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Fourier transform infrared spectroscopic study of ion binding and intramolecular interactions in the polar head of digalactosyldiacylglycerol

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Abstract. Lipid bilayers composed of digalactosyldiacylglycerol (DGDG), that is, Gal$p\alpha$1-6Gal$p\beta$1-3DAG, a non-ionic lipid of the thylakoid membrane of chloroplasts, aggregate in aqueous media containing mono- and divalent cations in amounts above a threshold concentration (C_t) of about 1.0, 4.7 and 10.0 mM for Ca^{2+}, Mg^{2+} and Na^+, respectively. In this work, we found that above C_t the DGDG membranes do not undergo fusion and that the aggregation can be reversed, or disrupted. This means that the perturbation induced by the salts results from adsorption, or complexation of the ions in the polar head of DGDG. To investigate this question, we used Fourier transform infrared (FTIR) spectroscopy to identify the molecular sites in DGDG which are modified by interaction, or adduct formation with $CaCl_2$, $MgCl_2$ and NaCl. We also determined whether the ions affect the intramolecular hydrogen bonding between the sn_2 ester C = O and the carbon-6 of the α-anomer of galactose (Gal). The major conclusions are: (*i*) the salts do not affect, at least directly, the ester carbonyl region of DGDG, (*ii*) the most probable sites of binding, or adsorption, for the ions are the ring oxygen, and (*iii*) the ring hydroxyls are the sites of either ion complexation or intra- and intermolecular H-bonding in interacting DGDG membranes. Within this framework, the complexation of the ions with Gal might induce total or partial dehydration of the galactolipid headgroup and thus provides the means to overcome the repulsive hydration forces that hinder aggregation of the DGDG membranes.

Key words: DGDG membranes – Digalactosyldiacylglycerol – FTIR – Divalent cations – Intramolecular H-bonding – Ion-binding – Membrane aggregation – Monovalent cations

Abbreviations: DGDG, digalactosyldiacylglycerol; EDTA, ethylenediaminetetracetic acid; FTIR, Fourier transform infrared; Gal, galactose; GlDG, D-glucosyldiacylglycerol; Glyc, glycerol; LHCII, chloroplast light harvesting complex II; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol

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Introduction

It has become evident in recent years that the thylakoid lipids play an important role in the organization and function of the thylakoid membrane of the chloroplast (see, e.g., Gounaris et al. 1983; Sprague et al. 1985; Murphy 1986; Huner et al. 1987; Pick et al. 1987; Webb et al. 1988; Siegenthaler et al. 1989; Murata et al. 1990; Fragata et al. 1991). However, it is recognized that a precise description of the relation between the molecular architecture of the lipid bilayer and the thylakoid function is still unavailable (see review in Webb and Green 1991). An unsolved question is the mechanism of reversible adhesion, or appression, in the thylakoid stacks. To date, two models have been used to explain the reversibility of appression; that is, the surface charge (SC) (Barber 1982; Staehelin and Arntzen 1983) and the molecular recognition (MR) hypotheses (Allen 1992 a, b) which aimed first at elucidating the functional characteristics of the phosphorylation of the chloroplast light-harvesting complex II (LHCII). The SC model postulates that the primary effect of LCHII phosphorylation is to increase the negative charge on the outer appressed surface of the thylakoid membrane, thereby overcoming the attractive forces that hold together the LHCIIs on appressed thylakoids in the grana. In the MR model one assumes that the phosphorylation of LHCII decreases the affinity of the protein complex for the photosystem II core antenna system, leading to structural changes which alter the complementarity of opposing faces of LHCIIs on adjacent thylakoid membranes, thus giving rise to unstacking.

The relevant mechanisms in the SC and MR models are essentially protein-protein interactions between phosphorylated and non-phosphorylated LCHIIs. An alternative mechanism of appression is suggested by recent studies on ionic effects in lipid-lipid interactions or aggrega-

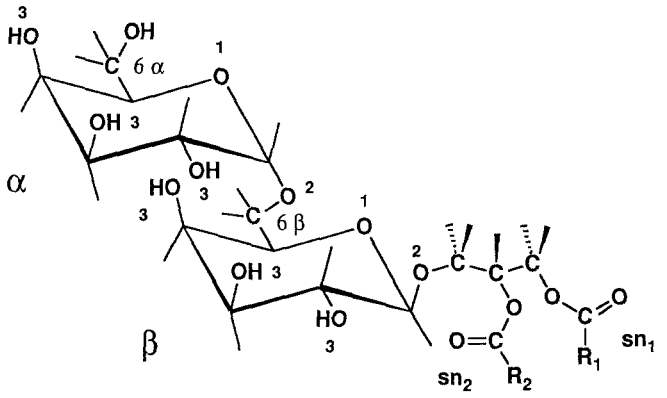

Fig. 1. Chemical structure of digalactosyldiacylglycerol (Gal$p\alpha$1-6 Gal$p\beta$1-3DAG; see IUPAC-IUB 1976, 1977) with indication of possible sites of intramolecular interaction (between sn_2 and $C_{6\alpha}OH$) and ion binding (1 to 3) (cf. Table 3)

tion (see, e.g., Webb et al. 1988; Fragata et al. 1991). The work of Webb et al. (1988), which we have confirmed in the present study (see Results), is directly related to this matter since it concerns interactions between vesicles composed of digalactosyldiacylglycerol (DGDG), a non-ionic thylakoid lipid that self-organizes into bilayer membranes (see, e.g., Quinn and Williams 1983; Murphy 1986). In brief, Webb et al. (1988) showed that DGDG vesicles aggregate in aqueous media containing mono- and divalent cations in amounts above a threshold concentration which is characteristic of each ion (cf. Figs. 1 and 3 of Webb et al. 1988). The significance of these findings to the function of a lipid-mediated mechanism of membrane appression stems from the fact that (*i*) DGDG constitutes about 29% of the total lipid content (TL) of the thylakoid membrane (Murata et al. 1990), (*ii*) among the other major thylakoid lipids, only phosphatidylglycerol (5% TL) gives rise to vesicles which are able to aggregate (see, e.g., Fragata et al. 1991), and (*iii*) the remaining thylakoid lipids, i.e. monogalactosyldiacylglycerol (MGDG; 56% TL), sulfoquinovosyldiacylglycerol (SQDG; 3% TL) and phosphatidylcholine (PC; 7% TL), are non-aggregating species which give rise either to hexagonal-II structures (MGDG; see Murphy 1986) or to bilayer membranes (SQDG, PC; see Webb et al. 1988).

On the basis of freeze-fracture electron microscopy studies, Webb et al. (1988) suggested that their turbidity data could be explained as the result of vesicle aggregation but not fusion (cf. their Fig. 1). We show here that the DGDG aggregation is reversed, or disrupted, upon addition of EDTA to the salt-containing incubation media (see, in this respect, Ohki et al. 1982; Düzgünes et al. 1987). Owing to the chelating properties of EDTA, the data indicate that the aggregated state of the DGDG membranes might be dependent on the adsorption, or binding of the ions in the lipid polar head. As a first step toward the investigation of this question, we used Fourier transform infrared (FTIR) spectroscopy for the localization of the particular groups, or molecular sites in DGDG which interact with $CaCl_2$, $MgCl_2$ or NaCl. We have also studied whether the ions affect the intramolecular hydrogen bonding which we detected in the polar head of the galactolipid.

Materials and methods

Chemicals

Digalactosyldiacylglycerol (Gal$p\alpha$1-6Gal$p\beta$1-3DAG, see Fig. 1) was purchased from Lipid Products (South Nutfield, UK) and purified according to the procedure described in next section. D_2O was obtained from Merck, Sharp and Dohme. The reagents for fatty acid analyses (see below) were from Pierce Chemical Company. $CaCl_2$, $MgCl_2$ and NaCl were from Sigma Chemical Company (St. Louis, MO) and were purified as described in Lessard and Fragata (1986); in brief, each salt was washed successively in three different organic solvents (methanol, benzene, chloroform), then dried at 150 °C for 24 h to remove any residual adsorbed solvent. Galactose was obtained from Fisher Scientific Company (Fair Lawn, NJ) and shown to be a mixture of α- and β-anomers on the basis of FTIR spectroscopic analyses of the type 2a and 2b vibrational bands around 837 and 893 cm^{-1}, respectively (see Parker 1971; Tu 1982). All other compounds were obtained from Sigma Chemical Company or Fisher Scientific Company.

DGDG purification and fatty acid analysis

DGDG was purified in an HPLC instrument from Waters Associates (Milford, MA), composed of two 510 pumps, a model G80 automatic gradient controller, a Rheodyne injector, model 7126 (Cotati, CA), equipped with a 1 ml loop and a programable UV-VIS detector monitored at 205 nm. Separation was achieved on a silica cation-exchange column operated in isocratic mode. All solvents (HPLC grade) used in the mobile phase were filtered through membrane filters of 0.2 μm pore size from Millipore (Bedford, MA), and degassed under vacuum prior to their introduction into the HPLC system. The mobile phase was a n-hexane:isopropyl alcohol:water mixture, 70:30:2 (v:v:v). The flow rate was 10 ml/min at 2 400 PSI. The samples were dried in a stream of nitrogen, then dissolved in the elution mixture to give a final concentration of 5 mg/ml and thereupon injected via the Rheodyne rotatory injector. The purified lipid was dissolved in chloroform.

The analysis of the fatty acid chains of the DGDG samples was done by gas chromatography of the methyl esters formed from methanolysis of the lipid (0.1 mg) with 1 ml of BF_3/MeOH 14% (w/v) (Pierce Chemical Company). Then, the mixture was heated 15 min at 100 °C. After approximately 10 min cooling, n-hexane and bidistilled water were added to separate the fatty acid methyl esters

which were dried by adding anhydrous Na_2SO_4 followed by a current of nitrogen. The residue was dissolved in 50 µl n-hexane. The fatty acid analysis was carried out in a Varian gas chromatograph, model 3700, equipped with a Shimadzu integrator, model C-R3A, using nitrogen as the carrier gas. The fatty acid chains compositions in mol% (in parenthesis) were 16:0 (8.5), 18:0 (0.7), 18:1 (3.5), 18:2 (3.1), and 18:3 (84).

Vesicle preparation

The DGDG vesicles were prepared according to the method of Huang (1969) with modifications described in L'Heureux and Fragata (1988). In brief, aliquots of the lipid dissolved in chloroform were dried under a current of nitrogen and then dispersed by vortex in an adequate volume of D_2O (see Results)[1] to give a final concentration of 1.5 mg/ml. This was followed by sonication of the solution (13 min) in a capped tube in a Heat Systems-Ultrasonics apparatus (Plainview, IL), model W-225R, set at about 160 W output, with nitrogen bubbling into the solution. In this way, the final suspension was not contaminated with titanium particles from the sonicator probe. The membranes were used as such in the aggregation studies (see next section).

Measurements of turbidity and turbidity reversibility

The aggregation of the DGDG vesicles was studied by observing the turbidity increase of the samples (see Day et al. 1980). The experiments were done in a UV-VIS double-beam spectrophotometer from SLM (Urbana, IL), model DW-2000, with the diffuser plate of the beam scrambler removed. The turbidity variations were observed at 600 nm. Aliquots of 1.0 M solutions of $CaCl_2$, $MgCl_2$ or NaCl were added to the incubation media in order to get the desired salt concentrations.

An ion chelator (EDTA) was used to measure the reversibility of turbidity of the preparations of aggregated DGDG membranes. This method was used successfully on several occasions (Ohki et al. 1982; Düzgünes et al. 1987) to study aggregation and fusion of small and large unilamellar vesicles composed of phosphatidylserine. The experiments reported below were performed with $CaCl_2$ and $MgCl_2$ only. The DGDG preparations were first treated with salt concentrations that induce maximum turbidity of the solutions, then 1 µl aliquots of a 1.0 M EDTA solution were added to the preparations and the turbidity decrease was measured.

FTIR measurements

First, DGDG membranes were prepared according to the method described above. Then, $CaCl_2$, $MgCl_2$ or NaCl were added to the vesicles preparations to make the final appropriate salt concentration. A drop (10–20 µl) of each of the samples was layered on 25 mm diameter BaF_2 plates and allowed to dehydrate over a period of about 24 h in an air-tight chamber. The FTIR measurements were performed in a BOMEM FTIR spectrometer, model DA 3.2, equipped with a HgCdTe detector (nitrogen cooled) and a KBr beam-splitter. In general, 100 interferograms were collected and co-added and the infrared spectra were obtained upon subtraction of the BaF_2 plate spectrum. Spectral resolution was 2 cm^{-1}. We note that the experiments were performed in D_2O to avoid overlapping of the ester carbonyl bands around 1 750–1 710 cm^{-1} with the H_2O absorption band in the 1 700–1 600 cm^{-1} region. We note, finally, that the identification of the band maxima frequencies was done in general by inspection of smoothed FTIR absorbance spectra and 2nd derivative spectra (see recent review in Bandekar 1992) obtained from calculations according to the Spectra-Calc version (Galactic Industries Corporation, Salem, NH) of the Savitzky-Golay convolution method (Savitzky and Golay 1964). In a few instances, 4th derivative calculations and Gaussian curve-fitting analyses were also performed.

Results

Reversibility of salt-induced turbidity of DGDG solutions

Figure 2a illustrates the turbidity changes observed in solutions of DGDG vesicles suspended in deuterium oxide[1] under the influence of various concentrations of mono- and divalent cations. It is seen that increasing amounts of cations cause an initial small turbidity increase that is followed by a sharp rise above a threshold concentration (C_t) which is characteristic of each type of metal ion. The curves drawn through the experimental points were obtained from a polynomial least-squares curve fitting analysis approximated to the 5th degree using a Cricket Graph program, version 1.3.2, from Cricket Software Inc. (Malven, PA). The C_t values for Ca^{2+}, Mg^{2+} and Na^+ were calculated to be about 1.0, 4.7 and 10.0 mM, respectively. These values are in good agreement with those reported previously by Webb et al. (1988), i.e. 1.0, 4.5, and 11.0 mM. The FTIR experiments described below were performed in the absence of salts and with salt concentrations above C_t.

Next, we studied the effect of EDTA on the turbidity of DGDG solutions induced by $CaCl_2$ and $MgCl_2$ (Fig. 2b). Figure 2b shows that the turbidity level in the absence of EDTA is almost completely suppressed upon addition of the chelator to the salt-treated DGDG preparations to give a final concentration of approximately 11–12 mM. The curves drawn through the experimental points were obtained as described above.

Vibrational characteristics of glycerol, galactose and DGDG

The vibrational spectra (absorbance, 2nd derivative) of galactose (Gal) and DGDG from 1 800 to 900 cm^{-1} are

[1] The turbidity experiments reported here were performed in D_2O, which was used to maintain uniformity with the FTIR spectroscopic studies

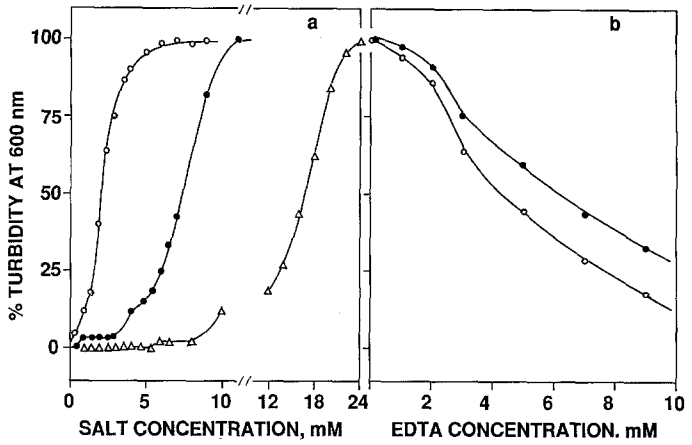

Fig. 2. **a** Turbidity at 600 nm of digalactosyldiacylglycerol (DGDG) vesicle suspensions vs. salt concentration. (○) $CaCl_2$; (●) $MgCl_2$; (△) NaCl. **b** EDTA-mediated reversibility of turbidity at 600 nm of DGDG vesicle suspensions. The DGDG vesicles were first treated with divalent cations as shown in Fig. 2a, then aliquots of 1.0 M EDTA were added to make the final appropriate concentration. (○) $CaCl_2$ pre-treated vesicles; (●) $MgCl_2$ pre-treated vesicles. EDTA, ethylenediaminetetraacetic acid

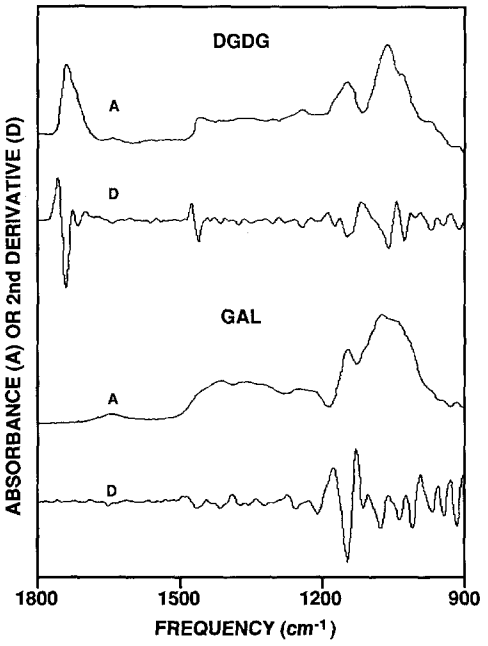

Fig. 3. Overview of absorbance and 2nd derivative spectra between 1 800 cm^{-1} and 900 cm^{-1} of digalactosyldiacylglycerol (DGDG) and galactose (Gal) solubilized in deuterium oxide, then dried on BaF_2 plates (see Materials and methods)

presented in Fig. 3, and the characteristic frequencies of their main bands as, well as those of glycerol (Glyc), are given in Table 1. The table identifies three main regions in the DGDG spectrum: (*i*) the ester C=O stretching modes between 1 750 and 1 710 cm^{-1}, (*ii*) the sugar bending modes in the 1 500–1 200 cm^{-1} range, and (*iii*) the sugar stretching modes from ~1 200 to 1 000 cm^{-1}, which usually contain some bending character.

The ester C=O region. In the 1 750–1 710 cm^{-1} region, the absorbance spectrum of DGDG has a band maximum at about 1 741 cm^{-1} (Fig. 4) assigned to the sn_1

Table 1. Characteristic frequencies (cm^{-1}) of the main bands in the infrared spectra in the 1 800–1 000 cm^{-1} region of glycerol, galactose and digalactosyldiacylglycerol (DGDG)

Molecule			Band assignment[d]	Refs.[e]
Glycerol[a]	Galactose[b]	DGDG[c]		
		1 741 m	ν(C=O), sn_1	1–4
		1 723 s	ν(C=O), sn_2	1–4
1 455	1 458	1 466 s	δ(OCH), δ(CCH), $\delta(CH_2)$	1, 2, 5–7
		1 453 m	δ(OCH), δ(CCH), $\delta(CH_2)$	1, 2, 5–7
1 415	1 418	1 419 m	δ(OCH), δ(CCH)	5, 6
		1 407 m	δ(OCH), δ(CCH)	5, 6
	1 374	1 378 m	δ(OCH), δ(CCH), δ(COH)	1, 2, 5, 6
	1 242	1 246 m	δ(OCH), δ(CCH), δ(COH)	5, 6
1 235		1 236 s	δ(OCH), δ(CCH), δ(COH)	5, 6
1 210	1 211	1 211 m	δ(OCH), δ(CCH), δ(COH)	5, 6
		1 175 s	ν(CO), ν(CC), δ(OCH), δ(CCH)	5, 6
		1 163 s	ν(CO), ν(CC), ν_{as}(COC)[f]	5–8
	1 147	1 149 m	ν(CO), ν(CC)	5, 6
1 111	1 116	1 117 d	ν(CO), ν(CC), δ(CCC)	5, 6
	1 055	1 062 m	ν(CO), ν(CC), δ(COH)	7
1 043	1 046	1 047 s	ν(CO), ν(CC), δ(CCO)	5, 6
		1 031 s	ν(CO), ν(CC)	5, 6

[a] From Pachler et al. 1988
[b] From Fig. 3
[c] From Figs. 4–7
[d] *Abbreviations:* d, dip; δ, binding; ν, stretching; m, maximum; s, shoulder
[e] 1, Fookson and Wallach 1978; 2, Mantsch et al. 1981; 3, Mushayakarara et al. 1982; 4, Blume et al. 1988; 5, Tajmir-Riahi 1988a, b; 6, Fringeli and Günthard 1981, 7, Parker 1971; 8, Marchessault and Liang, 1962
[f] Antisymmetric stretching vibration of the glycosidic oxygen bridge (see Parker 1971; Marchessault and Liang 1962)

ester C=O stretching mode (free C=O, see refs. in Table 1), and a shoulder at 1 716–1 715 cm^{-1} (see Fig. 4 inset) which gives rise to a distinct band in the 2nd derivative spectrum. However, we found with the aid of the 4th derivative spectrum and Gaussian curve-fitting (not shown) that the band maximum is actually at about 1 723 cm^{-1}. This low frequency vibration is identified with the sn_2 ester C=O stretching mode (bound C=O, see refs. in Table 1) and is attributed to hydrogen bonding between the carbonyl and the hydroxyls in DGDG (see Mannock et al. 1990). It is worth noting that the FTIR study by Mannock et al. (1990) of the α- and β-D-glucosyldiacylglycerols, i.e. Glc*p*α1-3DAG and Glc*p*β1-3DAG, indicates that Glc*p*α1-3DAG exhibits a single carbonyl ester stretching band at 1 730 cm^{-1}, whereas Glc*p*β1-3DAG displays two ester C=O bands at 1 737 and 1 715 cm^{-1}. The authors concluded that the low frequency band results from hydrogen bonding between the 2-hydroxyl of the β-anomer but not of the α-anomer, and the sn_2 ester C=O. In contrast to this, we will show (see Discussion) that the α-anomer in DGDG is also involved in intramolecular H-bonding with the sn_2 ester carbonyl.

The region between 1 500 and 1 200 cm^{-1}. In this spectral region, Glyc, Gal and DGDG display a large number of overlapping bands (cf. Fig. 3). This lack of fine structure

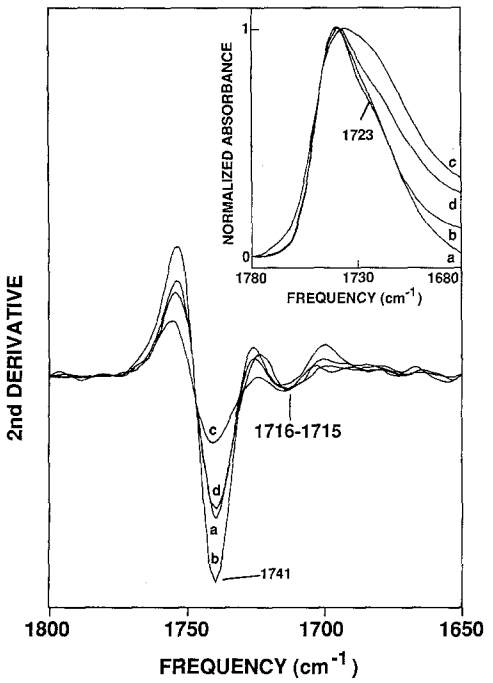

Fig. 4. Absorbance spectra (2nd derivative) of the ester C=O stretching mode for digalactosyldiacylglycerol preparations in D_2O in the absence of salt **a**, and in the presence of 18 mM NaCl **b**, 18 mM $MgCl_2$ **c** and 10 mM $CaCl_2$ **d**. The salt concentrations were above the threshold for membrane aggregation (see Fig. 2). *Inset*: Normalized absorbance spectra between 1 780 and 1 680 cm^{-1}. Conditions as above. The spectra were normalized at about 1 740 cm^{-1} (see text)

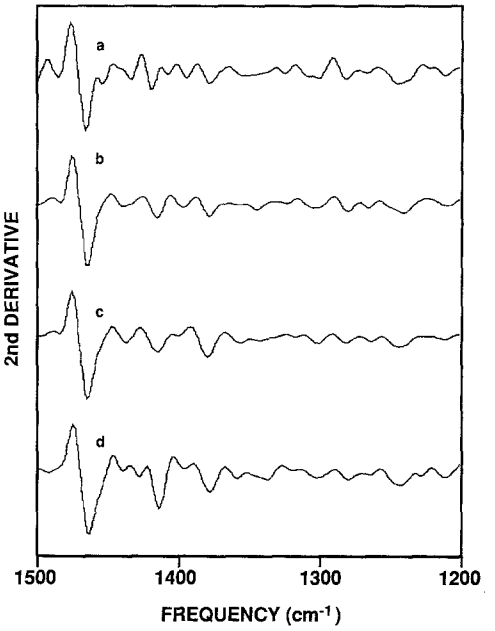

Fig. 5. Absorbance spectra (2nd derivative) between 1 500 and 1 200 cm^{-1} of digalactosyldiacylglycerol preparations in D_2O in the absence of salt **a**, and in the presence of 18 mM NaCl **b**, 18 mM $MgCl_2$ **c** and 10 mM $CaCl_2$ **d**. The salt concentrations were above the threshold for membrane aggregation (see Fig. 2)

does not favour the use of original absorbance spectra. Therefore, we restricted the identification of the vibrational bands to the 2nd derivative spectra (see Fig. 5), except where ambiguities persisted, in which case 4th derivative analyses were performed.

A survey of the literature (see refs. in Table 1) indicates that the vibrational bands in the 1 500–1 200 cm^{-1} region are combinations of deformation modes such as δ(OCH), δ(CCH), δ(COH) and $\delta(CH_2)$. The bands around 1 458–1 455 cm^{-1} and 1 418–1 415 cm^{-1} in Glyc and Gal result from δ(OCH), δ(CCH) and $\delta(CH_2)$ vibrations which are attributed to carbons 1 and 3 in Glyc and carbon 6 in Gal. In DGDG, the assignment of the frequencies at 1 466 and 1 453 cm^{-1} is more complicated. Previously, two bands at 1 465 and 1 457 cm^{-1} were attributed respectively to CH_2 scissoring vibrations (Mantsch et al. 1981) and gauche defects (Brumfeld et al. 1991) of the acyl chains of phosphatidylserine and phosphatidylethanolamine. However, the work of Parker (1971) and Tajmir-Riahi (1988 a, b) on the vibrational spectroscopy of carbohydrates suggests that at least the 1 466 cm^{-1} band can be assigned to deformation of the CH_2 groups in carbon-6 of either the α-anomer or the β-anomer of the Gal residues in DGDG. However, our study of the ionic effects on DGDG (see below) shows that the 1 466 and 1 453 cm^{-1} bands are related to the $C_{6\alpha}OH$ group.

The other interesting vibrations are (*i*) those at 1 419 and 1 407 cm^{-1} of δ(OCH) and δ(CCH) character which are most probably related to the ring oxygen, and (*ii*) the bands at 1 246, 1 236 and 1 211 cm^{-1} which are attributed to δ(OCH), δ(CCH) and δ(COH), and involve, therefore, the ring COH. Fringeli and Günthard (1981) suggested, in this respect, that strong H-bonding involving OH should affect the vibrational energy in the 1 400–1 200 cm^{-1} range. We show below that the molecular sites defined in (*i*) and (*ii*) are able to intervene in ion complexation and DGDG-DGDG interactions.

The region between 1 200 and 1 000 cm^{-1}. In this range, we note in particular the bands at ~1 163 cm^{-1}, 1 149–1 147 cm^{-1}, 1 062–1 055 cm^{-1} and 1 047–1 037 cm^{-1} (see Table 1) which are dominated largely by C–O and C–C single bond stretching modes in the non-planar conformations of the Gal and Glyc skeletons (see, e.g., Parker 1971; Fringeli and Günthard 1981). The vibrations at approx. 1 163 cm^{-1} are mixed, in addition, with an anti-symmetric stretching contribution, ν_{as} (COC), of the glycosidic bridges (see Marchessault and Liang 1962; Parker 1971) between the α and β anomers of Gal (cf. Fig. 1), and probably also between the β-anomer and Glyc. We note, furthermore, that the stretching vibrations at 1 062 and 1 047 cm^{-1} contain some bending character, that is, δ(COH) and δ(CCO) respectively, which may give rise to perturbations capable of affecting H-bonding strength (see below).

Ionic effects on the vibrational energies in DGDG

The effect of NaCl, $MgCl_2$ and $CaCl_2$ on the vibrational characteristics of DGDG are shown in Figs. 4, 5, 7, which

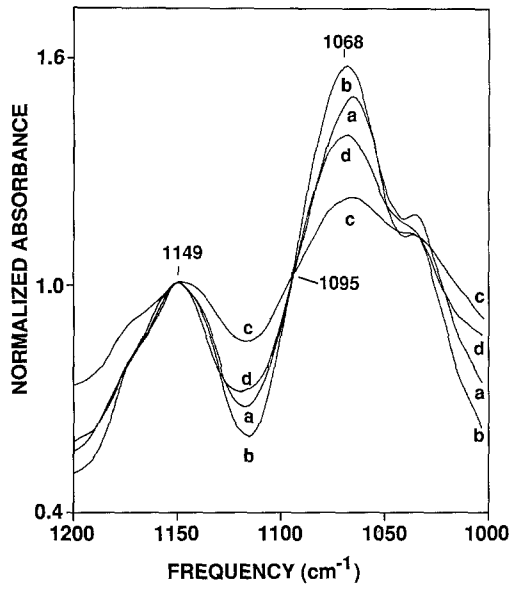

Fig. 6. Normalized absorbance spectra between 1 200 and 1 000 cm^{-1} of digalactosyldiacylglycerol preparations in D_2O in the absence of salt **a**, and in the presence of 18 mM NaCl **b**, 18 mM $MgCl_2$ **c** and 10 mM $CaCl_2$ **d**. The salt concentrations were above the threshold for membrane aggregation (see Fig. 2). The spectra were normalized at about 1 149 cm^{-1} (see text)

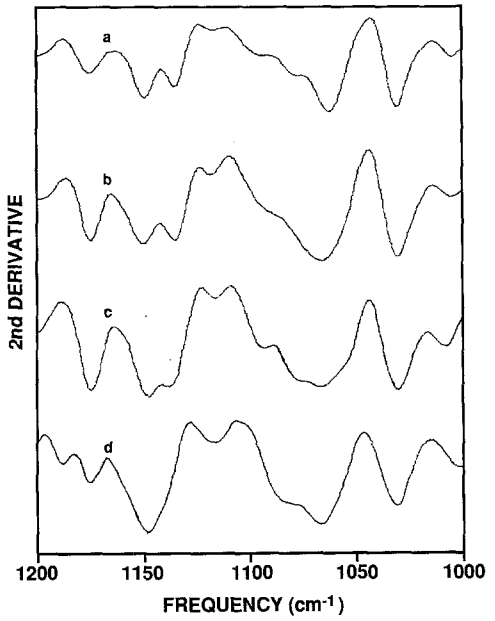

Fig. 7. Absorbance spectra (2nd derivative) between 1 200 and 1 000 cm^{-1} of digalactosyldiacylglycerol preparations in D_2O in the absence of salt **a**, and in the presence of 18 mM NaCl **b**, 18 mM $MgCl_2$ **c** and 10 mM $CaCl_2$ **d**. The salt concentrations were above the threshold for membrane aggregation (see Fig. 2)

display the 2nd derivative of the absorbance spectra, and in Fig. 4 inset and Fig. 6 that give the original absorbance spectra in the regions 1 780–1 680 cm^{-1} and 1 200–1 000 cm^{-1}, respectively. The most important features in the range from 1 800 to 1 000 cm^{-1} are summarized in Table 2. First, we note the presence of two main spectral regions where the vibrational frequencies are not affected by the mono- and divalent ions; that is, the ester C=O

Table 2. Effect of NaCl, $MgCl_2$ and $CaCl_2$ on selected vibrational energies (cm^{-1}) of the infrared spectrum in the 1 800–1 000 cm^{-1} region[a] of the polar headgroup of digalactosyldiacylglycerol

	Salt concentration[b]		
O	NaCl 18 mM	$MgCl_2$ 18 mM	$CaCl_2$ 10 mM
Ester C=O region			
1 741	1 741	1 741	1 741
1 723	1 724	1 724	1 723
1 500–1 200 cm^{-1} region			
1 466	1 466	1 466	1 466
1 453	1 453	1 454	1 455
1 419	1 415	1 415	1 415
1 407	1 398	1 400	1 398
1 378	1 379	1 380	1 379
1 246	1 251	1 248	1 250
1 236	1 240	1 240	1 242
1 211	1 209	1 213	1 213
1 200–1 000 cm^{-1} region			
1 175	1 175	1 175	1 175
1 163	1 159	1 159	1 160
1 149	1 150	1 149	1 149
1 117	1 117	1 116	1 116
1 062	1 066	1 065	1 067
1 047	1 057	1 053	1 057
1 031	1 031	1 031	1 031

[a] The band assignments are given in Table 1
[b] The salt concentrations were above the threshold for membrane aggregation (see Fig. 2)

region (1 750–1 710 cm^{-1}) and the region between 1 470 and 1 450 cm^{-1}. The small wavenumber shift (~1 cm^{-1}) observed at approximately 1 723 cm^{-1} (sn_2) would seem to indicate that no significant perturbation occurs at the interface between the acyl chains and the Glyc moiety of DGDG. However, the spectra in Fig. 4 inset (normalized at about 1 740 cm^{-1}) show clearly that the band intensities are substantially increased upon treatment of the DGDG membranes with Ca^{2+} and Mg^{2+}, the latter ion being the most effective. This is interesting since it is well established that the intensity of an infrared absorption band is dependent on the extent of electric dipole change during the vibrational displacement (see, e.g., Walker and Straw 1962; Painter et al. 1982). The result of calculations revealed that the ratio I_1/I_2, where I_1 and I_2 are respectively the integrated intensities of the sn_1 and sn_2 bands, is about 0.66 ± 0.12 ($CaCl_2$) and 0.60 ± 0.07 ($MgCl_2$) as compared to 1.06 ± 0.15 in absence of salts or in the presence of NaCl. This means that the ester C=O region is protected from the direct action of the salts, but not shielded from the electric field created by the presence of the divalent cations in the galactolipid-water interface. It is worth noting, in addition, that this finding is at variance with previous experiments performed with the anionic lipid phosphatidylglycerol (PG) showing a clear salt-mediated transformation of the sn_2 C=O (Nénonéné and Fragata, not published). The other bands of interest in DGDG which are unperturbed by the salts are at 1 466 and 1 453 cm^{-1} and these were assigned to the bending

Table 3. Schematic representation of cation-mediated effects in the polar headgroup of digalactosyldiacylglycerol (Gal$p\alpha$1-6 Gal$p\beta$1-3DAG)

Wavenumber[a] cm^{-1}	Assignment	Molecular site[b]	Wavenumber shift, cm^{-1}	Hydrogen bonding	Postulated effect
1 723 s	ν(C=O)	sn_2 C=O	NS	NA	No ion complexation
1 466 s, 1 453 m	δ(OCH), δ(CCH), $\delta(CH_2)$	$C_{6\alpha}OH$	NS	NA	No ion complexation
1 419 m, 1 407 m	δ(OCH), δ(CCH)	ring COC (1)[c]	↓	↓	Ion complexation
1 246 m, 1 236 s, 1 211 m	δ(OCH), δ(CCH), δ(COH)	ring COH (3)	↑	↑	Ion complexation and/or DGDG-DGDG interactions[d]
1 163 s	ν(CO), ν(CC), ν_{as}(COC)	bridge COC (2)	↓	↑	Ion complexation and/or DGDG-DGDG interactions[d]
1 062 m	ν(CO), ν(CC), δ(COH)	ring COH (4)	↑	?	[e]
1 047 s	ν(CO), ν(CC), δ(COH)	ring COH (5)	↑	?	[e]

[a] *Abbreviations:* δ, bending; m, maximum; NA, not affected; NS, no shift; ν, stretching; s, shoulder; ↓, decrease; ↑, increase
[b] The molecular sites were identified on the basis of the wavenumber assignments and other arguments given in the text
[c] The figures in parentheses are those used in Fig. 1 to identify the molecular sites
[d] These interactions are likely enhanced by salt-induced dehydration of the headgroup interface
[e] At this stage we cannot give a correct identification of the ion effect owing to mixing of ν and δ vibrations. However, the observed wavenumber shifts in these spectral regions (cf. Table 2) are most probably related to ion complexation

modes (OCH, CCH, CH_2) around the carbon-6 of the α-anomer (see Fig. 1). The above data suggest that hydrogen bonding occurs between sn_2 C=O and the hydroxyl in $C_{6\alpha}OH$ and that the presence of salts in the sugar interface cannot modify it (see Table 3 and Discussion).

The spectral changes observed in the 1 200–1 000 cm^{-1} region as the result of treatment of the DGDG preparations with NaCl, $MgCl_2$ and $CaCl_2$ are represented in the absorbance spectra in Fig. 6 and the 2nd derivative spectra of Fig. 7. To facilitate the study of the data, the spectra in Fig. 6 are normalized at about 1 149 cm^{-1} since this vibrational band does not undergo any appreciable wavenumber shift. Although the identification of certain frequency displacements is possible in Fig. 6 we found that the 2nd derivative spectra (Fig. 7) allow a better determination of the spectral shifts, which are summarized in Table 3. These salt-induced shifts are ascribed to interactions involving intra- and intermolecular H-bonding in the galactosidic headgroup, which might involve local dehydration of the molecular sites of interaction. The transitions are those referred to in Table 3 as 1 to 5, which originate from bending (1, 3), stretching (2) and mixtures of stretching and bending (4, 5) modes. We recall, in this respect, that (*i*) since H-bonds act as constraints to bending deformations, the force constants for these vibrations increase and the frequency is shifted to larger values, (*ii*) the absorption bands due to the stretching vibrations of the H-bond donor are shifted to lower frequencies, and (*iii*) the vibrational modes of the H-bond acceptor shift either to longer or shorter wavenumbers. Within this framework, the changes observed in transition 1 are interpreted as the result of ion complexation to the ring oxygen of Gal, thereby hindering H-bonding. The effect of the ions on transition 3 originates from ion complexation to the hydroxyls of the Gal skeleton, or to DGDG-DGDG interactions which are favoured by salt-mediated dehydration of the sugar headgroup. This same interpretation is given to transition 2, but for interactions occurring at the galactosidic oxygen bridge. Finally, transitions 4 and 5 cannot be identified correctly at this stage owing to mixing of stretching and bending vibrations. We hypothesize, nevertheless, that the salt effect results from ion complexation or DGDG-DGDG interactions at the site of the ring COH groups. We wish to remark, in addition, that here we observed also (as in the ester C=O region; see above) a considerable variation of relative intensities of the absorption bands (cf. Fig. 6), showing that the molecular environment of the OH groups in the headgroup of DGDG has been significantly altered by the presence of the salts. This is also clear upon close examination of the differing shapes of the 2nd derivative spectra displayed in Fig. 7, particularly in the 1 200–1 100 cm^{-1} range which has been identified as the bridge oxygen (COC) in the digalactosylglyceryl residue (cf. Table 3).

Discussion

Intramolecular H-bonding in the polar head of DGDG

In an infrared spectroscopic study of the α- and β-anomers of D-glucosyldiacylglycerol (GlDG), Mannock et al. (1987, 1990) observed that the α-anomer exhibits a single strong band at 1 730 cm^{-1} that was correctly assigned as an ester C=O stretching band, whereas the β-anomer displayed two bands at 1 737 and 1 715 cm^{-1} which are respectively the free and bound carbonyls, that is sn_1 C=O and sn_2 C=O. Based on filling model studies and conformational analysis data (Brown et al. 1970; Jarrell et al. 1987), Mannock et al. (1990) suggested that (*i*) the band at 1 715 cm^{-1} exhibited by the β-anomer originated from hydrogen bonding between the 2-hydroxyl of the sugar moiety and the sn_2 C=O, and (*ii*) the configuration of the α-anomeric centre would not permit H-bond-

ing interactions. These conclusions show the importance of intramolecular hydrogen bonding in the geometrical arrangement and the polymorphic phase behavior of bilayers composed of glycolipids.

We show below that internal H-bonding also takes place in the head group region of DGDG. However, the molecular sites in the Gal residues that interact with sn_2 C=O seem to differ from those determined by Mannock et al. (1990) in GlDG. We performed the following analysis based on a Dreiding stereomodel (Rinco Instrument Co.) study,[2] and DGDG data obtained from minimum energy modelling (Brasseur et al. 1983), monolayer studies (Bishop et al. 1980; Sen et al. 1981; Marra 1985; Ducharme et al. 1991), neutron diffraction (McDaniel 1988) and surface force measurements (Marra 1986). First, we considered the limiting area of DGDG obtained from surface pressure-area isotherms extrapolated to zero surface pressure (A_l). A_l is a measure of the molecular cross-sectional area under conditions of zero compression, i.e. when the molecules are in a closely packed arrangement in the absence of molecular deformations. Furthermore, A_l is also similar to the corresponding value obtained from X-ray diffraction of the crystalline material. For DGDG, the experimental A_l values are between 83 Å^2 (Bishop et al. 1980) and 87 Å^2 (Marra 1985), in good agreement with the theoretical determinations of Brasseur et al. (1983), that is, 85 Å^2. In this regard, it is noteworthy that these values may vary considerably with the biological origin of the lipid (see, e.g., Bishop et al. 1980), thereby indicating structural differences in their fatty acid chains. Next, we have considered the thickness of the head group region (T_{hg}). According to neutron diffraction data (McDaniel 1988) and surface force measurements (Marra 1986), T_{hg} is about 8 Å.

Now, the values of A_l and T_{hg} given above are similar to those determined in the present work with a Dreiding stereomodel, i.e. ~84 Å^2 and ~9 Å respectively, provided that the ester sn_2 C=O makes a hydrogen bond with either the OH group at the carbon-6 of the α-anomer of the Gal residues ($C_{6\alpha}OH$) or the OH group at the carbon-4 of Gal ($C_{4\alpha}OH$) (cf. Fig. 1). This means that the vibrational frequencies involved are those corresponding to the molecular sites $C_{6\alpha}OH$ and ring COH (3), respectively (cf. Table 3). However, the Dreiding stereomodel study shows that a better three-dimensional arrangement of the DGDG polar head is achieved with an interaction between sn_2 C=O and $C_{6\alpha}OH$.[2]

Salt-induced perturbations in the DGDG head group

Since the interaction between DGDG vesicles does not lead to membrane fusion (see Results), we assume that the onset of aggregation brings about some kind of ion adsorption, or binding in the lipid interface created upon dimerization of the vesicles. This is made clear by applying Born's expression for the total energy of charging an ion ($\Delta\mu$) to the case of a change in free energy on transferring the ion from a medium of low dielectric constant ε_1 to one of high dielectric constant ε_2 (see, e.g., Israelachvili 1985), i.e.

$$\Delta\mu = -\frac{Z^2 e^2}{8\pi\varepsilon_0 a}\left(\frac{1}{\varepsilon_1}-\frac{1}{\varepsilon_2}\right),$$

where Z is the valence of the ion, a its radius, e the electron charge, and ε_0 the permittivity of free space. For example, the diffusion of the ion out of the water-lipid interface where ε_1 is from 25 to 32 (cf. Lessard and Fragata 1986) into the bulk aqueous phase ($\varepsilon_2 \sim 78$) is energetically favourable, i.e. $\Delta\mu$ is between −27 and −18 kJ mol^{-1} which is a range that lies within the strength span of most hydrogen bonds, that is, from 10 to 40 kJ mol^{-1} (Israelachvili 1985). However, the observed aggregation of the DGDG membranes is a good indication that a thermodynamically driven extrusion of the ions might not occur. Therefore, this points to the occurrence of strong interactions between the ions and the chemical groups at the surface or inside the polar head of the DGDG membrane.

From the infrared data reported here, our first conclusion is that $CaCl_2$, $MgCl_2$ and NaCl might not interact with the ester carbonyl of DGDG, or, more specifically, with the sn_2 ester C=O, which is presumedly the molecular site of hydrogen bond formation with either $C_{6\alpha}OH$ or $C_{4\alpha}OH$, as was deduced above. This is interesting as we showed previously that in phosphatidylglycerol (PG), an anionic lipid which has a phosphorylglyceryl moiety connected to a diacylglycerol structural frame, these same salts interact strongly with the ester carbonyl (Nénonéné and Fragata, not published). These new findings are instrumental in fostering the investigation of the mechanisms of lipid function in the thylakoid membrane of the chloroplast.

In DGDG, the action of Ca^{2+}, Mg^{2+} and Na^+ is thus restricted to the α- and β-anomers of Gal. The most probable sites of binding, or adsorption, for the ions are between carbons 2 and 3 in the β-anomer, and between carbons 3 and 4 in the α-anomer (cf. Table 1 and Fig. 1). These assignments follow from the observed salt-induced variations of the vibrational energies in the 1 246–1 121 cm^{-1} and 1 163 cm^{-1} wavenumber regions (Table 2), and also from geometrical requirements which became apparent upon examination of the Dreiding stereomodel of DGDG as discussed above. Within this framework, the interaction of the ions with the Gal residues provides a means of inducing total or partial depletion of the head group of the DGDG membranes of the water molecules that participate in the structural organization of the lipid-water interface. A similar situation has been observed, for example, in phosphatidylserine (PS) monolayers, where adsorption of Ca^{2+} leads to strong dehydration of PS (Gruen et al. 1984; Kozlov et al. 1989). We conclude, therefore, that dehydration of the digalactosyl-water interface overcomes the repulsive hydration forces (see, e.g., Rand 1981) which hinder to a great extent the DGDG-DGDG interactions that bring about aggregation.

Now, a question that arises is how the aggregation process itself takes place. First, this concerns the mechanisms of binding or adsorption of the ions to the α- and

β-anomers of Gal. One possibility is hydrogen bonding of the dry ion [3] to the COH groups in Gal. Interactions of this type have been discussed extensively in the literature (see, e.g., Vinograd and Linnell 1971). One example is the structure of Cu(II)(dimethylglyoxime)$_2$ where the H-bond between the two monomers is long because the H-bonding acceptor oxygen in one of the monomers interacts with the Cu^{2+} of the second monomer (Vinograd and Linnel 1971). Second, the data discussed here indicate that the stabilization of the aggregated state upon ion binding or adsorption to Gal, is most probably mediated by H-bonds between the Gal residues in two approaching, or interacting membranes (see, e.g., Carpentier et al. 1983). We note, in addition, that the neutron diffraction data of McDaniel (1988) show that the digalactosyl group in the DGDG bilayer lies parallel to the plane of the membrane. This conclusion is in agreement with the flat orientation suggested for the head group of the glucosyldiglycerides isolated from *Acholeplasma laidlawii* (Wieslander et al. 1978), and with our three-dimensional simulations that suggest a quasi-flat orientation of Gal relative to the plane of a DGDG monolayer, i.e. $\sim 10°$ for the α-anomer and $\sim 15°$ for the β-anomer. In these conformers the dipole moments of four to six COH groups point out of the plane of the polar head of the galactolipid. The study of these molecular orientations will be approached more precisely by FTIR linear dichroism of DGDG aligned in oriented matrices (see, e.g., Holmgren et al. 1987).

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[2] This was confirmed recently (M. Fragata, A. Menikh and S. Robert, to be published) in a preliminary study of the energy minimization calculation of the DGDG molecule using the MM2 force field with the Chem 3D Plus program, version 3.0, from Cambridge Scientific Computing (Cambridge, MA)

[3] The interaction between the COH groups of Gal and hydrated ions may also take place (see Webb et al. 1988). However, at the lipid-water interface this may not occur since the dielectric constant (ε) of the interfacial region is quite low, that is, $\varepsilon \sim 25–32$ (see Lessard and Fragata 1986)

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