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Effects of neutral and anionic lipids on digalactosyldiacylglycerol vesicle aggregation

Murray S. Webb * and Beverley R. Green

Department of Botany, University of British Columbia, Vancouver (Canada)

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We have previously reported that large unilamellar liposomes made from the neutral galactolipid digalactosyldiacylglycerol (DGDG) will aggregate in the presence of monovalent or divalent cations, behavior that would not have been predicted for an uncharged lipid (Webb et al. (1988) Biochim. Biophys. Acta 938, 323–333). In this paper, the effects of including the other major thylakoid lipids on the Mg^{2+} concentration required for aggregation of DGDG vesicles has been examined. Addition of the neutral, hexagonal-II phase preferring lipid monogalactosyldiacylglycerol (MGDG) to DGDG up to 50 mol% had no effect, suggesting that the MGDG head group is as effective in causing aggregation as the DGDG head group. Addition of 0.5 to 5.0 mol% of either of the two anionic lipids phosphatidylglycerol (PG) or sulfoquinovosyldiacylglycerol (SQDG) inhibited the aggregation of DGDG vesicles, probably by the development of an electrostatic potential. Phosphatidylcholine (PC) in amounts up to 25 mol% did not inhibit or promote aggregation. Vesicles with a composition similar to that of thylakoids (DGDG/MGDG/SQDG/PG, 1:2:0.5:0.5) required 65 mM $MgCl_2$ in the presence of 200 mM KCl, i.e., higher concentrations than are present in the chloroplast stroma. If MGDG made up more than 25 mol% of any combination of lipids, vesicle aggregation could not be reversed by dilution. These results are consistent with cations playing a role in mediating the close approach of bilayers via an effect on head-group hydration and head-group interaction between bilayers.

Introduction

The lipid compositon of the higher plant thylakoid membrane is dominated by the uncharged galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) [1]. These lipids account for approx. 75% of the total thylakoid acyl lipid. The remainder of the lipid component is comprised of the anionic lipids phosphatidylglycerol (PG) and sulfoquinovosyldiacylglycerol (SQDG). It is now well-established that aqueous dispersal of DGDG, SQDG, and PG yields the lamellar liquid-crystalline phase while dispersal of MGDG gives the hexagonal-II phase (H_{II}) [2]. In spite of the fact that MGDG comprises up to 50% of the total lipid, the thylakoid membrane shows no evidence of non-bilayer structure in vivo, and much of the membrane area is involved in close membrane appresion (granal stacking). This behavior is thought to be mediated by interactions between the chlorophyll *a/b* light-harvesting complexes (LHC II) in adjacent bilayers [3].

In a previous paper [4], the reversible aggregation of vesicles composed solely of pure DGDG was demonstrated. The aggregation of uniform size populations of this neutral lipid could be triggered by the addition of various salts, e.g., 100 mM KCl or 10 mM $MgCl_2$. Anions and cations within the same valency group showed different efficacies at causing aggregation, and glycerol inhibited aggregation. These observations suggested that ions were exerting their effect by affecting the structure of water. In vivo, however, DGDG is only 20–30 mol% of the total thylakoid lipid [5]. Therefore, the effect of the other thylakoid lipids MGDG, SQDG, and PC, on the ability of DGDG vesicles to aggregate in the presence of salts was examined. Inhibition of bilayer close approach by the addition of either of the anionic lipids PG or SQDG to DGDG would be predicted due to electrostatic repulsion. On the other hand, if DGDG vesicle aggregation is due to head group-water interactions [4], then the MGDG head group may also

* Present address: Department of Agronomy, Cornell University, Ithaca, NY, U.S.A.

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

facilitate bilayer appression by a mechanism similar to that of DGDG.

Materials and Methods

Materials

Materials used were obtained as follows: KCl and $MgCl_2$ from Amachem; trypsin (bovine pancreas, EC 3.4.21.4, Type Xl) and egg PG (sodium salt, derived from egg PC) were obtained from Sigma and used without further purification. DGDG enriched in 18 : 2 was purchased from Serdary Research Laboratories (London, Ontario), and was purified as described for DGDG [4]. The purification of SQDG, MGDG, and PG has been described [4,6].

Vesicle reconstitution

Pure lipids or lipid mixtures were dried and dispersed into H_2O, salt, or buffer solutions at 10 mg lipid · ml^{-1} by a conventional vortexing/sonication method as described previously [4]. For most experiments vesicle dispersions were then extruded by ten passes through two 0.1 μm Nuclepore filters at 2000 kPa N_2 (The Extruder, Lipex Biomembranes Inc., Vancouver, Canada).

Measurement of vesicle sizes and ΔA_{50} values

Vesicles dispersed as outlined above were diluted to 1 mg lipid · ml^{-1} and sizes estimated by Quasi-Elastic Light Scattering (QELS) on a Nicomp 270 Submicron Particle Sizer. ΔA_{50} values for $MgCl_2$, the concentration of $MgCl_2$ giving 1/2 of maximal vesicle aggregation, were measured by turbidity at 600 nm as described in Webb et al. [4]. Reversibility of aggregation was measured by diluting the aggregated vesicles to 0.1 or 0.2 mg lipid · ml^{-1} and a final $MgCl_2$ concentration well below the ΔA_{50} value determined for that sample, then remeasuring by QELS.

Lipid analysis

In vesicles composed of mixtures of lipids the preparations were checked for complete incorporation of all of the added lipids as described previously [7]. Briefly, vesicles were re-extracted into $CHCl_3/CH_3OH$ (1 : 1, v/v) at the end of the experiment, then aliquots were spotted onto analytical silica gel plates (0.25 mm × 2.5 cm × 7.5 cm, Merck) and developed in acetone/benzene/water (91 : 30 : 8, v/v/v) [8]. Plates were sprayed with the phospholipid reagent of Allen and Good [1] and charred with a heat gun. After allowing the background blue color to fade, the plates were photographed on Polaroid P/N 665 film and the negatives scanned by densitometry (Helena Quick Scan). The recovery of each lipid in the dispersion was estimated by weighing the areas under the lipid peaks.

TABLE I

Recovery of lipids from vesicles

Vesicles were re-extracted into $CHCl_3/CH_3OH$ (1 : 1, v/v) after aggregation experiments. Aliquots were separated on silica TLC in acetone/benzene/water (91 : 30 : 8, v/v/v) and charred with the spray reagent of Allen and Good [1]. Plates were photographed then negatives scanned on a Helena Quick Scan gel scanner and recoveries estimated by weighing the areas under the lipid peaks. Data are the means (plus or minus standard deviation) of at least three separate preparations.

Mixture dispersed	% of recovered lipid				
	DGDG	MGDG	SQDG	PG	PC
DGDG	100	–	–	–	–
DGDG/PC	69.5	–	–	–	30.4
(3 : 1)	(6.9)				(6.7)
DGDG/PC	32.2	–	–	–	67.7
(1 : 3)	(6.4)				(6.4)
DGDG/MGDG/PC	29.5	46.0	–	–	24.5
(1 : 2 : 1)	(1.4)	(6.2)			(4.8)
DGDG/MGDG/SQDG	33.2	46.1	20.7	–	–
(1 : 2 : 1)	(6.5)	(4.4)	(2.0)		
DGDG/MGDG/PG	33.4	45.0	–	22.9	–
(1 : 2 : 1)	(3.3)	(0.1)		(1.3)	
DGDG/MGDG/SQDG/PG	30.9	44.0	13.0	12.0	–
(1 : 2 : 0.5 : 0.5)	(1.8)	(2.7)	(3.5)	(1.0)	

Fatty acid profiles were determined by GLC of fatty acid methyl esters as in Webb and Green [7].

Results

Fatty acid and lipid compositions

The fatty acid compositions for the purified thylakoid lipids have been published previously [7]. Both MGDG and DGDG were enriched in the polyunsaturated fatty acids 16 : 3 and 18 : 3. SQDG also contained a large proportion (50 mol%) of 18 : 3 as well as significant levels of 16 : 0 (43 mol%). PG purified from spinach leaves was rich in 16 : 0 and 18 : 3 but also contained high levels of the unique fatty acid *trans*-3-hexadecanoic acid. The fatty acid composition of commercial DGDG was very similar to that reported for DGDG purified from wheat flour [9], being highly enriched in 18 : 2 (73%) and depleted in 18 : 3.

The lipid composition of vesicles re-extracted into $CHCl_3/CH_3OH$ (1 : 1, v/v) after aggregation experiments is given in Table I. This data shows that the final lipid compositon of the vesicles was very similar to that of the dispersed lipid mixture. This result was obtained even when high levels (50 mol%) of MGDG were dispersed into the vesicles. Similar results with thylakoid lipid dispersions have been reported previously [7]. This data is in contrast to that of [10,11] who reported that MGDG could not be quantitatively transferred into the aqueous phase by conventional hydration methods. The reason for this difference is not clear.

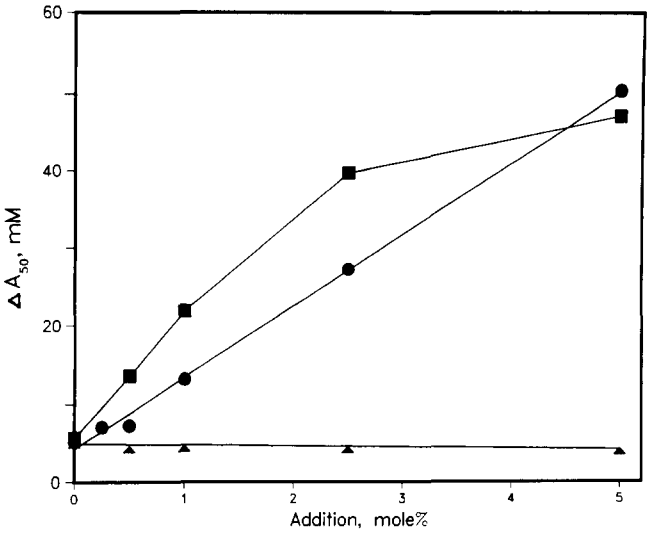

Fig. 1. Effect of the addition of 0–5 mol% egg PG (■), SQDG (●), or MGDG (▲) to DGDG on the ΔA_{50} values (mM $MgCl_2$) required for vesicle aggregation. Data are means of three determinations from a representative experiment. Standard deviations are about 1 mM.

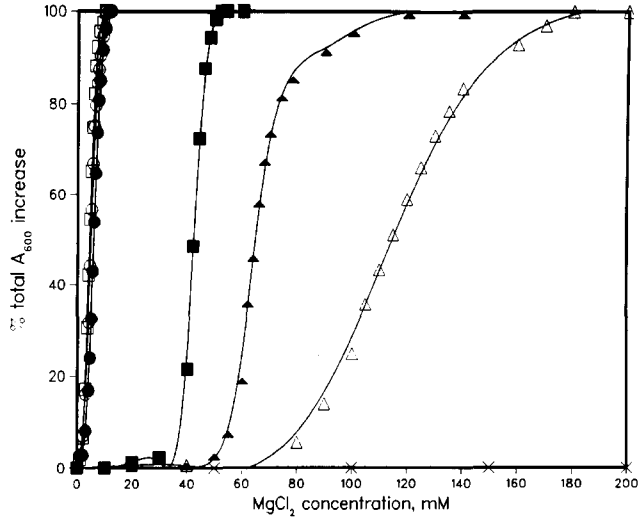

Fig. 2. Plot of turbidity (% total A_{600} increase) against added $MgCl_2$ for various lipid mixtures. Vesicles of DGDG (□); DGDG/PC, 3:1 (●); DGDG/PC, 1:3(×); DGDG/MGDG/PC, 1:2:1 (○); DGDG/MGDG/PG, 1:2:1, (■); DGDG/MGDG/SQDG, 1:2:1 (△); and DGDG/MGDG/SQDG/PG, 1:2:0.5:0.5 (▲) are shown. Data are the means of three determinations from a representative experiment.

Effects of other lipids on DGDG aggregation

In our previous work with vesicles of pure DGDG we measured the increase in light scattering at 600 nm as a function of salt concentration [4]. The salt concentration giving half maximal increase in absorbance was denoted ΔA_{50}. In this work the impact of other lipids on the extent of salt-induced DGDG vesicle aggregation in $MgCl_2$, was examined. The effect of the addition of small amounts of egg PG, SQDG, or MGDG to DGDG vesicles is shown in Fig. 1. Addition of either anionic lipid significantly increased the ΔA_{50} value required for aggregation. The presence of 1 mol% egg PG or SQDG raised the ΔA_{50} from 5.5 mM for pure DGDG to 12 and 20 mM, respectively. Further lipid addition to 5 mol% increased the ΔA_{50} value linearly for SQDG and in an asymptotic curve for PG (Fig. 1 and see below). In contrast to the anionic lipids, the addition of MGDG in the 0.5–5.0 mol% range had no effect on DGDG aggregation (Fig. 1). Further addition of MGDG up to 50 mol% also had no significant effect on the ΔA_{50} values for the binary mixtures (data not shown).

TABLE II

Size and aggregation characteristics of lipid mixtures

Lipid mixtures were dispersed in H_2O and extruded to make 100 nm unilamellar vesicles. Diameters were measured by QELS at 1 mg lipid·ml^{-1} in H_2O. Vesicles were aggregated by the sequential addition of $MgCl_2$ to determine the ΔA_{50} value (mM $MgCl_2$) and sizes measured again by QELS. Then vesicles were diluted to 0.1 or 0.2 mg lipid·ml^{-1} and to the final $MgCl_2$ concentration (mM) shown and sizes measured again by QELS. Data represents mean (plus or minus standard deviation) from representative experiments of 2–4 separate trials.

Lipid mixture	Size in H_2O (nm)	Aggregated vesicles		Diluted vesicles	
		size (nm)	ΔA_{50} (mM)	size (nm)	$MgCl_2$ (mM)
DGDG	124 (46)	>1000	5.4	143 (37)	1.2
DGDG/PC (3:1)	121 (22)	>1000	4.0	126 (29)	1.2
DGDG/PC (1:3)	115 (30)	133 (46)	>200	147 (60)	40
DGDG/MGDG/PC (1:2:1)	121(22)	>1000	4.1	>1000	1.2
DGDG/MGDG/SQDG (1:2:1)	137 (53)	>1000	115	>1000	20
DGDG/MGDG/PG (1:2:1)	134 (45)	>1000	42	>1000	12
DGDG/MGDG/SQDG/PG (1:2:0.5:0.5)	132 (45)	>1000	65	>1000	28

Addition of the neutral dipolar lipid PC to DGDG in amounts up to 25 mol% of total lipid (Fig. 2) did not prevent salt-induced aggregation. However, when the ratio of DGDG to PC was reversed (DGDG : PC, 1 : 3), the vesicles did not aggregate even in 200 mM $MgCl_2$. The effect of MGDG on aggregation in ternary and quaternary mixtures was then investigated (Fig. 2 and Table II) by substituting it for DGDG in vesicles with other lipids. The DGDG/MGDG/PC (1 : 2 : 1) mixture resulted in vesicles with a ΔA_{50} of 4.1 mM. This value was identical to that obtained from DGDG/PC (3 : 1) vesicles, both samples composed of 75 mol% galactolipid. This result suggests that MGDG and DGDG are equally active at promoting vesicle aggregation in the presence of salts. If MGDG behaved like PC in vesicle aggregation, then the DGDG/MGDG/PC (1 : 2 : 1) vesicles should not aggregate even at very high $MgCl_2$ levels, like those made from the DGDG/PC (1 : 3) mixture.

These results also suggest that the neutral PC head group has no inhibitory effect on galactolipid vesicle aggregation, provided that it is not the dominant lipid species. In contrast, the replacement of PC in the DGDG/MGDG/PC (1 : 2 : 1) mixtures with either spinach PG (DGDG/MGDG/PG, 1 : 2 : 1) or SQDG (DGDG/MGDG/SQDG, 1 : 2 : 1) increased the ΔA_{50} values for these mixtures to 42 and 115 mM, respectively (Table II and Fig. 2). A mixture with equal proportions of PG and SQDG (DGDG/MGDG/SQDG/PG, 1 : 2 : 0.5 : 0.5) yielded an intermediate ΔA_{50} of 65 mM $MgCl_2$. Therefore, both PG and SQDG actively inhibit vesicle close approach, probably by electrostatic repulsion. These data also show different degrees of inhibition of aggregation for the two anionic lipids; the SQDG mixture required higher $MgCl_2$ concentrations for complete charge screening than did the PG mixture. This is shown in Fig. 1 in which the 5 mol% PG mixture peaked at a ΔA_{50} value of 43 mM while the 5 mol% SQDG value was 51 mM and continued to increase up to 115 mM at 25 mol% (Fig. 2). These results strongly suggest that the negatively charged groups of these two lipids are interacting differently with ions in the aqueous phase.

Effect of cation mixtures on ΔA_{50} values

An attempt was made to determine the effect of cation mixtures on galactolipid vesicle aggregation. Recently, Schroppel-Meier and Kaiser [12] measured K^+ and Mg^{2+} concentrations in spinach chloroplasts at 180 and 18 mM respectively. DGDG/MGDG/SQDG/PG (1 : 2 : 0.5 : 0.5) vesicles were dispersed in 0, 100, and 200 mM KCl and the ΔA_{50} values for $MgCl_2$ measured (Table III). These data show an 8 mM decrease in $MgCl_2$ required for aggregation with each 100 mM increase in KCl concentration. This is strong evidence that electrostatic repulsion is the primary force preventing close approach in these vesicles. The 10 : 1 efficacy ratio for divalent : monovalent cations in screening surface charges [13], arising from the ion valency raised to exponential terms in the Boltzmann distribution, is similar to the 100 : 8 ratio reported here. Slight differences in these ratios probably originate in some degree of ion binding to the bilayer surface.

TABLE III

Effect of KCl on vesicle aggregation in $MgCl_2$

Mixtures of DGDG/MGDG/SQDG/PG (1 : 2 : 0.5 : 0.5) were dispersed in H_2O, 100, or 200 mM KCl and extruded to make 100 nm unilamellar vesicles. Diameters were measured by QELS at 1 mg lipid·ml^{-1} in H_2O. Vesicles were aggregated by the sequential addition of $MgCl_2$ to determine the ΔA_{50} value (mM $MgCl_2$) and sizes measured again by QELS. Finally, vesicles were diluted to 0.1 or 0.2 mg lipid·ml^{-1} and to the final $MgCl_2$ concentration (mM) shown and sizes measured again by QELS. Data represents mean plus or minus standard deviation from representative experiments of 2–4 separate trials.

KCl (mM)	Size in KCl (nm)	Aggregated vesicles		Diluted vesicles	
		size (nm)	ΔA_{50} (mM)	size (nm)	$MgCl_2$ (mM)
0	132 (45)	>1000	65	>1000	28
100	114 (27)	>1000	57	>1000	20
200	113 (22)	>1000	49	>1000	20

Effect of head group and fatty acid modification

In view of the above effects, it was of interest to determine if specific aspects of galactolipid structure are required for vesicle aggregation. Commercially available DGDG had a fatty acid composition highly enriched in 18 : 2 and depleted in 18 : 3 [6], typical of wheat flour DGDG [9]. The turbidity of 18 : 2-enriched DGDG vesicles during the addition of MgCl was compared to that of spinach-DGDG and a slightly increased ΔA_{50} of 8.6 mM was found, compared to 5.5 mM for spinach DGDG vesicles (data not shown). Given the extreme sensitivity of the ΔA_{50} to low levels of charged contaminants (Fig. 1) it was not possible to attribute this difference specifically to the change of fatty acid unsaturation. Interpolation of Fig. 1 indicates that charged contaminants in the 0.25–0.5 mol% range would be sufficient to raise the ΔA_{50} to about 9 mM $MgCl_2$.

Modification of DGDG vesicle aggregation by trypsin digestion was attempted because of the extensive use of this enzyme to investigate the role of proteins in mediating granal stacking. In addition, trypsin possesses some activity against non-protein substrates, including fatty acid esters [14]. However, no effect of extensive trypsin digestion on the ΔA_{50} of DGDG vesicles was noted, nor were lipid degradation products visible by TLC after the experiment (data not shown).

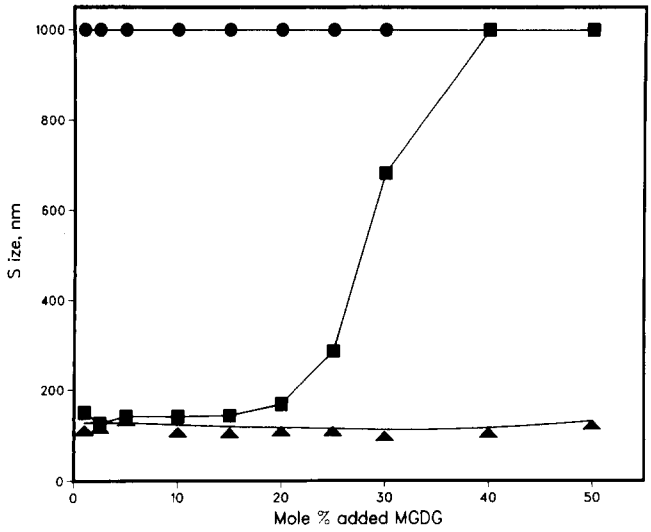

Fig. 3. Effect of MGDG on the reversibility of DGDG vesicle aggregation. Plot of vesicle or aggregate size (measured by QELS) for vesicles dispersed in H_2O (▲), after aggregation in $MgCl_2$ (●), and of re-diluted vesicles (■). Representative data from three experiments.

Reversibility of vesicle aggregation

The addition of small amounts (< 5 mol%) of the lipids PG, SQDG, and MGDG to DGDG vesicles (Fig. 1) had no effect on the reversibility of vesicle aggregation (data not shown) during subsequent dilution of $MgCl_2$. In all cases, vesicles released from the aggregates during dilution regained their original mean diameters of about 120 nm. However, in vesicle systems containing 50 mol% MGDG, aggregation was not reversible despite the dilution of $MgCl_2$ to levels well below the ΔA_{50} value (Tables II and III). Irreversibility was observed even in the presence of 25 mol% anionic lipids PG and SQDG. The aggregated vesicles were > 1000 nm in diameter, as measured by QELS, even after dilution (Tables II and III). These observations are consistent with the proposed role of inverted micellar structures, adopted by H_{II} phase lipids such as MGDG, as intermediates in bilayer fusion [15,16].

In order to determine how much H_{II} phase lipid is needed to make aggregation irreversible, various binary mixtures of DGDG and MGDG were dispersed into H_2O, aggregated by $MgCl_2$ addition, then diluted with H_2O (Fig. 3). Addition of MGDG to DGDG up to 50 mol% had no effect on the size of extruded vesicles in H_2O, nor on the minimum size of aggregated vesicles (Fig. 3) as measured by QELS. Irreversible galactolipid vesicle aggregation was only observed in vesicles containing more than 25 mol% MGDG. A significant increase in the size of released vesicles occurred at 25–30 mol%; below this value the original 120 nm vesicles were re-released from the aggregates. Formation of very large vesicles, > 1000 nm in diameter, required 40 mol% MGDG in DGDG.

Discussion

The experiments described in this paper were undertaken to gain some insight into the salt-induced aggregation of pure DGDG vesicles [4] and to determine if this phenomenon occurs in physiologically relevant lipid and salt mixtures. The replacement of DGDG with MGDG made no difference to the level of Mg^{2+} required for aggregation, i.e. the two galactolipids appear to interact to the same extent with cations (Figs. 1 and 2, Table II). In contrast, the tendency of galactolipid vesicles to aggregate in $MgCl_2$ is strongly inhibited by the presence of even small amounts of the anionic lipids PG and SQDG. The simplest interpretation is that higher amounts of added salt are required to suppress electrostatic repulsion between bilayers containing these lipids. That electrostatic repulsion is the primary force preventing close approach is supported by the near 10 : 1 efficacy ratio of divalent : monovalent cations in Table III [13]. These results are consistent with theoretical and empirical data showing the decrease of adhesion energy between vesicles of DGDG with only 2–4% added dioleoyl-PG [17,18]. Our results may explain the failure of previous workers to observe DGDG vesicle aggregation in salt solutions. The presence of only 1 mol% charged lipid contaminant would develop sufficient electrostatic potential to prevent vesicle close approach and aggregation in 10 mM $MgCl_2$ [19] and possibly in 10 mM $CaCl_2$ [20]. As expected from electrostatic double-layer theory, the addition of the neutral lipid PC (up to 25 mol%) did not inhibit aggregation (Figs. 1 and 2).

The different curves obtained for PG and SQDG (Fig. 1 and Table II) suggest that these lipids interact in different ways with the Mg^{2+} ion. The ΔA_{50} increased linearly with the proportion of PG in DGDG at low levels (Fig. 1) but appeared to reach a limiting value between 5 and 25mol% PG, i.e., the ΔA_{50} value was the same for 5 mol% PG in DGDG as for 25 mol% PG in the DGDG/MGDG/PG (1 : 2 : 1) mixture (Table II). A similar relationship was reported for the binding of Ca^{2+} to PC-PG liposomes over the range of 20–80 mol% PG, with saturation between 50 and 100 mM Ca^{2+} [21].These concentrations are in the same range as the apparent saturation with Mg^{2+} seen here. The 1 : 1 association constant for Mg^{2+} : PG and Ca^{2+} : PG are almost identical: 6 M^{-1} and 8.5 M^{-1}, respectively, as calculated from the zeta potential [22,23]. In contrast, the SQDG molecule required higher Mg^{2+} levels to achieve similar suppression of the electrostatic potential, and the amount of $MgCl_2$ required for aggregation increased in proportion to SQDG up to 25 mol% (Fig. 1 and Table II). This argues that the negatively-charged

sulphonate group of this lipid does not form complexes with Mg^{2+}, and is simply being screened according to electrical double-layer theory [24].

Aggregation induced by cations was examined using liposomes with a lipid composition approximating that of the thylakoid membrane (DGDG/MGDG/SQDG/PG, 1:2:0.5:0.5), in order to determine the relevance of this phenomenon to thylakoid appression. Table III shows that the ΔA_{50} of such liposomes ranged from 65 mM in pure water to 49 mM in 200 mM KCl. Schroppel-Meier and Kaiser [12] reported stromal concentrations of 18 mM Mg^{2+} and 180 mM K^+ for spinach chloroplasts, which would mean that the Mg^{2+} concentration is much lower than what is required for vesicle aggregation in our system. This suggests that forces between thylakoid lipids may play a minor role, if any, in the appression of thylakoid membranes in vivo. However, it should be pointed out that ions may be concentrated at membrane surfaces by double-layer interactions and their activities in the highly viscous aqueous stromal phase are not known. Due to the negative surface charge density of thylakoids and the exponential variation of valency in the Boltzmann distribution, divalent cations and Mg^{2+} in particular are expected to be highly concentrated above bulk levels at the thylakoid surface [13]. Furthermore, aggregation was tested here at 1 mg lipid $\cdot$ ml^{-1} while the concentration of lipid in the chloroplast may be significantly higher [4]. Although intrinsic membrane proteins are definitely the major factor in thylakoid appression, attractive forces between the galactolipid bilayers may also be making a contribution.

Data presented here suggest the possibility that MGDG may promote vesicle fusion. The presence of MGDG at 50 mol% in ternary and quaternary mixtures (Fig. 2 and Table II) as well as at > 25 mol% in binary mixtures with DGDG (Fig. 3) led to irreversible vesicle aggregation. While this is not an unambiguous assay for vesicle fusion, a similar method using the irreversibility of turbidity changes in phosphatidylserine vesicles gave results very similar to those obtained by a more rigorous assay using the fluorescent measurement of vesicle contents mixing [25]. Gounaris et al. [19] reported that small (30–50 nm) vesicles made from total bean thylakoid polar lipids (50% MGDG) did fuse to form larger vesicles on the addition of $MgCl_2$, as judged from freeze-fracture electron micrographs. However, they did not examine binary mixtures of MGDG and DGDG. It is possible that the latter vesicles simply adhere more strongly with a higher content of MGDG, consistent with the two-fold higher adhesion energies measured for MGDG monolayers compared to DGDG monolayers [26]. On theoretical grounds, it is expected that MGDG should promote fusion because it is an H_{II}-phase preferring lipid, and has a strong tendency to form the inverted micelles that have been proposed as intermediates in the fusion process [15,16]. However, further work will have to be done to establish whether the irreversible aggregation we have observed is due to bilayer fusion.

In this paper, we have shown that the rather surprising observation made in our previous work [4], namely, that mono- and divalent cations can cause the aggregation of liposomes made from the unionizable neutral lipid DGDG applies also to another galactolipid, MGDG. In both papers, we have shown that galactolipid vesicle aggregation was not due to the presence of small quantities of charged lipid contaminants, since deliberately adding such lipids increased the salt concentration required for aggregation. Aggregation could not be produced by simply lowering the pH, and there was no change in lamellar spacing with salt concentration [4]. Freeze-fracture micrographs showed that aggregated DGDG vesicles were flattened [4], which could be explained by the force of adhesion causing deformation and loss of volume [17,18]. The relative efficacy of different cations in promoting aggregation suggested that they were acting to destabilize water structure and thereby reduce the repulsive hydration forces which keep bilayers apart [27]. An attractive hydration force due to inter-bilayer hydrogen-bonded water bridges has recently been proposed [27,28]. Both MGDG and DGDG should be able to make multiple H-bonds between bilayers, depending on head-group orientation and degree of hydration [27–30]. It remains to be explained how these interbilayer interactions are increased in the presence of both monovalent and divalent cations.

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