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Composition and phase behaviour of polar lipids isolated from *Spirulina maxima* cells grown in a perdeuterated medium

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Abstract

The lipid composition of *Spirulina maxima* cells grown in a perdeuterated medium was determined by using nuclear magnetic resonance spectroscopy, fast atom-bombardment–mass spectrometry, gas chromatography–mass spectrometry as well as conventional chemical methods. The extent of deuteration was determined by mass spectrometry and was superior to 97.5%. The major lipids identified in the strain were: non-polar lipids (9%), monogalactosyldiacylglycerol (5%), digalactosyldiacylglycerol (22%), phosphatidylglycerol (31%), sulfoquinovosyldiacylglycerol (32%), phosphatidylinositol (traces). The major fatty acids were 16:0 (80%) and 18:1 (15%). These results demonstrate that the adaptation of the cells to D_2O did not imply a profound modification of the lipid composition. The perdeuterated polar lipid mixture dispersed into an excess of water organises spontaneously in a lamellar phase as seen by ^{31}P and deuterium solid state NMR and can therefore be used to prepare perdeuterated model membranes with a well defined composition. Liposomes made using these lipids have a gel to liquid-crystalline phase transition in the range 15–27°C and are in a fluid L_α phase above this temperature.

Keywords: Cyanobacterium; Deuterated biomass; Lipid composition; NMR, solid state, ^2H- and ^{31}P-; FAB-MS; GC-MS ; LSIMS; (*S. maxima*)

1. Introduction

Perdeuterated molecules are often required for modern structural biology, specially in NMR or neutron diffraction experiments [1–5]. Although they can be obtained by chemical synthesis [6–8], they are best produced by growing micro-organisms on labelled medium. Since the pioneering work of Katz and Crespi in the sixties, we know that it is possible to grow on perdeuterated medium bacteria (*Escherichia coli*, *Bacillus tiberius*, *Azotobacter agilis*,...), yeasts (*Torulopsis utilis*, *Saccharomyces cerevisiae*,...), algae (*Chlorella vulgaris*, *Scenedesmus obliquus*), cyanobacteria (*Spirulina maxima*...) and, with more difficulties, superior eukaryotic cells [9–13]. However, very little is known about the lipid composition of the biomass produced in these conditions.

The blue-green algae (or cyanobacteria) represent a large group within the prokaryotic kingdom [14]. They are classified as Gram-negative bacteria possessing a cell envelope composed of an outer membrane, a peptidoglycan layer and a plasma membrane. *Spirulina maxima*, a group of cyanobacteria that inhabits carbonate-rich lakes in torrid zones, has attracted much attention because of their utilisation as human and animal nutritional protein source [15,16]. Since it can be grown on perdeuterated minimal medium consisting mainly of D_2O, mineral salts, and CO_2 as a sole carbon source, *S. maxima* is a good choice for the production of perdeuterated biomass. It is well established that the growth rate is lower in D_2O, and that the adaptation of *S. maxima* in a perdeuterated environment has to be made in a stepwise manner, by progressive adaptation to increasing percentage of deuterium in the medium. However, although this organism has been used for the production of perdeuterated biomass for many years by different laboratories, very little is known about the metabolic changes associated with the adaptation to a deuterated environment. This is an important question both from a physiological as from a practical point of view. Detailed knowledge of metabolical differences could help to characterise the cellular response to the stress related to growth in a perdeuter-

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ated environment and could lead to an improvement of growth rate and biomass under these conditions by changing the nutrients, pH or temperature. We therefore undertook a detailed analysis of the lipid composition of perdeuterated biomass of *S. maxima*.

Another motivation for this work is that perdeuterated lipids can be used to prepare model membranes, which are required for specific NMR experiments. We previously used such models, using synthetic lipids, for the determination of the membrane bound conformation of neuropeptides by the transferred NOE technique [17,18]. However, having the possibility to use natural lipid mixtures would be an important improvement, provided that one can use a mixture of well defined composition. The second goal of this work was therefore to establish precise data on the polar lipid composition of the most easily obtainable perdeuterated lipid mixtures, and to characterise by solid state NMR the model membranes formed by hydration of these lipids.

2. Materials and methods

2.1. Culture conditions

Spirulina maxima cells were grown in a perdeuterated medium (D_2O 99.8%) kindly supplied by Dr. Mermet-Bouvier (CEA Paris). The culture medium was the same as the one used in a previous study [19].

2.2. Lipid extraction

Lipids were extracted from the cell suspensions using the modified method of Bligh and Dyer [20] and yielding 70 g of total lipids obtained from 500 g dry cells. The total lipids were fractionated by Me_2CO precipitation to remove the pigments and neutral lipids: to 1 g of lipid extract dissolved in 2 ml $CHCl_3$, 200 ml of Me_2CO were added and the mixture was stored for 24 h at 4°C. Polar lipids were precipitated by centrifugation at $4000 \times g$ for 10 min.

2.3. Purification of lipids

The different lipids were purified on a DEAE-cellulose column (30 g, 200 ml volume) (DEAE, Bio-Rad, Anion-exchange cellulose Cellex D). This purification resulted in the identification of several distinct compounds on TLC ($CHCl_3/CH_3OH/HOAc/H_2O$, 65:25:4:1): GL1 (R_f 0.85), GL2 (R_f 0.6), GL3 (R_f 0.35), GL4 (R_f 0.13) and GL5 (R_f 0.06) and two phospholipids PL1 (R_f 0.4) and traces of PL2 (R_f 0.06).

The optimum elution sequence was found to be (%MeOH in $CHCl_3$ volume, ml): 0/400 (non-polar lipids); 10/400 (GL1 and PL2), 20/400 (GL2), 30/400 (GL4), 40/400 (PL1 and GL3), 50/400 (GL3); 100/800, salts/600 (PL1). The salt solution consisted of $CHCl_3$/MeOH 4:1, NH_4OH 2%, NH_4OAc 0.02 M.

TLC was carried out on silica gel plates, Kieselgel 60F254, Merck. The coated plates were washed once by ascending chromatography in $CHCl_3/CH_3OH/HOAc/H_2O$ (65:25:4:1), air dried and activated at 110°C for 12 h.

Lipids were detected by spraying the TLC plates with anthrone for glycolipids, and Dittmer–Lester reagent for phospholipids [21].

Fractions exhibiting the same TLC profile were pooled and final purification of the compounds was achieved by chromatography on prep. TLC (elution solvent $CHCl_3/MeOH/HOAc/H_2O$, 65:25:4:1) or by column chromatography on Bio-Sil A (Bio-Rad) using a $CHCl_3$/MeOH gradient.

2.4. Determination of the polar lipid components

2.4.1. Hydrolysis of polar lipids

2 mg of polar lipids were dissolved in TFA 2 M (200 μl) and stirred for 2 h at 110°C in a sealed tube and the hydrolysis products were dried down under a N_2 stream. Et_2O/H_2O (1:1) partition yielded an aqueous phase containing monosaccharides and glycerol while the fatty acids were recovered in the upper organic phase.

2.4.2. Fatty acids analysis

Fatty acids were dissolved in Et_2O and diazomethane was added until persistence of the yellow colour. The mixture was kept for 45 min at 4°C and then the diazomethane was dried down under nitrogen. Fatty acid methyl esters were redissolved in Et_2O and analyzed by GC and GC-MS.

2.4.3. Trimethylsilylation of monosaccharides and glycerol

Monosaccharides and glycerol were transformed into TMSi derivatives: 200 μg of compounds were dissolved in 100 μl of pyridine/hexamethyldisilazane/trimethylchlorosilane (Tri-Sil reagent, Pierce). After 15 min at room temperature in a sealed tube and drying, derivatives were resuspended in light petrol. TMSi-erythritol was added as an internal standard for the GC analyses.

2.4.4. Propionylated derivatives

200 μg of monosaccharides and glycerol, obtained after hydrolysis of GLs, were treated by 0.1 ml of pyridine/propionic anhydride (1:1) in a sealed tube for 2 h at 45°C. The mixture was then dried down and resuspended in light petrol.

2.5. Glycosyl linkage analyses

2.5.1. Methylation of glycolipids

GLs were O-methylated according to Hakomori [22]: 2 mg of GL were suspended in 100 μl of DMSO and

sonicated for 15 min; then 100 μl of dimethylsulfinyl carbanion in DMSO were added and the mixture was stirred for 1 h at room temp. CH_3I (100 μl) were slowly added and the suspension stirred for 1 h. Addition of the base and CH_3I was repeated twice. The reaction mixture was partially dried down under a N_2 stream and then, the excess of iodine was removed by adding an equal volume of an aqueous solution of $Na_2S_2O_3$. The product was chromatographed using Sep-Pak cartridge (conditioned with 8 ml acetonitrile and 8 ml water). O-Methylated products were obtained by eluting the cartridge with 8 ml water and 5 ml acetonitrile.

Hydrolysis of per-O-methylated GL was performed with 2 M TFA at 110°C for 2 h. The reaction was stopped by drying under a N_2 stream.

Reduction was performed using 100 μl of $NaBH_4$ (10 mg/ml in 1 M NH_4OH/EtOH 99% (1:1)). The reaction was stopped after 1 h at room temperature, by addition of 100 μl HOAc. The reaction mixture was then dried down under a N_2 stream and twice with 0.5 ml of HOAc/CH_3OH (9:1, v/v) and twice with 0.5 ml CH_3OH.

2.5.2. Per-O-acetylation of alditols

The partially O-methylated alditols were O-acetylated using 100 μl Ac_2O and 100 μl dry pyridine at 110°C for 1 h. After drying, the partially O-methylated partially O-acetylated alditols were resuspended in light petrol and analyzed by GC and GC-MS.

2.6. Analyses

IR spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrophotometer.

GC-MS of partially O-methylated alditol acetates was carried out on a HP-1 glass column programmed from 80 to 290°C (15°C/min) and interfaced with a Hewlett–Packard 5989A mass spectrometer. Positions of the O-methyl groups on the carbohydrates were assigned by reference to the MS fragmentation patterns with those of authentic standards.

GC analyses of TMSi and propionyl derivatives of monosaccharides and fatty acid methyl esters were performed on a OV1 WCOT column (25 m × 0.22 mm) programmed from 100 to 280°C (3°C/min).

FAB-MS was performed on a ZAB-HS (VG analytical, Manchester) instrument with triethylamine as a matrix. Ions were produced by bombardment with a beam of Xe atoms (8 keV) in a spectrometer operating in the negative ion mode.

LSIMS spectrometry of native GL2 was obtained on a Autospect mass spectrometer (Fisons Instruments, Manchester) (8 keV for accelerating voltage).

^{13}C-NMR analyses were performed on a Bruker AMX 500 spectrometer with an Aspect X 32 calculator. The chemical shifts were measured relative to the CD_2 signal positioned at 28.6 ppm (as determined once with TMS as internal reference). Samples were prepared as follows: GL1 (11 mg in 2.5 ml of $CDCl_3$), GL2 (25 mg in 2.5 ml of $CDCl_3$), GL3 (28 mg in 2.5 ml of $CDCl_3$/MeOH (2:0.5)) and PL1 (35 mg in 2.5 ml of $CDCl_3$).

2.7. Solid state NMR experiments

Aqueous multilamellar dispersions for ^{2}H- or ^{31}P-NMR were prepared as follows: polar lipids (100 mg) were dissolved in deuterium-depleted water (2.5 ml) and hydrated at 30°C with vortex mixing to homogeneity. The liposomes were transferred to a 5 mm NMR tube. The ^{31}P-NMR experiments were performed on a MSL200 Bruker spectrometer, using a Hahn-echo pulse sequence and inverse gated broad band proton decoupling (spectral width 50 kHz, decoupling power 7 W, 90°C pulse of 4 μs, interpulse delay of 50 μs, recycle time 1 s). The ^{2}H-NMR experiments were performed on a Bruker AMX500 equipped for static solid state experiments, using a quadrupolar-echo pulse sequence (7 mm solenoid coil, 90° pulse of 5.4 μs, interpulse delay of 60 μs, recycle time 1 s, spectral width 500 kHz). Deuterium NMR spectra were acquired at temperatures ranging from 3 to 60°C in order to determine the stability of the lamellar phase and the phase transition temp of the lipid mixture.

3. Results and discussion

3.1. Composition of polar lipids

Polar lipids were first fractionated by DEAE-cellulose chromatography and final purification was done either by prep. TLC or by column chromatography. When analyzed for their phosphorus content, one major phospholipid (PL1) and traces of a second one (PL2) were present. When analyzed for their glycoconjugate content, five components readily reacted with the anthrone reagent on the silica gel TLC (GL1–GL5). Three of them, GL1–GL3, exhibited chromatographic mobilities similar to those of the previously described glycolipids of *S. maxima* grown on hydrogenated medium; these glycolipids have been tentatively identified as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) [23]. The two remaining unidentified more polar components (GL4 and GL5) had R_f values corresponding to previously described molecules. They have been postulated to correspond to trigalactosyldiacylglycerol and tetragalactosyldiacylglycerol [23,24]. This was not confirmed in the present study: IR spectra of GL4 and GL5 showed a strong hydroxyl absorption band (3350 cm^{-1}) but the characteristic absorption bands corresponding to the glycosyldiacylglycerol structure were very weak. GC analysis of the TMSi and per-O-methylated derivatives revealed the presence of disaccharide. Analysis of the acid hydrolysis products demonstrated the presence of glucose

as the only monosaccharide constituent. No galactose that may derive from the hydrolysis of tri- and tetragalactosides was detected. Furthermore, fatty acid could be detected neither by GC, GC-MS, ^{13}C-NMR nor by fast atom bombardment-MS analyses (data not shown). Therefore it was concluded that GL4 and GL5 were not glycolipids but rather corresponded to non-lipidated substances. These molecules were not further studied.

Quantitative analysis of lipids showed the presence of non-polar lipids (9%), GL1 (5%), GL2 (22%), GL3 (32%), PL1 (31%), PL2 (traces).

Polar lipids were hydrolysed and their fatty acid constituents were analyzed by GC. The average fatty acid composition was as follows: 16:0 (80%), 18:0 (2.5%), 16:1 (2.5%), 18:1 (15%). The relatively large proportion of 18:1 is in agreement with a previous report [25].

3.2. Structural analyses of the different glycolipids and phospholipids

3.2.1. IR

The IR spectra of GL1 and GL2 showed the typical absorption patterns for alcohol and ester functions: strong absorption at 3371 cm^{-1} (O-H), 1728 and 1268 cm^{-1} (due to ester functions C = O and C-O), and 1078 cm^{-1} (C-O of alcohol functions). On the other hand, we observed a frequency shift for the CD_2 and CD_3 absorption bands, that may be calculated by the Hooke law [26]. We consequently attributed the observed bands at 2197, 2091 and 1071 cm^{-1} to CD_2 and CD_3 (instead of 2940, 2860 and 1460 cm^{-1} for CH_2 and CH_3).

3.2.2. Determination of the GL1 and GL2 components

GL1 and GL2 were hydrolysed and the resulting products were partitioned between $CHCl_3$ and H_2O. The aqueous phase was dried, trimethylsilylated and analyzed by GC. Galactose (Gal) and glycerol (Gro) were the two water-soluble components present in the acid hydrolysis products (the retention times of deuterated products were almost identical to those of hydrogenated compounds used as standards). The molar ratios of Gro:Gal, determined by GC of the propionylated derivatives (due to the high volatility of TMSi-Gro), were found to be equal to 1:1 and 1:2 for GL1 and GL2, respectively.

3.2.3. Fatty acid analysis

The fatty acid methyl esters derived from the hydrolysis of GL1 and GL2 were identified by GC by using cochromatography with a set of standards. 16:0 and 18:1 were the two compounds present in both glycolipids. GC-MS analyses of the samples confirmed the structure of the fatty acids: molecular ions were seen at m/z 301 and 329, respectively, for deuterated 16:0 and 18:1. In addition, peaks corresponding to $(M-31)^+$ ions (loss of methoxy) and an intense ion at $m/z = 77$ corresponding to the Mac Lafferty rearrangement for methyl esters [27] of deuterated fatty acids (instead of m/z 74 for hydrogenated) were also observed in both spectra.

3.2.4. ^{13}C-NMR spectroscopy of GL1

The spectrum of GL1 showed the presence of the anomeric carbon resonance of galactose residue at 103.4 ppm demonstrating the *O*-β-D-Gal-(1 → 1′)-2′,3′-di-*O*-acyl-D-Gro structure [28]. Other resonances were also observed at 173.8 and 173.6 ppm for the two carbonyl carbons, 129.5 for C = C, 77.6, 77.01, 76.7, 73.9, 72.8, 71.1, 68.4, 62.1 for C2–C6 of the β-Gal residue and for C1–C3 of Gro, 33.37, 29.71, 28.65, 28.49, 28.35, 28.19, 28.03, 23.94 for CD_2-CD_3.

3.2.5. FAB-MS of GL1

The predominant molecular ion observed in the spectrum at $m/z = 800$ corresponds to a monogalactosyldipalmitoylglycerol.

3.2.6. Glycosyl linkage composition of GL2

GC and GC-MS analyses of the partially O-methylated, partially O-acetylated alditols derived from GL2, showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (characteristic peaks at m/z = 103,119,132,149,165 and 210) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol (characteristic peaks at m/z = 103, 119, 132, 164, 193 and 238). Accordingly, a 1 → 6 digalactosyl linkage was demonstrated. For comparison, analysis of the corresponding derivatives from GL1 gave only a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol.

3.2.7. ^{13}C-NMR spectroscopy of GL2

The anomeric configuration of the glycoside residues of GL2 was determined by ^{13}C-NMR. The spectrum showed two anomeric carbon resonances at 103.4 and 99.7 ppm, assigned, respectively, to a β-Galp1 → Gro and α-Galp(1 → 6)Galp. In addition, the presence of two carbonyl signal resonances at 173.8 and 173.5 ppm proved the presence of two ester functions. Others signals at 129.3 for C = C; 127.6, 125.1, 119.6, 72.9, 69.5, 68.4, 67.4, 62.2, 61.1 for C2–C6 of Gal and C1–C3 of Gro; 33.4, 30.6, 26.2, 23.9, 21.4, 13.6 for CD_2-CD_3 were also observed in the spectrum.

3.2.8. The positive ion liquid secondary ion mass spectrometry (LSIMS)

The LSIMS spectrum of the native GL2 revealed the presence of two intense pseudo-molecular ions $[M + Na^+]$ separated by 28 daltons at m/z 996 and 1024 (Fig. 1). They correspond to digalactosyldiacylglycerol containing, respectively, two 16:0 and 16:0, 18:1. For the assignment of other ions observed in the spectrum, see the legend to Fig. 1.

From the spectrum, it was possible to deduce the extent of deuteration from the analysis of peak intensities [29]. It corresponded to a deuterium labelling of 97.5% (after correction for the ^{13}C natural abundance).

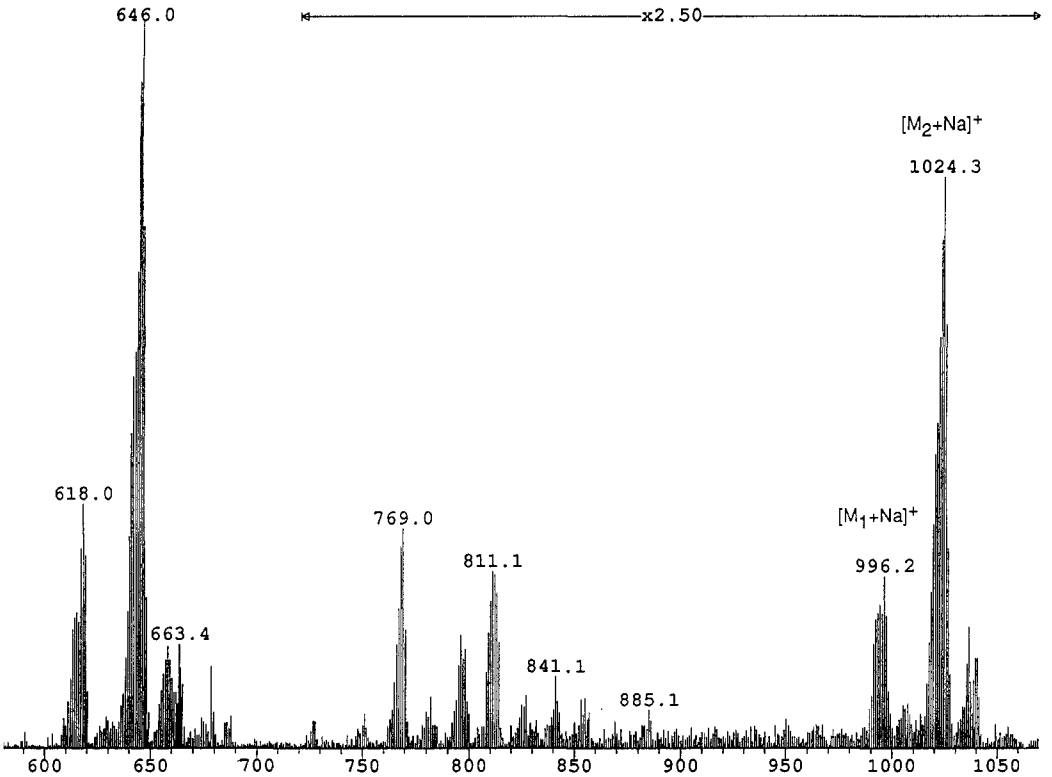

Fig. 1. LSIMS spectrum of GL2. Due to the isotopic distribution, a complex spectrum was observed for each fragment, and a statistical analysis of these patterns provides the average extent of deuteration of the molecule (97.5%). Two peaks corresponding to the loss of diacylglycerol were identified at m/z 618 (two 16:0) and at m/z 646 (one 16:0 and one 18:1).

3.2.9. Structural analysis of GL3

GL3 gave a purple spot on TLC after spraying with the anthrone reagent and exhibited the R_f of SQDG. Analysis of the ^{13}C-NMR spectrum confirmed the α anomeric linkage ($\delta = 98.00$ ppm) between the sulfoquinovose and the glycerol. Other signals were observed in the spectrum: δ 174.6–174.3 for C = O; 130 for C = C; 76.9, 75.3, 73.7, 71.8, 69.2, 66.3, 56.4 for C2–C6 of SQDG and C1–C3 of Gro; 38–25 for CD_2-CD_3.

FAB-MS of this polar lipid showed two molecular species at m/z 862 (90%) and 894 (10%) corresponding to SQDG molecules containing 16:0 and 18:1, and 18:0 and 18:1, respectively.

3.2.10. Structural analysis of PL1

The major phospholipid PL1 was first identified to PG by TLC analysis using the Dittmer–Lester reagent and standard compounds. FAB-MS of this phospholipid showed two intense molecular ions at $m/z = 793$ and 822, corresponding, respectively, to molecules containing two moles of 16:0, and one mole of 16:0 and one mole of 18:1. This interpretation was consistent with the data obtained by conventional GC and GC-MS analyses of fatty acids showing that palmitic and oleic acids were the main acids detected (see above).

3.2.11. ^{13}C-NMR spectroscopy of PL1

The ^{13}C-NMR spectrum showed several resonances establishing the structure of PG δ(ppm): 174.3, 173.9 for C = O; 129.6 for C = C; 70.6, 70.0, 66.3, 62.1, 61.9 for C1–C3 of Gro; 33.6, 30.9, 30.7, 29.9, 28.6, 26.3, 24.0, 21.6, 13.2 for CD_2-CD_3.

Taken together, the structural data obtained in the present study clearly identified the major lipid constituents of *S. maxima* (MGDG, DGDG, SQDG and PG) and show that the lipid composition of cells grown in a perdeuterated medium is identical to that of normal cells [23,30,31].

3.3. Analysis of the phase diagram by solid state NMR

Upon dispersion into an excess of water, the lipid mixture extracted from the *S. maxima* biomass, organises spontaneously in liquid crystalline Lα phase. This is demonstrated by both phosphorus and deuterium solid state NMR.

The ^{31}P-NMR spectrum at 25°C (Fig. 2A) is an axially symmetrical spectrum characteristic of a lamellar organisation of phospholipids [32–34], with a chemical shift anisotropy $\Delta\sigma = 45$ ppm (measured as the frequency separation between the main peak and the low-field shoulder).

The ^{2}H-NMR spectrum of this lipid mixture consists of a superposition of powder patterns, each doublet being characteristic of the quadrupole splitting of a specific

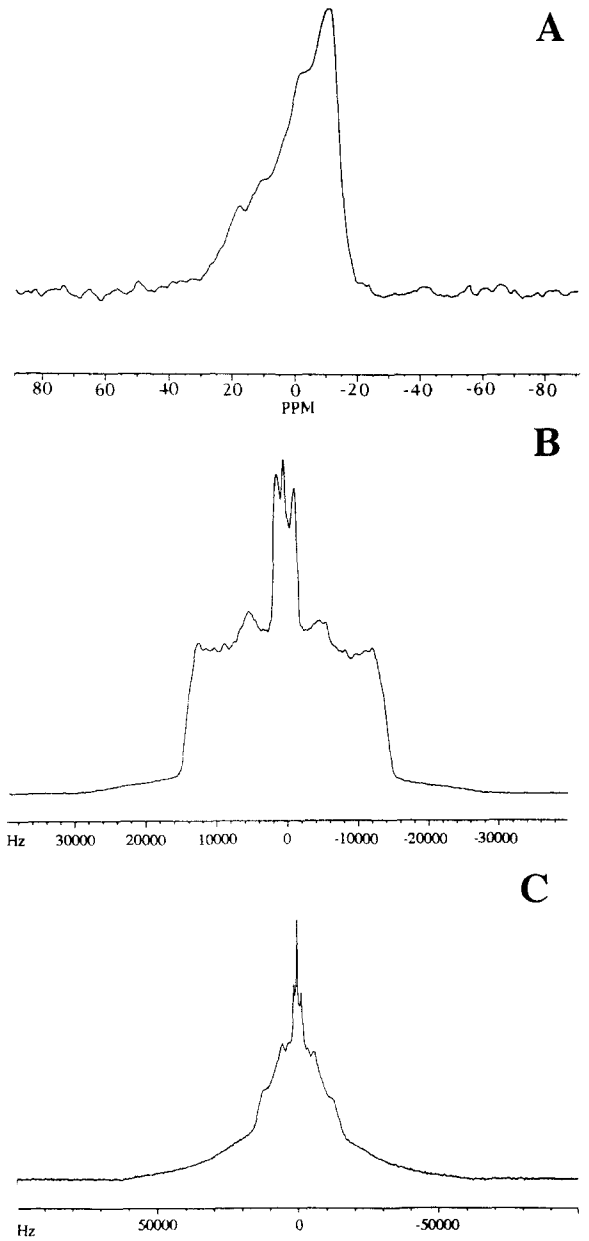

Fig. 2. NMR spectra of the mixture of polar lipids: (A) ^{31}P-NMR spectrum; (B) ^{2}H-NMR powder spectrum of liposomes at 30°C, i.e., above the phase transition; (C) ^{2}H-NMR spectrum of lipids at 10°C, i.e., below the phase transition (gel phase).

position in the molecule [35,36]. The spectrum shown in Fig. 2B is characteristic of a powder spectrum obtained classically with an unoriented dispersion of lipids (liposomes) in a Lα lamellar phase. Since we are observing a complex mixture of perdeuterated lipids, there is a large number of deuterated atoms, each of which giving rise to a Pake doublet: We therefore observe only an envelope with very few individual resolved splittings. Deuterium NMR is also widely used to determine phase transitions, in particular gel to liquid-crystalline phase transitions [33,37,38]. We have measured the deuterium NMR spectra of this lipid mixture, for temperatures ranging from 3°C to 60°C. The spectrum is unchanged between 27°C and 60°C, apart from the classical reduction of the quadrupole splitting for increasing temperatures, which is due to an increased mobility of the CD bond in the molecules. Between 3°C and 15°C, the spectrum is characteristic of lipids organised in a gel phase (Fig. 2C). Gradual changes in the spectra are observed between 15°C and 27°C, which indicate that this lipid mixture displays a gel to liquid-crystalline phase transition in this range of temperature.

4. Conclusion

We have shown that *S. maxima*, when adapted to growth in a perdeuterated medium (97.5%), has a lipid composition made of MGDG, DGDG, SQDG and PG which is highly similar to that of cells grown in a hydrogenated environment. Moreover, we proved that two compounds initially described as tri- and tetragalactosyldiacylglycerol were not lipidic compounds.

The crude mixture of polar lipids isolated from the perdeuterated biomass was shown to form liposomes spontaneously. The detailed characterisation of both the chemical composition and phase behaviour of this lipid mixture now makes it the system of choice for experiments requiring perdeuterated model membranes. Samples of this lipid mixture may be obtained from the authors.

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