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Salt-mediated interactions between vesicles of the thylakoid lipid digalactosyldiacylglycerol

Murray S. Webb [a], C.P.S. Tilcock [b] and B.R. Green [a]

[a] *Department of Botany and* [b] *Department of Biochemistry, University of British Columbia, Vancouver (Canada)*

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Interbilayer interactions between large unilamellar vesicles of the plant thylakoid galactolipid digalactosyldiacylglycerol in aqueous salt solutions have been examined by light scattering, freeze-fracture electron microscopy, and X-ray diffraction. When suspended in aqueous salt solutions, vesicles of 100 nm diameter were found to aggregate in a rapid and reversible manner to yield aggregates greater than 1000 nm in diameter. Freeze-fracture electron microscopy showed these aggregates to consist of appressed, but not fused, vesicles. Quasi-elastic light scattering and turbidity experiments showed that aggregation was not due to charged impurities of the lipid behaving in accordance with electrostatic double double layer theory. Experiments testing the efficacies of various chloride salts indicated a strong correlation existed between ionic radius and ability of the salt to promote aggregation. Similar experiments examining the effect of sodium salts, glycerol, and pH on vesicle aggregation implicate an interaction between the digalactosyldiacylglycerol head group and structured water as underlying the aggregation process. The results suggest that digalactosyldiacylglycerol may contribute to close membrane approach of thylakoids in higher plant chloroplasts.

Introduction

The lipid composition of higher plant photosynthetic membranes is dominanted by the neutral galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These lipids comprise 70–80 mol% of the total thylakoid lipid, the remainder being composed of the anionic lipids sulfoquinosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) [1]. Dispersal of DGDG, SQDG, or PG in excess water at room temperature spontaneously gives liquid-crystalline lamellar phases, whereas MGDG yields a hexagonal-II phase [2,3]. The contribution of these various lipids to the overall organization of thylakoids and function of chlorophyll-protein complexes is not known, although a number of ideas have been proposed (for reviews, see Refs. 4 and 5). Recently, the preferential activation of reconstituted chloroplast ATP synthase by MGDG has been reported [6].

The thylakoid lipids exist in a membrane system which, in vivo, shows large areas of close membrane approach (grana). The interaction of neighbouring thylakoid membranes, referred to as 'stacking', is believed to be mediated by interactions of units of the chlorophyll *a/b* light-harvesting complex (LHC II) [7]. It has been proposed that the role of the galactolipids in this inter-

Correspondence: B.R. Green, Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, B.C., Canada V6T 2B1.

bilayer interaction is merely as a neutral, non-charged, lipid matrix serving to maintain a low surface charge density and, hence, minimize electrostatic repulsion between adjacent bilayers [4]. While there have been some suggestions for a role of thylakoid PG and its unique fatty acid *trans*-3-hexadecanoic acid in granal stacking [8] this seems unlikely in view of genetic evidence showing normal stacking in a mutant of *Arabidopsis thaliana* lacking *trans*-3-hexadecanoic acid [9] and reduced stacking in a barley mutant with normal *trans*-3-hexadecanoic acid levels [10].

We describe here the characterization of well-defined DGDG vesicles and their behavior in dilute salt solutions. Further, we present evidence suggesting that DGDG may play a role in close membrane approach in thylakoids.

Materials and Methods

Materials

All solvents (BDH reagent grade) were redistilled before use. Other compounds were obtained as follows: galactose and NaCl from BDH; $CaCl_2$, $MgCl_2$, KCl and glycerol from Amachem; NaBr from Anachem; NH_4Cl, $NaClO_4$, glucose, and succinate from Fisher; CsCl from Calbiochem; RbCl from ICN; LiCl from Baker-Adamson; $NaNO_3$, NaSCN from MCB; oleic acid, egg phosphatidylethanolamine, egg phosphatidylcholine, dioleoylphosphatidic acid, dipalmitoylphosphatidylglycerol, valinomycin, and tricine from Sigma. Atomic absorption analysis of the distilled water from our lab has indicated metal levels < 10 ng $\cdot$ ml^{-1} of Cu, Mn, and Zn.

Lipid purification

A preliminary description of our purification procedure for thylakoid lipids has been published [11]. Briefly, 2–2.5 kg of deveined spinach leaves were ground in 6.5 l of $CHCl_3/CH_3OH$ (1 : 2, v/v) with a Polytron homogenizer and the lipid phase recovered as previously described [12]. This extract was concentrated under vacuum and separated by liquid chromatography (Waters Prep LC 500) on silica (Waters PrepPak-500) in $CHCl_3/CH_3OH/H_2O$ (60 : 30 : 3, v/v) at 150 ml $\cdot$ min^{-1}. The DGDG-enriched fraction was loaded onto a 2.5×20 cm carboxymethyl-cellulose (Whatman CM-52) column pre-equilibrated in $CHCl_3$. Pigments, MGDG, and phosphatidylcholine (PC) were eluted with eight column volumes of 7% CH_3OH in $CHCl_3$ (v/v) at 4 ml $\cdot$ min^{-1}. DGDG and some phosphatidylethanolamine (PE) were recovered with eight volumes of 11% CH_3OH in $CHCl_3$ (v/v). Residual PE was removed using silica on a circular, spinning, chromatography system (Chromatotron, Harrison Research, Palo Alto, CA) with $CHCl_3/CH_3OH/H_2O$ (65 : 45 : 1, v/v) at 3.5 ml $\cdot$ min^{-1}.

For SQDG purification, the SQDG enriched fraction from the first column was loaded onto the carboxymethyl-cellulose as described above. Pigment, PC, PG, PE, MGDG and DGDG were removed by washing with 18% CH_3OH in $CHCl_3$ (v/v), then SQDG was eluted with 22% CH_3OH in $CHCl_3$ (v/v). Residual PG was removed from the SQDG by silica chromatography on the Chromatotron system in $CHCl_3/CH_3OH/H_2O$ (65 : 45 : 1, v/v) at 3.5 ml $\cdot$ min^{-1}.

Lipid identity and purity were verified by extensive TLC, GLC of fatty acid methyl esters, ^{13}C-NMR, as well as galactose and phospholipid assays (Webb et al., in preparation).

Vesicle reconstitution

Aliquots of lipids in $CHCl_3$ were dried under a stream of N_2 and residual solvent removed under reduced pressure overnight at 4 °C. Lipids were dispersed at 10 mg $\cdot$ ml^{-1} either by vortexing and sonication (20–30 s total in 5-s bursts followed by cooling on ice, under N_2; Branson bath sonicator) or by a reverse-phase evaporation method using Freon-11 [13]. Resultant dispersions were converted to large unilamellar vesicles by repeated extrusion (The Extruder, Lipex Biomembranes) through two stacked 0.1 μm Nucleopore polycarbonate filters at 2000 kPa N_2 [14]. When DGDG was dispersed in salt solutions, higher N_2 pressures, up to 4500 kPa, were required for extrusion due to vesicle aggregation. No differences in aggregation properties were observed using the reverse-phase or vortexing/sonication methods of lipid dispersal.

For the turbidity (A_{600}) standard curve, vesicles in H_2O were extruded through 0.4 μm filters at 350 kPa and an aliquot removed for A_{600} reading. The remainder was extruded at 0.2 μm (350 kPa),

then 0.1 μm (2000 kPa), then 0.05 μm (2500 kPa) and finally 0.01 μm (4500 kPa) [15] and assayed for A_{600}.

For the valinomycin control, the valinomycin in $CHCl_3$ was mixed with DGDG in $CHCl_3$ at 1 $\mu g \cdot \mu mol^{-1}$ DGDG (1113:1, mol:mol), solvent removed as above and vesicles dispersed in water.

Turbidity measurements

Turbidity readings at 600 nm were obtained on a Varian Cary 210 spectrophotometer using non-aggregated 100 nm vesicles in H_2O as blanks. Vesicles were diluted to 1 $mg \cdot ml^{-1}$ with H_2O, then small amounts of concentrated salt solutions added and A_{600} recorded immediately.

X-ray diffraction

X-ray powder patterns were recorded using Cu K_α radiation generated on a Rigaku RU-200 microfocus rotating anode X-ray machine coupled, via Franks optics, to the Princeton SIV area detector [16–18]. Radial densitomerization of the images was performed as previously described [19,20]. Data are presented as the basis vector length of the lamellar lattice versus temperature. Repeat spacings were calibrated against lead stearate and are accurate to ±0.05 nm.

DGDG was dispersed at 50% (w/w) in 5 mM EDTA, 100 mM KCl, or water and loaded into acid-cleaned X-ray capillaries which were sealed with an epoxy plug. Samples were ramped in temperature from 0°C to 50°C in 10°C steps with a 10 min equilibration at each temperature. Diffractions were typically collected over a 60 s exposure.

Quasi-elastic light scattering (QELS)

Vesicle and aggregate diameters were estimated by quasi-elastic light scattering (QELS) using the Nicomp Submicron Particle Sizer 270 at lipid concentrations of 0.1, 1.0, or 10 $mg \cdot ml^{-1}$. Samples were irradiated by a 5 mW Helium-Neon laser at 632.8 nm and the autocorrelation function converted to mean vesicle diameter as described previously [15].

Freeze-fracture electron microscopy

Vesicles at 10 $mg \cdot ml^{-1}$ in water were aggregated by the addition of salt to 100 mM KCl, 10 mM $MgCl_2$, or left in water, then made to 20 or 25% glycerol (v/v) and frozen in Freon-22 cooled with liquid nitrogen. Samples were fractured in a Balzers 400 instrument at −107°C and $\leqslant 8 \cdot 10^{-7}$ torr then coated with 2.0 (±0.1) nm Pt followed by 20 (±4) nm carbon as measured by a quartz crystal thickness monitor. Replicas were cleaned with commercial bleach or 70% chromic acid for several hours, rinsed, and examined in a Zeiss EM-10 electron microscope at 80 kV accelerating voltage.

Results

Aggregation of large unilamellar vesicles by salt

The addition of salt to hydrated vesicles at lipid concentrations of 10 $mg \cdot ml^{-1}$ was observed to result in very rapid appearance of a cloudy, readily precipitable, particulate suspension. As shown in Table I, aggregation could be obtained either by dispersing the lipid directly into salt solutions, or by the addition of salt to vesicles suspended in water. The aggregation process was found to be fully reversible (Table I). That is, 100 nm DGDG vesicles in water could be aggregated by the addition of KCl or NaCl to yield particles of more

TABLE I

QUASI-ELASTIC LIGHT SCATTERING (QELS) DETERMINATIONS OF THE DIAMETERS OF DGDG VESICLES DISPERSED IN VARIOUS SALT SOLUTIONS

DGDG was dispersed at 10 $mg \cdot ml^{-1}$ in 0.1 M NaCl or 0.1 M KCl and extruded to give 100 nm diameter large unilamellar vesicles. Diameters were measured by QELS before and after 1:10 or 1:100 dilution with water. Vesicles dispersed in water were extruded to 100 nm, sized by QELS before and after addition of salt, then diluted with water and diameters measured again by QELS. Data represents mean ± S.D. from representative experiments of 5–8 trials. n.a., not applicable; n.d., not determined.

Vesicles made in	Diameter in H_2O (nm)	Outside solution made to	Diameter (nm)	
			after salt addition	after 1:10 or 1:100 dilution
H_2O	121 ± 21	0.1 M KCl	>1000	103 ± 23
H_2O	121 ± 20	0.1 M NaCl	>1000	n.d.
0.1 M KCl	n.a.	0.1 M KCl	>1000	104 ± 23
0.1 M NaCl	n.a.	0.1 M NaCl	>1000	129 ± 32

than 1000 nm diameter, and the original 100 nm vesicles could be released by subsequent 1 : 10 to 1 : 100 dilution with water. This release is easily observable by light microscopy. Vesicle appression followed by fusion would be expected to yield vesicles with diameters larger than 100 nm, and it would be unlikely for vesicles with the original size distribution to be formed upon dilution. This result strongly suggested that the structure of the aggregate was of appressed, but not fused, vesicles.

This was confirmed for DGDG aggregation in 100 mM KCl and 10 mM $MgCl_2$ by freeze-fracture electron microscopy (Fig. 1). DGDG vesicles in water (Fig. 1a) showed typical vesicle appearance for liquid-crystalline phase lipid dispersions. Fractured lipid surfaces were smooth, and vesicles were never observed to be in direct contact. Measurement of vesicle diameters from micrographs gave values in the 100–120 nm range for equatorial fractures. This was verified by a QELS determination of 121 (± 30) nm diameter for this preparation. Similarly, the bilayer surface appearance of DGDG vesicles aggregated in 100 mM KCl (Fig. 1b) or 10 mM $MgCl_2$ (Fig. 1c) was identical to that of DGDG in water. Single vesicles in aggregates had equatorial diameters between 80 and 120 nm. No vesicles were observed to be significantly larger than this range, confirming the lack of vesicle fusion, despite close membrane approach.

These preparations showed several striking features. First of these was the close adhesion of the vesicles. While it is beyond the resolution of freeze-fracture to determine if adjacent polar surfaces were in direct contact, it is clear that bilayers are stable within several nanometers of each other. A second interesting aspect was the extensive vesicle flattening. We suspect that this

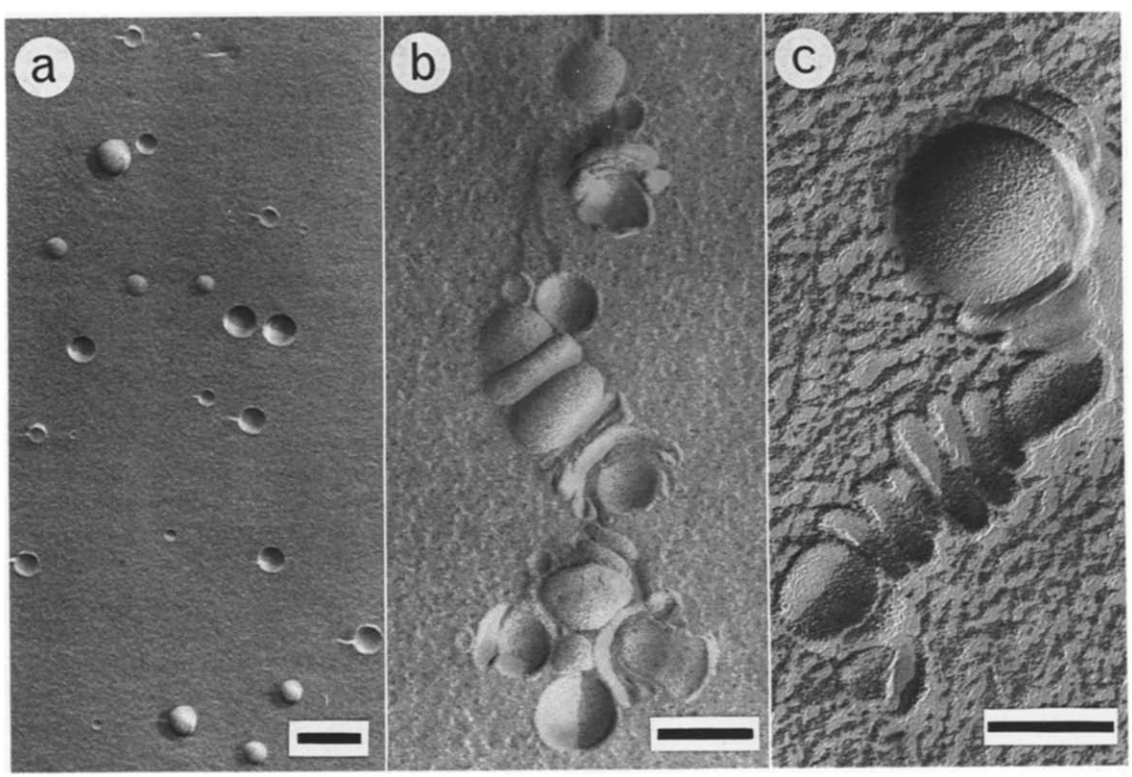

Fig. 1. Electron micrographs of freeze-fracture replicas of 100 nm DGDG vesicles at 10 $mg \cdot ml^{-1}$ dispersed in water (1a) or dispersed in water then aggregated by the external addition of salt to give 100 mM KCl (1b) or 10 mM $MgCl_2$ (1c). Samples were diluted to 25% (1a,b) or 20% (1c) (v/v) glycerol before freezing from 22° C. Bars represent 200 nm (1a) or 100 nm (1b and 1c).

results in part from osmotic stress due to external salt addition. A consequence of this flattening is the small radius of curvature at the ends of some vesicles (Fig. 1b) that was estimated at 5 nm.

Since the salt-induced aggregation of an uncharged lipid was an unexpected finding, we were concerned that there might be a charged impurity in the DGDG preparation that was behaving in accordance with electrostatic double layer theory. To test this, 2.0 or 2.5 mol% of possible contaminants were added to egg PC, the mixture dispersed at 10 $mg \cdot ml^{-1}$ in 100 mM KCl, extruded at 0.1 μm and vesicle or aggregate diameters measured by QELS. As shown in Table II, egg PC with 2.0 mol% egg PE, oleic acid (18 : 1), dioleoylphosphatidic acid (DOPA), dipalmitoylphosphatidylglycerol (DPPG) or 2.5 mol% SQDG did not aggregate in 100 mM KCl. Further, pure SQDG dispersed in 100 mM KCl or 10 mM $MgCl_2$ did not aggregate. Gas-liquid chromatography analysis of the fatty acid methyl esters of DGDG re-extracted after the experiments indicated that no significant change in the fatty acid profile of the DGDG occurred during the experiments. Thin-layer chromatography of the re-extracted DGDG showed only one compound in these samples. Therefore, it is unlikely that breakdown products of the galactolipid were causing vesicle aggregation.

TABLE II

QUASI-ELASTIC LIGHT SCATTERING (QELS) DETERMINATIONS OF DIAMETERS OF VARIOUS LIPID MIXTURES DISPERSED IN SALT SOLUTIONS

Lipid mixtures were dispersed in the solutions indicated and extruded to make 100 nm unilamellar vesicles. Diameters were measured by QELS as in Table I. In the DGDG and valinomycin experiments the vesicles were dispersed at 10 $mg \cdot ml^{-1}$ in water, extruded to 100 nm, diluted 1 : 10 with water and sizes measured by QELS. Concentrated KCl was then added to a final concentration of 100 mM KCl and sizes measured again by QELS. Data represent means ± S.D. from representative experiments.

Lipid	Addition (mol%)	[Lipid] ($mg \cdot ml^{-1}$)	Salt	Diameter (nm)
Egg PC	none	10	100 mM KCl	117 (±24)
Egg PC	2% egg PE	10	100 mM KCl	121 (±37)
Egg PC	2% 18:1	10	100 mM KCl	125 (±35)
Egg PC	2% DOPA	10	100 mM KCl	124 (±36)
Egg PC	2% DPPG	10	100 mM KCl	127 (±36)
Egg PC	2.5% SQDG	10	100 mM KCl	129 (±46)
SQDG	none	10	H_2O	119 (±34)
SQDG	none	10	100 mM KCl	104 (±26)
SQDG	none	10	10 mM $MgCl_2$	100 (±32)
DGDG	none	1	H_2O	125 (±30)
DGDG	none	1	100 mM KCl [a]	>1000
DGDG	valinomycin	1	H_2O	109 (±28)
DGDG	valinomycin	1	100 mM KCl [a]	>1000

[a] Added outside vesicles.

We have also examined the lamellar repeat distances for DGDG in water, 100 mM KCl, and 5 mM EDTA by X-ray diffraction. As seen in Table III, there was no difference in the DGDG lamellar repeat for any of these solutions between 0 °C and 50 °C. If a charged contaminant were present, the lamellar spacing should have decreased in the presence of salt due to charge screening. This strongly suggested that aggregation was not due to a charged contaminant. In summary, these and other reasons (see Discussion) have led us to conclude that aggregation was not due to charged impurities of the DGDG.

Another possibility was that aggregation might have been due to the generation of a diffusion potential across the bilayers. While aggregation occurred with 100 mM KCl both inside and outside the vesicles (Table I) this possibility was further checked by the addition of valinomycin to DGDG vesicles. The presence of valinomycin did not inhibit KCl-mediated aggregation (Table II). We have also verified that osmotic effects were not causing aggregation by the external addition of 0.2 M glucose or galactose. Neither sugar triggered vesicle precipitation (not shown).

Effect of different salts on aggregation

We have examined the efficacy of different salts in causing DGDG vesicle aggregation. Quasi-elastic light scattering has been shown [14] to give very accurate size determinations for vesicles in the 30 to 200 nm range when compared to measurements by freeze-fracture electron mi-

TABLE III

X-RAY DIFFRACTION MEASUREMENT OF THE LAMELLAR SPACING OF DGDG IN WATER, 100 mM KCl, OR 5 mM EDTA

DGDG was dispersed at 50% (w/w) in either water, 100 mM KCl, or 5 mM EDTA and X-ray powder patterns recorded from 0 to 50 °C. Data represent the lamellar lattice repeat distance and are accurate ± 0.05 nm.

Temperature (°C)	Lamellar spacing (nm)		
	Water	100 mM KCl	5 mM EDTA
0	5.17	5.21	5.22
10	5.20	5.19	5.21
20	5.15	5.17	5.19
30	5.14	5.12	5.15
40	5.13	5.13	5.12
50	5.10	5.07	5.12

croscopy. However, the large, polydisperse, and unstable nature of the aggregates made unambiguous size determination by QELS difficult. Instead, turbidity (A_{600}) was used as a measure of the degree of aggregation by light scattering.

The variation of A_{600} with vesicles of defined size is shown in Fig. 2. Absorbance was seen to vary sigmoidally, rather than linearly, with increasing particle size. The increase of absorbance was nearly linear for vesicles with diameters between 50 and 200 nm, but leveled off with vesicles above 200 nm diameter. Since our starting poin was 100 nm vesicles, this indicates that turbidit can be used as a sensitive measure of the stabl aggregation of the first few vesicles.

Turbidity changes during the titration o DGDG vesicles with various chloride salts ar shown in Fig. 3a for Ca^{2+} and Mg^{2+} and in Fig 3b for the monovalent salts. For most salts th turbidity increased sharply with a narrow con centration range of 2–3 mM for the divalent salt and 5–20 mM for the monovalent salts. Aggrega tion was strongly dependent on the ionic specie within a valence group. Plotting of the ion con centration required for 50% of total A_{600} increas (ΔA_{50}) against hydrated ionic radii (Fig. 4) show that cation efficacy was strongly correlated t

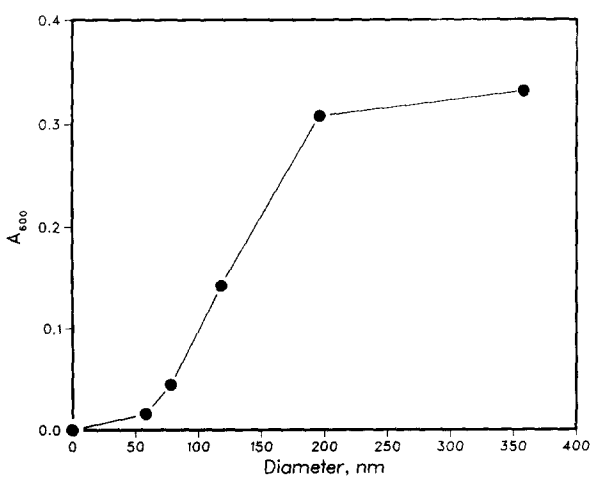

Fig. 2. Variation of the absorbance at 600 nm with mean diameters of DGDG vesicles extruded to various sizes in water. Values are representative data from two experiments.

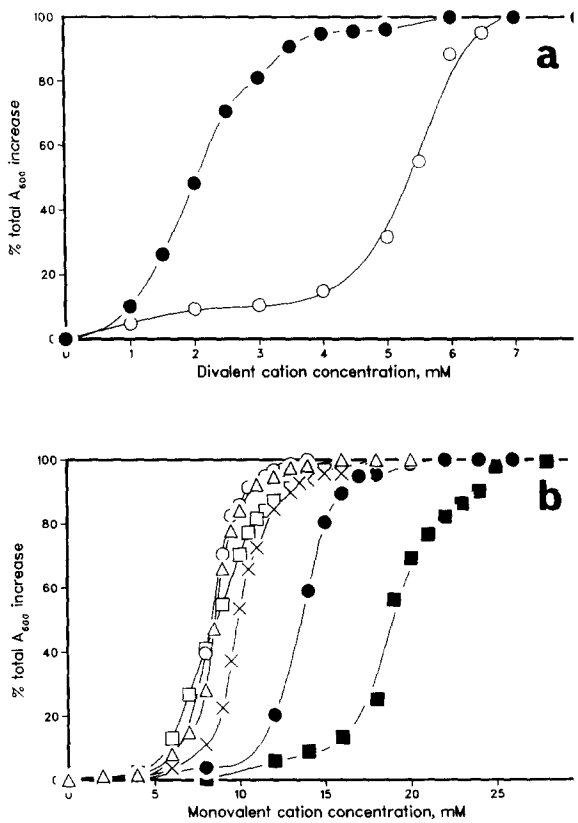

Fig. 3. Variation of A_{600} (light scattering) during the sequenti addition of divalent (3a) or monovalent (3b) chloride salts t 100 nm DGDG vesicles dispersed in water. In Fig. 3a th Ca^{2+} (●) and Mg^{2+} (○) salts are shown. In Fig. 3b the sal are Cs^+ (△), Rb^+ (×), NH_4^+ (○), K^+ (□), Na^+ (●), and Li (■). Data show the means of three or four replicates from representative experiment.

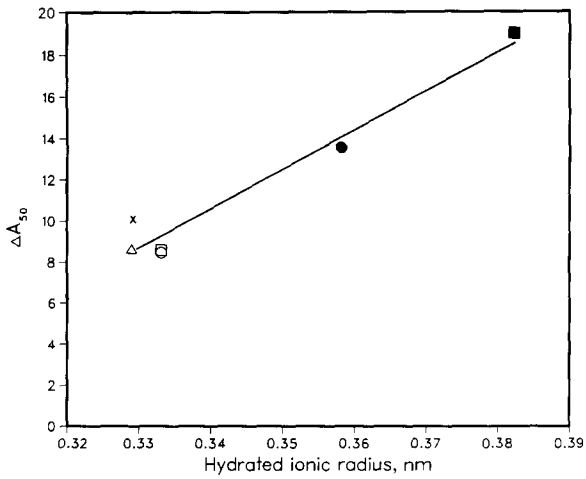

Fig. 4. Relationship between ion concentration required for 50% of maximal A_{600} increase (ΔA_{50}) and hydrated ionic radii of various monovalent chloride salts. The ΔA_{50} values were interpolated from Fig. 3b, the radii data from Ref. 41. Symbols are the same as given in Fig. 3b.

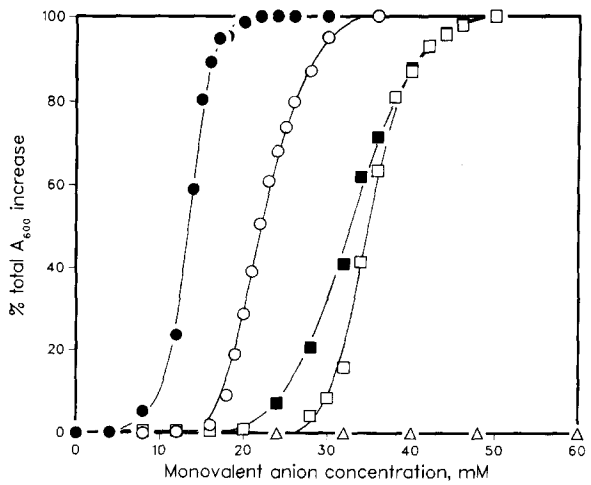

Fig. 5. Variation of A_{600} during sequential addition of monovalent sodium salts Cl^- (●, data from Fig. 3b for comparison), NO_3^- (○), Br^- (□), ClO_4^- (■), and SCN^- (△) to 100 nm DGDG vesicles dispersed in water. Data represent means of three or four replicates from a representative experiment.

ionic radius. The most effective cations were those with small hydrated radii or large crystal radii (Cs^+ and Rb^+), while the least effective were those with large hydrated radii or small crystal radii (Na^+ and Li^+). This relationship holds for Ca^{2+} and Mg^{2+} chloride salts (not shown). It should be added that the standard deviations of the turbidity readings, omitted for clarity, were usually 10–15%, yielding standard deviations of ΔA_{50} of about 1 mM.

The possibility that anions could be involved in aggregation was also examined. Turbidity changes during the titration of DGDG vesicles with various sodium salts are shown in Fig. 5. Of those examined, Cl^- was the strongest aggregation-promoting anion. Replacement of Cl^- with NO_3^-, Br^-, ClO_4^-, or SCN^- led to higher salt concentrations being required for aggregation. Aggregation was never observed with the SCN^- ion up to 100 mM under the conditions described here. An analysis similar to that in Fig. 4 for the anions was not possible due to the lack of available ionic radii for polyatomic anions.

During the preparation of samples for freeze-fracture electron microscopy it was noticed that the addition of glycerol as a cryoprotectant caused a clearing of the normally opaque nature of aggregated vesicles. The possibility that glycerol might inhibit or reduce aggregation was, therefore, examined. The addition of glycerol resulted in disruption of $MgCl_2$-induced aggregates (Fig. 6). A similar effect was observed with 100 mM KCl-induced aggregates (not shown). Addition of iden-

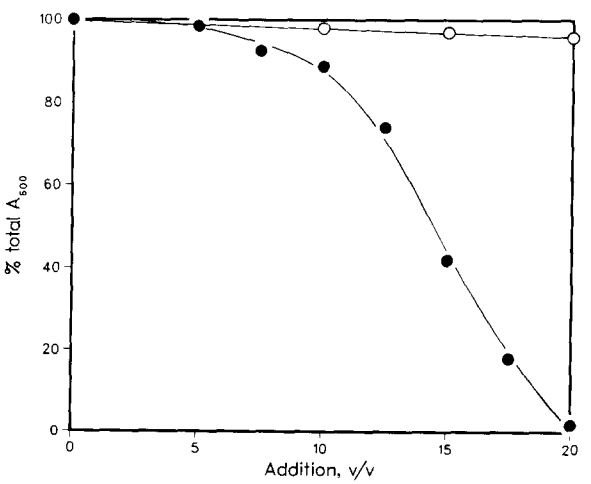

Fig. 6. Variation of A_{600} (light scattering) during the sequential addition of water (○) or glycerol (●) to 100 nm DGDG vesicles at 1 mg·ml^{-1} in water aggregated by the addition externally of 10 mM $MgCl_2$. Data show the means of three or four replicates from a representative experiment.

tical volumes of water showed that observed A_{600} decreases in glycerol were not due to dilution of scattering particles. This effect of glycerol explains the apparent discrepancy between QELS determination of aggregate diameters as > 1000 nm for DGDG in KCl or $MgCl_2$ (Table I) and the freeze-fracture micrographs indicating aggregate diameters in the 200–500 nm diameter range (Figs. 1b and 1c) in the presence of 20–25% glycerol.

The effect of changing the bulk solution pH on $MgCl_2$ concentrations required for vesicle aggregation is shown in Fig. 7. There was no aggregation in the absence of Mg^{2+} at any pH. Decreasing the pH from 7.5 to 5.0 decreased the ΔA_{50} for $MgCl_2$ from 6 mM to 2 mM. Further pH decrease to 3.5 correlated with a decrease of ΔA_{50} to 1 mM. It is clear that $MgCl_2$-induced aggregation occurred in DGDG vesicles over the pH range experienced by thylakoids in vivo. The small decrease of ΔA_{50} between pH 5.0 and pH 3.5, a range that brackets the pK_a of thylakoid membranes [21], suggests that charge neutralization of surface ionizable groups in this pK_a range was not responsible for aggregation.

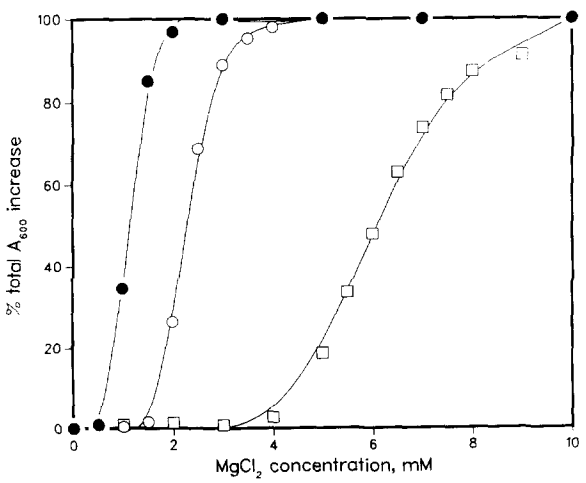

Fig. 7. Effect of pH on the concentration of $MgCl_2$ required to aggregate DGDG vesicles. Lipid was dispersed at 10 mg·ml^{-1} in water then diluted with succinate-HCl (pH 3.5) (●), succinate-HCl (pH 5.0) (○), or Tricine (pH 7.5) (□) to final buffer concentrations of 1 mM and lipid concentrations of 1 mg·ml^{-1}. Data show means of three or four replicates from a representative experiment.

Discussion

We have presented evidence showing that large unilamellar vesicles of the neutrally charged thylakoid galactolipid DGDG aggregate in the presence of a variety of salts. Since this has not been reported previously to our knowledge and the result was somewhat surprising, we were careful to eliminate charged impurities as the causal agent in aggregation. Several lines of evidence support this conclusion. Firstly, no phospholipid contaminants could be detected in the DGDG preparation at greater than 0.25 mol% by phospholipid assay. No other compounds could be detected at about 2 mol% by ^{13}C-NMR and thin layer chromatography (Webb et al., in preparation). Secondly, the addition of plausible contaminants to egg PC bilayers did not trigger aggregation (Table II), nor did pure SQDG vesicles aggregate under conditions used here. Thirdly, the fact that vesicle aggregation at pH 3.5 still required the addition of salts (Fig. 7) suggests that charge neutralization or charge screening by protons was not a prerequisite for aggregation. Charged contaminants with pK_a values above 3.5, once neutralized by protons, would be expected to aggregate without salt addition in this pH range. Fourthly, electrostatic double layer theory holds that charge screening is valence-dependent, but ionic species-independent within a valence group [21,22]. We have shown strong dependence on the ion species within both monovalent and divalent cation groups (Fig. 4) and the monovalent anions (Fig. 5). Furthermore, the efficacies of the monovalent cations shown here does not follow that known for the association of these ions with charged phospholipid membranes [23]. Thus, these vesicles were not behaving as if a charged impurity was being screened as predicted by electrostatic double layer theory. Fifthly, and in a related vein, the activity of both anions (Fig. 5) and cations argues against electrostatic effects or specific ion adsorption to the bilayer surface [24]. Finally, the lack of a change in the lamellar repeat distance of DGDG between water, 100 mM KCl, or 5 mM EDTA strongly suggests that aggregation was not due to a charged contaminant (Table III). If a charged contaminant had been present in these bilayers, then screening of the charged groups by

the addition of either KCl or EDTA would be expected to reduce the magnitude of electrostatic repulsion and allow closer bilayer approach under the influence of attractive Van der Waals forces. Such a change would have been observed as a decrease in the lamellar repeat distance in KCl or EDTA [25], but was not seen in this system (Table III).

Given the above considerations, the mechanism involved in salt-induced DGDG vesicle aggregation is not clear. The strong correlation between cation efficacy and effective ionic radius (Fig. 4) suggests that a water interaction may be involved. The observed sequence:

$$Cs^+ \cong Rb^+ \cong NH_4^+ \cong K^+ > Na^+ > Li^+$$

correlates well with the ability of these cations to break the structure of water as indicated by infrared spectroscopy [26] and arguments based on the extent of hydration of the ions [27]. For the cations, the structure-breaking ions were also the most effective at promoting DGDG vesicle aggregation. On the other hand, while the efficacy of anions at promoting aggregation agrees well with the lyotropic series for the salting-out of proteins [28]:

$$Cl^- > NO_3^- > Br^- \cong ClO_4^- > SCN^-$$

the orientation is opposite to the cations, with the structured-breaking anions (SCN^-, ClO_4^-) being the least effective at causing bilayer aggregation (Fig. 5). Similar sequences have been observed for other hydrophobic colloidal systems [29] in which oxygen atoms are an important component. Eagland [29] has suggested that anions probably interact preferentially with the hydration of hydrophilic groups while the cations will interact more strongly with the hydration of hydrophobic methylene groups. As a result of these tendencies, the effect of structure-breaking anions was expected to be the reverse of structure-breaking cations, as observed in our data. Furthermore, these interactions were expected to be additive [29].

Interactions between approaching membranes in aqueous solutions are dominated by attractive Van der Waals forces, repulsive electrostatic forces, and repulsive hydrostatic forces [30]. Given the lack of electrostatic repulsive forces in uncharged DGDG bilayers, the major repulsive force preventing DGDG aggregation is the hydration force. If DGDG were to show a specific interaction with structured water, and that interaction could be disrupted by dissolved ions, then it is possible that the steeply rising hydrostatic repulsive force observed between phospholipid bilayers [24] could be reduced between approaching DGDG bilayers. Supporting evidence for such a view has been obtained by Johnston et al. [31] who observed glycolipid–ion interactions in cerebroside monolayers and concluded that ion-induced changes in water structure could explain observed effects of salts on gluco- and galacto-cerebroside monolayer expansion. Wieslander et al. [32] have reported effects of $CaCl_2$ and $MgCl_2$ on the degree of hydration of *Acholeplasma laidlawii* diglucosyldiacylgycerol as detected by ^{2}H-NMR. Tomoaia-Cotisel et al. [33] have observed effects of salts on the monolayer properties of distearoyl derivatives of MGDG and DGDG. These authors interpreted their data as indicating that hydrated ions were penetrating into the region between adjacent headgroups and causing monolayer expansion. Since these investigators reported no difference between the efficacies of Na^+ and Mg^{2+} and observed that Cl^- was the least effective of the anions, it is likely that they were investigating a different phenomena from that reported here. A direct analogy between these results and ours is, or course, difficult because of the differences in structures of the glycolipids. Clearly, however, glycolipid-ion interactions are extensive.

On the other hand, an effect of DGDG on attractive forces between bilayers is also possible. It is known that the magnitude of Van der Waals forces acting between bilayers, as reflected by the Hamaker coefficient, may vary as much as 10-fold depending on the lipid composition of the interacting bilayers [24]. Direct measurements of the forces acting between approaching DGDG bilayers in water [34,35] has shown the Hamaker coefficient for DGDG to be 6-fold higher than that obtained for dipalmitoylphosphatidylcholine. At present, we are unable to explicitly describe a mechanism for salt-induced DGDG aggregation. The possibility exists that the DGDG head group has a weak electrostatic charge or dipole moment that is being screened by salt addition. However,

the effect of glycerol (Fig. 6) on aggregation and the correlation between ion efficacy in breaking water structure and in causing vesicle aggregation strongly implies head group–water interactions as underlying the aggregation process.

Interacting particles may aggregate in either a primary energy minimum with a small interparticle separation, or in a secondary energy minimum existing at larger interparticle distances [30]. While most aggregation processes are considered to be due to interaction in the primary minimum, aggregation of phosphatidylserine vesicles in the secondary energy minimum has been reported [36]. Marra [34] has identified an energy minimum between approaching DGDG bilayers in water at 1.3 nm separation; however this was not identified as a primary or secondary minimum nor was the effect of salts on the position and depth of the minimum examined. Although appressed DGDG vesicles appear to be in very close contact (Figs. 1b and 1c), the limits of resolution of freeze-fracture electron microscopy do not allow us to measure the intervesicle separation in these aggregates. These structures may represent loose aggregates of vesicles condensed in a secondary energy minimum.

We have performed calculations that indicate that the lipid concentration of the chloroplast stroma is in the 2–4 $mg \cdot ml^{-1}$ range. These calculations were based on: Barber's [21] estimate of 200 m^2 of thylakoid surface area per m^2 of leaf surface area; an area per DGDG molecule of 0.7 nm^2; assuming approx. 50% of exposed thylakoid surface area is due to lipid; and average leaf thickness of about 1 mm; approx. 50% of total leaf volume being occupied by photosynthetically active cells, and 10% of mesophyll cell volume is occupied by chloroplasts. While it is obvious that many of these values are both approximate and species-dependent, the calculations nonetheless indicate that the lipid concentrations used in this study are close to those observed in the chloroplast. Similarly, the ion concentrations used here are in line with the concentrations found in isolated chloroplasts [37–39] and those used for reversible experimental unstacking and restacking of isolated thylakoids [7]. It appears, then, that DGDG vesicles reversibly aggregate in lipid and salt concentrations relevant to those found in vivo.

This type of aggregation has not been specifically reported in the plant lipid literature. This may, in part, be due to the disruptive effect of glycerol on aggregation (Fig. 6). The routine addition of glycerol to vesicle suspensions as a cryoprotectant would prevent such structures from being visible by freeze-fracture electron microscopy. We have found that binary and trinary mixtures of plant lipids that are shown to be aggregated, by QELS, appear as discrete vesicles by freeze-fracture due to the effects of glycerol (Webb et al., unpublished data).

The effect of salts on the interactions of vesicles made from total chloroplast lipids has been examined by Gounaris et al. [40]. These authors observed increased turbidity and vesicle size upon incubation of total lipid dispersions in salts. These authors interpreted their results as indicating the screening of the surface ionizable groups of PG and SQDG by cations and protons in agreement with classic electrostatic double layer theory. However, Gounaris et al. [40] obtained ion-specific differences in effectiveness of Ca^{2+} and Mg^{2+} similar to those reported here, but not expected from electrostatic double layer theory. Since we did not observe the biphasic nor pH effects seen by those authors in our purified DGDG vesicles, it seems likely that the results of Gounaris et al. [40] were a consequence of using a mixture of thylakoid lipids showing different tendencies to aggregate as specific ion concentrations. This would include the pH-dependent but ionic species-independent aggregation due to screening of the anionic lipids PG and SQDG [40], as well as the pH-independent (Fig. 7) but ionic-species dependent (Figs. 3–5) aggregation reported here.

In summary, we have presented evidence indicating that vesicles of the thylakoid galactolipid digalactosyldiacylglycerol aggregate strongly in the presence of physiologically relevant levels of aqueous salt solutions. Further, our data suggest that the mechanism by which vesicle aggregation occurs is probably related to the degree of hydration of the bilayer surface. Work is currently underway to determine if this interaction plays a significant role in granal stacking in higher plant chloroplasts, and the effect of other thylakoid components on DGDG aggregation.

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