlawii*-PG show four splittings (Fig. 4A) and the  $\Delta\nu_q$  values obtained from dePaked spectra are presented in Table 2. There is only a small difference between the two largest splittings and these are therefore difficult to distinguish in the powder spectrum. However, two splittings larger than 20 kHz are clearly resolved in a dePaked NMR spectrum (see the lower panel of Fig. 5, trace A). Moreover, these are observed at 30 and 60°C for both the PG sample (Fig. 4A) as well as for the two mixtures of PG and DGlcDAG (Fig. 4B,C).

The  $^2\text{H}$ -NMR spectrum of DGlcDAG shows only two splittings (Fig. 4D and Table 2), which from a lineshape analysis of dePaked spectra were found to have an intensity ratio of 3:1.

The lineshapes from mixtures of PG and DGlcDAG are rather complex. It is obvious from Fig. 4 and Table 2 that the number of splittings is larger than expected. All the splittings of the individual PG and DGlcDAG molecules are present and the

smaller splitting of DGlcDAG overlaps with the second smallest one of PG (Table 2). Moreover, as the molar ratio of DGlcDAG/PG is increased, the intensities of the signals of the individual lipids change accordingly (Fig. 4). Thus, it is concluded that the individual lipids retain their  $\Delta\nu_q$  values even when they are mixed.

The mixtures of PG and MGlcDAG form more than one phase (see Section 3.4) and it is therefore difficult to assign the observed splittings to separate lipids in the different phases (Fig. 5). Assuming that PG retains approximately the same  $\Delta\nu_q$  values in the  $L_\alpha$  phase of these mixtures as for the PG/DGlcDAG mixtures, it seems plausible that the  $\Delta\nu_q$  values at about 20 kHz originate from MGlcDAG in the  $L_\alpha$  phase (Table 3) and that the expected smaller  $\Delta\nu_q$  value of MGlcDAG overlaps with the PG splitting at 16 kHz. This indicates that the quadrupole splittings of MGlcDAG in the  $L_\alpha$  phase are similar to those of DGlcDAG. Finally, the  $\Delta\nu_q$  values of GPDGlcDAG closely resemble those of DGlcDAG (Fig. 6A and Table 2). To summarise the results, the sugar-containing lipids DGlcDAG, MGlcDAG and GPDGlcDAG exhibit a different quadrupole splitting pattern in the  $L_\alpha$  phase than PG, both individually and in the mixtures.

### 3.3. Phase behaviour of individual lipids

*A. laidlawii*-PG forms an  $L_\alpha$  phase in the temperature interval studied in this work, namely 30 and 60°C. The phase behaviour was determined by  $^{31}\text{P}$ -NMR, giving the well known lineshape of a powder pattern exhibiting a chemical shift anisotropy of a uniaxial lamellar system [37]. The formation of the

Table 2

Quadrupole splittings obtained by  $^2\text{H}$ -NMR on DGlcDAG, PG, GPDGlcDAG and DGlcDAG/PG mixtures containing  $\alpha$ -deuterated acyl chains

Sample	Quadrupole splittings (kHz)			
DGlcDAG <sup>a</sup>			18	16
DGlcDAG/PG (75:25, mol/mol) <sup>b</sup>	25	23	18	16
DGlcDAG/PG (50:50, mol/mol) <sup>b</sup>	25	24	19	16
PG <sup>b</sup>	25	24		15
GPDGlcDAG <sup>a</sup>			18	16

<sup>a</sup>The spectrum was recorded at 30°C.

<sup>b</sup>The spectra were recorded at 35°C.

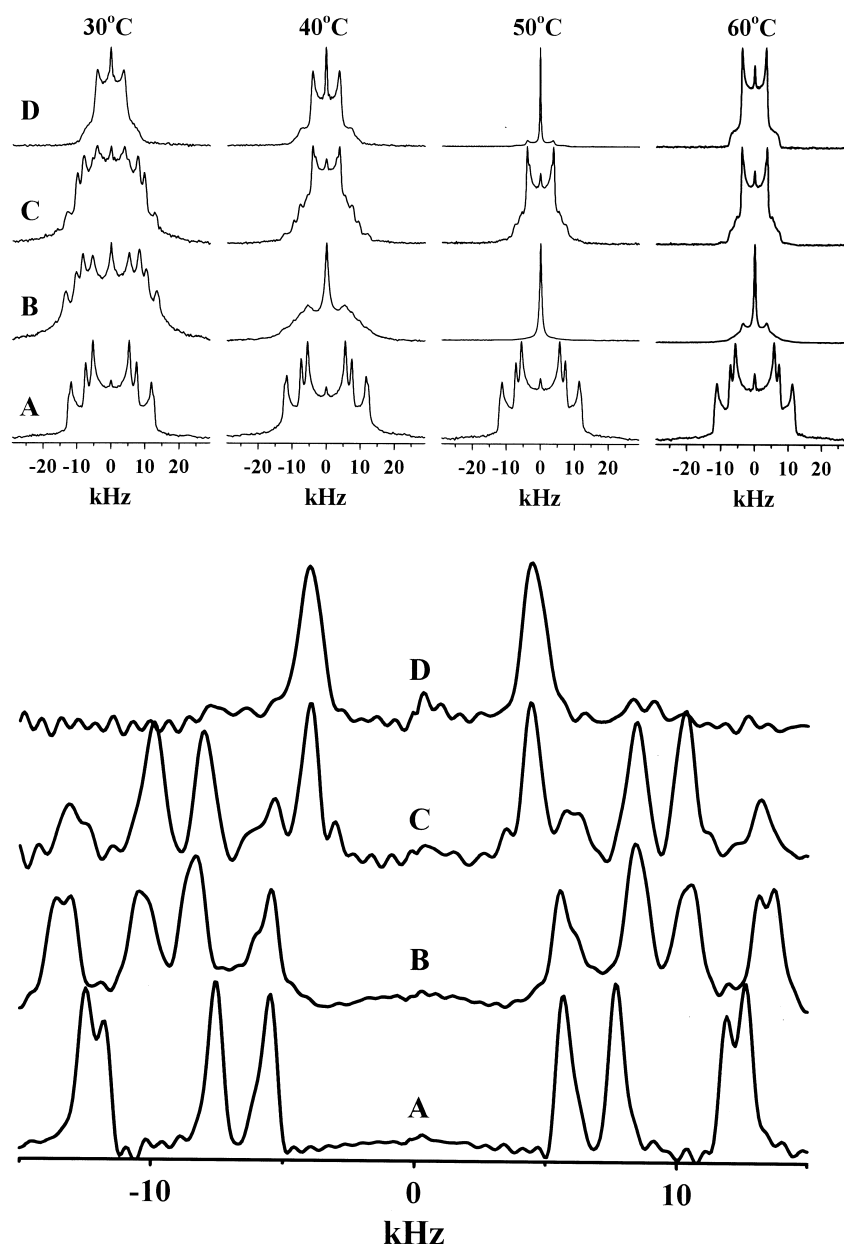


Fig. 5.  $^2\text{H}$ -NMR spectra of lipids isolated from *A. laidlawii* A-EF22 cells supplemented with 18:1c- $d_2$ . The water content was 20 wt%. Upper panel: (A) PG. (B) MGlcDAG/PG (50:50, mol/mol). (C) MGlcDAG/PG (75:25, mol/mol). (D) MGlcDAG. Lower panel: De-Paked spectra at 30°C.

$L_\alpha$  phase was confirmed by SAXS, which shows diffraction peaks with distance ratios of 1:1/2:1/3. At 30 and 60°C, the repeat distances were 48.4 and 47.0 Å, respectively.

The  $^2\text{H}$ -NMR spectra recorded from DGlcDAG reveal quadrupole splittings similar in magnitude to those of PG (Fig. 4D and Table 2) and it is con-

cluded that an  $L_\alpha$  phase is formed at temperatures up to at least 60°C.

Fig. 5D illustrates  $^2\text{H}$ -NMR spectra of MGlcDAG, where the width of the lineshape is reduced by approximately a factor of 2 as compared with the spectra from the  $L_\alpha$  phase of PG (Fig. 5A and Table 3). This shows that an  $H_{II}$  phase is predominantly

Table 3

Quadrupole splittings obtained by  $^2\text{H}$ -NMR on MGlcDAG, PG and MGlcDAG/PG mixtures containing  $\alpha$ -deuterated acyl chains

Sample	Quadrupolar splitting $\Delta\nu_q$ (kHz)			
MGlcDAG <sup>a</sup>				8 <sup>d</sup>
MGlcDAG/PG (75:25, 26 mol/mol) <sup>a</sup>	25	20 <sup>c</sup>	16	12
MGlcDAG/PG (50:50, 27 mol/mol) <sup>b</sup>	26	21 <sup>c</sup>	17	12
PG <sup>a</sup>	25	24	15	11

<sup>a</sup>The spectrum was recorded at 35°C.

<sup>b</sup>The spectrum was recorded at 30°C.

<sup>c</sup>Suggested to originate from MGlcDAG in an  $L_\alpha$  phase.

<sup>d</sup>From MGlcDAG in an  $H_{II}$  phase.

formed between 30 and 40°C. At 50°C, a major part of the lineshape represents an isotropic phase, while at higher temperatures, an  $H_{II}$  phase is again formed.

GPDGlcDAG at 30°C gives rise to  $^2\text{H}$ -NMR spectra corresponding to an  $L_\alpha$  phase (Fig. 6A). At 50 and 60°C, a very small isotropic component emerges in the spectrum in addition to the components originating from the  $L_\alpha$  phase. X-ray diffraction studies of GPDGlcDAG indicated that only an  $L_\alpha$  phase is formed at temperatures between 30 and 60°C. The repeat distances were 63.2 Å at 30°C and 59.2 Å at 60°C.

### 3.4. Phase behaviour of lipid mixtures

$^2\text{H}$ -NMR spectra recorded from DGlcDAG/PG (50:50, mol/mol) and DGlcDAG/PG (75:25, mol/mol) show that the mixtures form an  $L_\alpha$  phase within

the temperature range 30–60°C (Fig. 4B,C). This was also confirmed by SAXS on the sample with 75 mol% DGlcDAG, giving reflections corresponding to bilayer repeat distances of 52.3 and 50.0 Å at 30 and 65°C, respectively (Fig. 7A).

The  $^2\text{H}$ -NMR spectra recorded from mixtures of MGlcDAG and PG (50:50 and 75:25, mol/mol) are shown in Fig. 5B,C. At 30°C, an  $L_\alpha$  phase was formed with 50 mol% PG, and a coexistence of  $L_\alpha$  and  $H_{II}$  phases was present with 25 mol% PG. When the temperature was raised to 40°C, an  $H_{II}$  phase dominated in the latter sample, while the former sample gave a spectrum that probably originates from a coexistence of  $L_\alpha$  and isotropic phases. Both MGlcDAG/PG mixtures formed isotropic and/or  $H_{II}$  phases at 50 and 60°C in similarity to MGlcDAG. Thus, the influence of up to 50 mol% of PG on the phase equilibria of MGlcDAG is rather modest. The sample with 75 mol% MGlcDAG showed hysteretic behaviour. A freshly prepared sample only gave  $L_\alpha$  and  $H_{II}$  phases when heated for the first time (Fig. 5C). This was confirmed by both  $^{31}\text{P}$ -NMR and SAXS, the latter giving a repeat distance of an  $L_\alpha$  phase equal to 52.8 Å and a lattice parameter,  $a$ , for the  $H_{II}$  phase equal to 66.8 Å at 30°C (Fig. 7B). At 60°C, only reflections corresponding to an  $H_{II}$  phase with  $a$  equal to 62.2 Å were observed. By repeatedly going up and down in temperature, an increasing fraction of an isotropic phase was formed, and after such a procedure this phase was present independent of the temperature between 30 and 60°C. It has previously been observed that reversed cubic phases may form when an  $L_\alpha$ -forming lipid is mixed with an  $H_{II}$ -forming lipid, and that

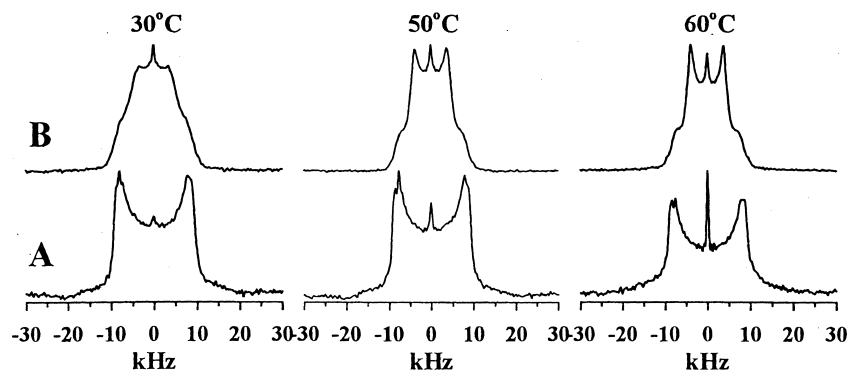


Fig. 6.  $^2\text{H}$ -NMR spectra of lipids isolated from *A. laidlawii* A-EF22 cells supplemented with 18:1c- $d_2$ . The water content was 20 wt%. (A) GPDGlcDAG. (B) MADGlcDAG/GPDGlcDAG (90:10, mol/mol).

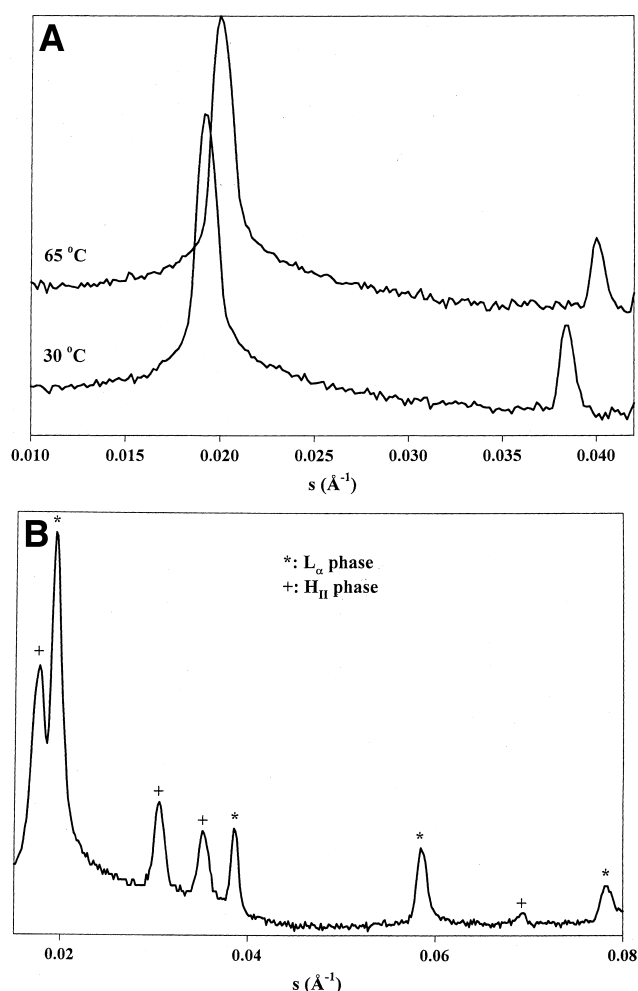


Fig. 7. Small angle X-ray powder diffraction patterns of lipid mixtures isolated from *A. laidlawii* A-EF22 cells supplemented with 18:1c-d<sub>2</sub>. The water content was 20 wt% in both samples. (A) DGlcDAG/PG (75:25, mol/mol) forming an L<sub>α</sub> phase at 30 and 65°C. (B) MGlcDAG/PG (75:25, mol/mol) forming a coexistence of L<sub>α</sub> (\*) and H<sub>II</sub> (+) phases at 30°C.

repetitive heating/cooling cycles can induce the formation of these phases [46]. A sample with 75 mol% MGlcDAG but with 40 wt% water showed the following phase sequence: L<sub>α</sub>–isotropic–H<sub>II</sub> upon a temperature increase from 30 to 60°C, and no effect of hysteresis was observed. The  $\Delta v_q$  values for the mixtures of PG and MGlcDAG are presented in Table 3.

An H<sub>II</sub> phase is mainly formed by a sample of MADGlcDAG with 10 mol% GPDGlcDAG in 20 wt% water. Possibly, a small fraction of an L<sub>α</sub> phase is also obtained, as judged by the rather high shoulders of the <sup>2</sup>H-NMR lineshape. It should be

noted, however, that these shoulders do not change significantly with temperature (Fig. 6B).

## 4. Discussion

### 4.1. Verification of the structure of PG from *A. laidlawii*

Both the mass and NMR spectra recorded from the membrane phospholipid isolated from *A. laidlawii* strain A-EF22 unequivocally show that the lipid is PG. The somewhat deviating phase behaviour and quadrupole splitting pattern obtained by the *A. laidlawii*-PG as compared to synthetic PG (see Section 3.1) may be explained by the heterogeneous acyl chain composition of the PG from natural origin. The mass spectra show that both the synthetic DOPG and the *A. laidlawii*-PG are able to form dimers under the conditions used to analyse the lipids. To the authors' knowledge, this phenomenon has not been observed for any membrane lipid before.

### 4.2. Quadrupole splittings of lipid mixtures in the L<sub>α</sub> phase

First of all it is important to stress the fact that both SAXS and <sup>31</sup>P-NMR gave evidence of *only one lamellar phase* for PG, DGlcDAG and mixtures thereof (Fig. 7A), confirming that the observed splitting patterns indeed originate from a single lamellar phase.

<sup>2</sup>H-NMR quadrupolar splittings of deuterated  $\alpha$ -methylenes have been studied earlier [41], and it is generally found that the *sn*-1 chain gives rise to one splitting while the *sn*-2 chain deuterons, being magnetically inequivalent, give rise to two splittings [37,47–50]. The magnetic inequivalence of C–<sup>2</sup>H<sub>2</sub> deuterons has also been observed in the glycerol backbone [51,52] and the headgroup [51–54] of phospholipids as well as in the acyl chain of cerebrosides [55]. The quadrupole splittings are determined by the average orientation of the C–<sup>2</sup>H bond and by the fluctuations around this direction [3], and it should be noted that very small structural alterations of the lipids can result in rather large changes in the magnitude of the quadrupole splitting because of its profound dependence on the angle between the bilayer

normal and the C–<sup>2</sup>H bond. The quadrupole splittings of methylene segments close to the headgroup can thus be used to monitor changes in lipid organisation.

From Figs. 4–6 it is clear that the pattern of the quadrupole splittings of PG in the L<sub>α</sub> phase differs from those of MGlcDAG, DGlcDAG and GPDGlcDAG. PG gives rise to four splittings of 11, 15, 24, and 25 kHz (Table 2); the two smaller ones are assigned to the *sn*-2 deuterons, and the two, barely resolved, larger ones arise from the *sn*-1 deuterons. The occurrence of two separate splittings from the *sn*-1 deuterons has not been observed earlier.

The <sup>2</sup>H-NMR spectrum from DGlcDAG in the L<sub>α</sub> phase at 30°C is characterised by two quadrupole splittings equal to 18 and 16 kHz (Table 2). The integrated intensity of the two splittings has the ratio 3:1, and this suggests that the outer splitting consists of two overlapping signals, namely two deuterons from the *sn*-1 acyl chain and one deuteron from the *sn*-2 chain. The inner splitting with the lowest intensity corresponds to the remaining *sn*-2 deuteron. These findings are in accordance with earlier studies on *A. laidlawii* glucolipids with α-deuterated chains [1,35]. From a comparison of the deviant splitting patterns of PG and DGlcDAG, it is suggested that DGlcDAG must differ in the organisation of the glycerol backbone compared to that of PG. The difference in the conformation of the backbone then results in the observed contrast in the quadrupole splittings of the α-methylene groups of the acyl chains. Thus, it may be concluded that the polar headgroup plays a crucial role for the conformation or orientational organisation of the glycerol backbone of the lipid. Moreover, this interpretation is supported by the results obtained for GPDGlcDAG, since this lipid gives a similar splitting pattern as DGlcDAG (Table 2). Thus, it is the sugar moiety rather than the phosphate group that determines the backbone organisation of GPDGlcDAG.

Following the same route as above concerning the assignment of the quadrupole splittings to individual lipids, it is suggested that those observed from mixtures of MGlcDAG and PG can be assigned as presented in Table 3. Note that although MGlcDAG only forms non-lamellar phases, addition of PG to MGlcDAG eventually results in the formation of an

L<sub>α</sub> phase at 30 and 35°C (Fig. 5 and Table 3). The spectra from the samples forming more than one phase exhibit a splitting that is tentatively assigned to MGlcDAG in the L<sub>α</sub> phase. The expected smaller splitting from MGlcDAG is overlapped by the splitting from PG at 16 kHz. Thus, MGlcDAG has a pattern of quadrupole splittings that is similar to that of DGlcDAG in an L<sub>α</sub> phase, but with the interesting difference that the splittings from MGlcDAG are *significantly larger* than for DGlcDAG [56]. In summary, it seems that a sugar-containing headgroup changes the organisation of the region close to the glycerol backbone as compared to PG. Furthermore, the molecular ordering of the α-methylene groups of MGlcDAG in a lamellar phase is larger than that for DGlcDAG.

#### 4.3. Phase behaviour of individual lipids

The phase behaviours of DGlcDAG, MGlcDAG, PG, GPDGlcDAG and MADGlcDAG have been examined previously. Both PG and DGlcDAG forms an L<sub>α</sub> phase in accordance with earlier studies [1,5,56,57]. The MGlcDAG studied in this work has an acyl chain composition similar to the MGlcDAG examined by Andersson et al. [1], and the phase behaviours are comparable. In previous phase equilibria studies of GPDGlcDAG isolated from both strain A-EF22 and B-PG9, the water concentrations were significantly higher (≥95 wt% water) and an L<sub>α</sub> and/or normal micellar solution phase was detected [58,59]. In the present study, GPDGlcDAG with 20 wt% water was found to form an L<sub>α</sub> phase. The small isotropic component observed in the <sup>2</sup>H-NMR spectra (Fig. 6A) might represent a reversed cubic phase, but it cannot be verified with certainty, since there was no indication of any other phases, but an L<sub>α</sub> phase in the X-ray diffractograms. The repeat distances in the L<sub>α</sub> phase are 12–15 Å larger for GPDGlcDAG than for PG at 20 wt% water, which most probably depends on the considerably larger polar headgroup of the former lipid.

#### 4.4. Phase behaviour of lipid mixtures

It has previously been observed that mixtures of the two lipids dioleoylphosphatidylcholine (DOPC)

and monoolein (MO) can form either a reversed cubic phase or an  $H_{II}$  phase under conditions where the individual lipids form an  $L_{\alpha}$  phase [60]. It was shown later that the hydration of the ester carbonyl groups increases in the ternary system, and that the *sn*-3 hydroxyl group of MO forms a hydrogen bond with the phosphate group of DOPC [61]. Both these factors induce a curling of the lipid monolayers that leads to a transition to reversed non-lamellar phases. A similar hydrogen bond interaction could possibly occur between the *A. laidlawii* glucolipids and PG, since the mean area per molecule is smaller for total lipid extracts than for the individual lipids [11]. However, the two DGlcDAG/PG mixtures studied in this work formed an  $L_{\alpha}$  phase at temperatures between 30 and 60°C just like the individual lipids (Fig. 4). Thus, there seems to be no interactions between the polar headgroups that are strong enough to enable reversed non-lamellar phases to be formed with DGlcDAG/PG in water.

It is interesting to note that an  $L_{\alpha}$  phase is not stabilised to a significant extent even in the equimolar mixture of MGlcDAG and PG (Fig. 5). This result is in line with the observation that a total lipid extract from *A. laidlawii* strain A-EF22 forms reversed non-lamellar phases at almost the same temperature as a corresponding lipid extract where PG and the two phosphoglucolipids have been removed [6]. However, this finding is in sharp contrast to the ability of anionic phospholipids to stabilise an  $L_{\alpha}$  phase upon addition to phosphatidylethanolamine (PE). Soya PE forms an  $H_{II}$  phase above 0°C [62], but binary lipid mixtures containing either 20 mol% egg PG, 33 mol% bovine heart diphosphatidylglycerol, 30 mol% soya phosphatidylserine, or 20 mol% phosphatidylinositol, form essentially solely an  $L_{\alpha}$  phase at 30°C [7–10].

The phase equilibria of MADGlcDAG are only marginally influenced by the presence of 10 mol% GPDGlcDAG (Fig. 6). This result is also in sharp contrast to the ability of GPDGlcDAG to stabilise an  $L_{\alpha}$  phase when mixed with dielaidoyl-PE. This PE species transforms from an  $L_{\alpha}$  to an  $H_{II}$  phase at 66°C, and addition of 7.5 mol% GPDGlcDAG raises the transition temperature to at least 90°C [12].

Taken together, the phase behaviour of the different lipid mixtures indicate that an anionic lipid has a larger ability to transform an  $H_{II}$  phase to an  $L_{\alpha}$

phase when the  $H_{II}$  phase is formed by PE as compared to a glucolipid.

#### 4.5. Packing of glucolipids in bilayers

It is evident from the above discussion that the phase behaviour of the non-ionic MGlcDAG is marginally affected by the presence of the anionic PG, and that the  $^2H$ -NMR quadrupole splittings of the acyl chains are observed for the individual lipids in MGlcDAG/PG and DGlcDAG/PG mixtures. Moreover, since PG and the glucolipids reside in a single  $L_{\alpha}$  phase, and since the splittings are quite different in the two classes of lipids, this strongly indicates that the glycerol backbone region of the glucolipids has a different conformation than that of PG. This can be a consequence of the more extensive hydration of the anionic lipid than that of the non-ionic lipids [21,40,63]. Thus, MGlcDAG and DGlcDAG are somewhat less hydrophilic than PG.

The formation of reversed non-lamellar structures can be understood from a simple model involving the critical packing parameter [64]. Since MGlcDAG forms an  $H_{II}$  phase, its packing parameter is larger than one, corresponding to a wedge-shaped molecule. Therefore, MGlcDAG introduces a closer mean packing of the acyl chains when added to PG or DGlcDAG. This interpretation is supported by the finding that the quadrupole splittings of PG and DGlcDAG are larger in the presence of MGlcDAG (Table 3 and [56]), which parallels the effect of PE on PG, where PE is the non-lamellar-forming lipid [65]. However, it should be noted that a relatively small fraction of PG in PE produces an  $L_{\alpha}$  phase, while a considerably larger fraction of PG is needed to accomplish this transition with a non-lamellar phase of MGlcDAG. This unique behaviour of mixtures between glucolipids and phospholipids is presently investigated in more detail in our laboratory (G. Orädd, L. Rilfors and G. Lindblom, to be published).

The phosphoglucolipid GPDGlcDAG exhibits properties that are a mixture of those characterising DGlcDAG and PG. The quadrupole splitting pattern is nearly identical to that of DGlcDAG which indicates that the glycerol backbone region has the same organisation in both lipids. However, in similarity with PG, the phosphoglucolipid has a pronounced

ability to stabilise an  $L_{\alpha}$  phase when mixed with PE. It can be suggested that the glycerophosphoryl moiety is located quite far out in the water phase, lending GPDGlcDAG the property to stabilise an  $L_{\alpha}$  phase.

The previously obtained result from a monolayer study by Andersson et al. [11], namely that the mean area per molecule is smaller for *A. laidlawii* total lipid extracts than for the individual lipids, can also be explained by the present results, suggesting a tighter packing of the mixed molecules. However, another consequence of the difference in packing between the two classes of lipids can be that hydrogen bonding between the hydroxyl groups of the glucolipids and the phosphate group of PG is facilitated. Thus, interactions between the glucolipids and PG can be an additional explanation to the monolayer results.

The particular phase behaviour of the lipids in the membrane of *A. laidlawii* gives the bacterium more flexibility. The anionic membrane lipids are needed for various cell functions [66,67], but they are not able to significantly shift or change the molecular packing in the membrane at the concentrations occurring in *A. laidlawii* [1,6]. The molecular packing in turn is important for other functions of the membrane like keeping the spontaneous curvature at a more or less constant value and maintaining a barrier towards the surroundings.

## Acknowledgements

This work was supported by the Swedish Natural Research Council and the Knut and Alice Wallenberg Foundation. P.E.A. acknowledges the Swedish Medical Research Council Grant 11565.

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