Biochimica et Biophysica Acta, 984 (1989) 41–49
Elsevier

BBAMEM 74514

Permeability properties of large unilamellar vesicles of thylakoid lipids

Murray S. Webb * and Beverley R. Green

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

(Received 16 November 1988)
(Revised manuscript received 8 May 1989)

Key words: Digalactosyldiacylglycerol; Phosphatidylcholine; Permeability; Unilamellar vesicle; Thylakoid; Fluidity

The permeability properties of large unilamellar vesicles of the chloroplast thylakoid lipid digalactosyldiacylglycerol (DGDG) to $^{86}Rb^+$, $^{36}Cl^-$, and D-[^{3}H]glucose have been determined. In addition, the permeabilities of binary, ternary, and quaternary mixtures of thylakoid lipids to $^{86}Rb^+$ have been measured. Vesicles of DGDG were found to be two orders of magnitude more permeable to Rb^+ and at least 50-fold less permeable to Cl^- than phosphatidylcholine (PC) vesicles. Vesicles of DGDG and PC were similar in glucose permeability. Electron spin resonance measurement of DGDG bilayer fluidity with the spin probes 5-doxylstearate and 12-doxylstearate indicated that DGDG bilayers were not significantly more fluid than PC bilayers, suggesting that fluidity differences could not account for the observed differences in ion permeability. The addition of 50 mol% of monogalactosyldiacylglycerol (MGDG) to DGDG vesicles had no effect on Rb^+ permeability, suggesting that the hexagonal H_{II} phase preference of MGDG does not increase bilayer permeability. The addition of sulfoquinovosyldiacylglycerol (SQDG) lead to a large increase in Rb^+ permeability. The calculated permeability coefficient to Rb^+ for a DGDG/MGDG/SQDG/phosphatidylglycerol (1:2:0.5:0.5) mixture similar to that of thylakoid membranes was $2.0 \cdot 10^{-9}$ cm·s^{-1}. This value is three orders of magnitude higher than that for phospholipid systems, and 10-fold higher than that for vesicles of DGDG. The relationship between galactolipid vesicle permeability and thylakoid membrane permeability is discussed.

Introduction

Higher plant thylakoid lipids are comprised mostly of the uncharged glycolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) [1]. Thylakoids also contain significant proportions of the anionic lipids sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). An outstanding feature of these lipids is the unusually high degree of polyunsaturation of the constituent acyl chains. Typcially the fatty acids of MGDG and/or DGDG are at least 75% linolenic acid, and may reach as high as 90% in the MGDG of some species [2]. The phase behavior of purified thylakoid lipids has been extensively studied and it is now widely accepted that aqueous dispersal of MGDG yields the hexagonal-II phase while the dispersal of DGDG, SQDG, or PG gives the liquid-crystalline lamellar phase [3,4]. Promotion of the hexagonal-II phase by unsaturation of MGDG acyl chains has been demonstrated [5,6], and suggests that the phase behavior of thylakoid lipids may be rationalized by the 'shape concept' as used for phospholipid systems [7,8].

A major function of the thylakoid is the development and maintenance of a transmembrane proton gradient of approximately three pH units. It is clear that, in order to avoid short-circuiting the electrical portion of the protonmotive gradient, the thylakoid must be an effective permeability barrier to ions. Analysis of the permeability properties of phospholipid vesicles has shown that the phospholipid bilayer is the primary barrier to transmembrane movement of charged solutes and is sufficiently impermeable to potassium and sodium to maintain concentration gradients for time periods up to days [9]. Typical permeability coefficients for these ions are in the 10^{-12} to 10^{-13} cm·s^{-1} range [9]. Corresponding values have not been reported for the plant

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* Present address: Department of Agronomy, Cornell University, Ithaca, NY 14853, U.S.A.

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

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

thylakoid lipids; although a recent report by Foley et al. [10] suggested that the permeability of DGDG and DGDG/MGDG mixtures to KCl was similar to that of phosphatidylcholine (PC).

We have assessed the permeabilities of a uniform-size population of large unilamellar vesicles of the galactolipid DGDG to $^{86}Rb^+$, $^{36}Cl^-$, and [^{3}H]glucose, and of mixtures of thylakoid lipids to $^{86}Rb^+$. We present data showing that the permeability characteristics of DGDG are significantly different from those of PC. Further, the effect of adding other thylakoid lipids on permeability of DGDG vesicles is shown and the importance of these results to the function of the thylakoid is discussed.

Materials and Methods

Materials

Materials used in this work were obtained as follows: 5-doxylstearate and 12-doxylstearate from Aldrich; KCl from Amachem; ^{86}RbCl and Na^{36}Cl from Amersham; NaCl, sodium acetate and all solvents (reagent grade and re-distilled before use) from BDH; D-glucose from Fisher; mannitol from Mallinckrodt; Freon-11 from Matheson; RbCl from Merck; D-[2-^{3}H]glucose and [^{14}C]cholesterol from New England Nuclear; Sephadex G-50 (coarse) from Pharmacia, and all others from Sigma. Soybean PC (Type III-S, Sigma) was judged pure by analytical TLC and used without further purification.

Lipid purification and analysis

The extraction (from *Spinacea oleracea*), purification and analysis of MGDG, DGDG, PG, and SQDG on carboxymethyl-cellulose has been described [11,12]. The galactolipids and PG were at least 99.5 mol% pure, and SQDG 98 mol% pure. The fatty acid compositions were determined by gas–liquid chromatography of fatty acid methyl esters using pentadecanoic acid as an internal standard. Phosphatidylcholine was assayed by the method of Stewart [13], galactolipids by the method of Roughan and Batt [14].

In vesicles composed of mixtures of lipids the preparations were checked for complete incorporation of all of the added lipids by re-extracting the vesicles into $CHCl_3/CH_3OH$ (1 : 1, v/v) at the end of the experiment. 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) [15]. Plates were sprayed with the phospholipid reagent of Allen and Good [2] 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.

Vesicle reconstitution

Lipids in $CHCl_3/CH_3OH$ were mixed, dried rapidly under a stream of N_2 at 40 °C then any residual solvent was removed under vacuum overnight. If included, valinomycin (in $CHCl_3$), nystatin (CH_3OH), or gramicidin D (CH_3OH) were added at an ionophore/lipid ratio of 1 : 500 (mol/mol).

Lipids were dispersed at 10 $mg \cdot ml^{-1}$ into 1 ml of salt solution or buffer (see below) by vortexing and sonication. These multilamellar dispersions were converted to large unilamellar vesicles of approximately 100 nm diameter by ten extrusions through two 0.1 μm Nuclepore filters (The Extruder, Lipex Biomembranes, Vancouver, B.C., Canada) at 2000 kPa N_2. Vesicles were periodically checked for size by light scattering (Nicomp 270 Submicron Particle Sizer) and were always at 120 ($\pm$30) nm diameter. Increased dispersion turbidity associated with vesicle fusion or aggregation [12] was not observed.

Rb^+ flux

Lipid or lipid mixtures, containing nystatin, were dispersed as described above into 1 ml H_2O containing 1–5 μCi ^{86}RbCl, usually at 110 μM RbCl. External $^{86}Rb^+$ was removed by passing the vesicles over a 10 ml (wet bed) column of Sephadex G-50 (packed into a 10 ml disposable syringe) pre-equilibrated in equal concentration of NaCl and pre-spun at $100 \times g$ for 30 s. Vesicles were eluted from this column by spinning at $100 \times g$ for 30 s and the timing of the experiment was started at the end of this spin. At various times 100-μl aliquots of the vesicles were loaded onto a 1 ml Sephadex G-50 column, equilibrated and pre-spun as above, but packed into Bio-Rad Econo columns. External tracer was removed by another spin at $100 \times g$ for 30 s. Label remaining in the vesicles was assayed by Liquid Scintillation Counting (LSC). All efflux experiments were performed at 30 °C.

Measurement of the vesicle recovery from the 1 ml columns was chekced using DGDG vesicles labelled with [^{14}C]cholesterol. Results indicated routine recovery of 90% of the vesicles with $\leqslant 1.5\%$ carry-over of external label. In initial experiments it was ensured that $^{86}Rb^+$ co-eluting with the vesicles was trapped within the vesicles, rather than surface bound, by addition of valinomycin (1 μl in $CHCl_3$) to 100–150 μl of vesicles to a final valinomycin/lipid ratio of about 1 : 250. These vesicles were incubated for a further 15 min at 30 °C then co-eluting $^{86}Rb^+$ measured as above. This treatment invariably reduced the co-eluting label to background levels. An identical 1 μl volume of $CHCl_3$ did not cause leakage of Rb^+ from these vesicles under the same conditions. This indicated that vesicle structure remained intact during valinomycin addition and therefore, that co-eluting $^{86}Rb^+$ was trapped within the vesicle rather than surface bound.

Cl^- flux

Efflux of $^{36}Cl^-$ was measured as described for $^{86}Rb^+$ except that vesicles, containing gramicidin D in place of nystatin, were dispersed into 5 μCi of $Na^{36}Cl$ at 9.30 mM. The external solution was changed to 9.19 mM NaCl, 0.11 mM sodium acetate and sampled as described above.

Glucose flux

The efflux of D-glucose was measured as above except that lipid, without added ionophore, was dispersed into 1 ml of 50 mM D-glucose, 5 mM Tricine-NaOH (pH 7.5) containing 5 μCi D-[^{3}H]glucose. The external solution was changed to 50 mM mannitol, 5 mM Tricine-NaOH (pH 7.5), and sampled as described above.

Calculation of permeability coefficients

Under conditions of the flux of an electroneutral solute, or the non-electrogenic flux of ionic solutes by movement of ion pairs, the permeability coefficient (P, $cm \cdot s^{-1}$) may be simply calculated from:

$$P = J/(A \cdot \Delta C)$$

where J is the flux in $\mu mol \cdot s^{-1}$, ΔC is the concentration gradient experienced by the solute of interest ($\mu mol \cdot cm^{-3}$), and A is the total surface area of the vesicle preparation (cm^2) [9]. With the specific activity of the internal contents of the vesicles, the initial slope of the efflux curve (μmol remaining vs. time) gives J. Lipid measurement by assay for galactose and/or phospholipid gives the area term by assuming 0.7 nm^2 per lipid molecule [16].

Alternatively, the permeability coefficient was calculated using:

$$P = (kV/A) \cdot (C/\Delta C)$$

where k is the first-order rate constant of solute efflux in s^{-1}, V is the trapped volume of the vesicles determined from the zero time sample ($\mu l/\mu mol$ lipid), A is the total membrane surface area determined from the lipid assay as above, and C was the internal concentration of the solute of interest ($\mu mol \cdot cm^{-3}$). The rate constant, k, was determined from the slope of a least-squares linear regression of $\ln(\%_0/\%)$ vs. time (seconds) as described previously [17], where $\%_0$ and % represent the % solute remaining trapped within vesicles at time zero and time t, respectively. For chloride efflux the initial external Cl^- concentration $\neq 0$, therefore the rate constant, k, was determined by a modified $\ln(\%_0/\%)$ expression [17]. For most of these regressions the r^2 value was $\geqslant 0.97$, indicating first-order kinetics for the efflux. For both methods of calculation an area value equal to one-half the total area determined from the lipid assay has been used in order to account for the single monolayer of lipid 'seen' by the diffusing ion. Statistical significance refers to a two-tailed 'Student's' test at a $a = 0.05$ significance level.

Electron spin resonance

Vesicles were made from 2.7 mg of DGDG, combined with spin labels 5-doxylstearic acid (I[12,3]) or 12-doxylstearic acid (I[1,14]) at molar probe/lipid ratio of 1 : 200, by the reverse phase evaporation method [18] and dispersed into 0.2 ml of 150 mM KCl and 0.5 mM Tris-HCl (pH 8.0).

Spectra were recorded on a Varian Model E9 EPR spectrometer at 21°C with a microwave power of 10 mW, a time constant of one second, a 4 min scan time and a modulation amplitude of 1.25 gauss (G). For the slow anisotropic motion of the I[12,3] probe the order parameter, S, was calculated from [19]:

$$S = \{(A_{\parallel} - A_{\perp} - C)/(A_{\parallel} - 2A_{\perp} - 2C)\} \cdot 1.723$$

where:

$$C = 1.4G - 0.053(A_{\parallel} - A_{\perp})$$

and $A_{\parallel}$ and $A_{\perp}$ equal one-half of the separation, in gauss, of the outer and inner extrema, respectively, of the I[12,3] spectrum. For the relatively rapid anisotropic motion of the spin label I[1,14] the order parameter was not calculable, however, the rotational correlation time, τ_0, was calculated from [20]:

$$\tau_0 = 6.5 \cdot 10^{-10} \cdot \omega_0 [(\sqrt{h_0/h_{+1}}) - 1]\ s$$

where ω_0 is the width of the midfield peak and h_0 and h_{+1} represent the heights of the midfield and highfield peaks, respectively, of the I[1,14] spectrum.

Results

Lipid and fatty acid composition of vesicles

The fatty acid compositions of spinach DGDG, MGDG, SQDG, and PG are given in Table I. The fatty acid composition of the thylakoid lipids was similar to that reported previously for these lipids isolated from spinach [2]. The galactolipids were enriched in the polyunsaturated fatty acids linolenic acid (18 : 3), linoleic acid (18 : 2), and palmitolenic acid (16 : 3). The fatty acid profiles of SQDG and PG were enriched in palmitic acid (16 : 0) and 18 : 3, with PG also showing high levels of *trans*-Δ3-hexadecenoic acid (16 : 1). Finally, the fatty acid composition of commercially obtained soy PC was similar to that expected for this lipid, being enriched in 16 : 0, oleic (18 : 1) and linoleic acids.

Given a previous report [18] of the preferential retention of MGDG on the walls of test tubes during the dispersal of binary galactolipid mixtures, we have checked the lipid composition of vesicles at the end of

TABLE I

The fatty acid composition of lipids used

Fatty acid methyl esters were prepared with BF_3/methanol and analyzed by gas–liquid chromatography. Data presented are the mean (±S.D.) from four determinations.

Lipid	mol% fatty acids						
	16:0	16:1 [a]	16:3	18:0	18:1	18:2	18:3
MGDG	0.9	0.1	24.7	0.5	0.4	1.0	72.4
	(±0.1)		(±0.6)	(±0.1)	(±0.2)	(±0.1)	(±1.4)
DGDG	7.6	–	3.2	0.9	0.8	2.4	85.0
	(±0.2)		(±0.1)	(±0.1)	(±0.1)	(±0.1)	(±0.3)
SQDG	43.2	0.6	1.4	1.5	1.0	4.5	46.7
	(±0.7)	(±0.1)	(±0.1)	(±0.2)	(±0.2)	(±0.4)	(±1.1)
PG	18.2	34.7 [a]	–	3.3	0.8	3.8	39.1
	(±0.2)	(±0.3)		(±0.8)	(±0.2)	(±0.1)	(±0.5)
Soy PC	15.5	–	–	4.3	10.1	64.8	5.3
	(±0.25)			(±0.13)	(±0.1)	(±0.7)	(±0.2)

[a] *trans*-3-Hexadecanoic acid.

the experiment by TLC. The results in Table II show that there was no preferential loss of MGDG and, in general, the lipid composition of the vesicles was very similar to that of the composition of the dispersed mixture. The slightly decreased levels of PG and SQDG in the quaternary mixtures was likely due to the preferential charring of unsaturated fatty acyl chains by the reagent. Such residues are present in lower proportions in PG and SQDG than in the galactolipids (Table I). This is indicated by our inability to detect disaturated PG in these dispersions (not shown) by charring even though the presence of this phospholipid was indicated by positive reaction with the molybdenum reagent [2]. It is possible that more rapid removal of bulk organic solvent from stock lipid mixtures prevented phase separation of the lipids, particularily MGDG, during drying onto the walls of the test tube and before dispersal into vesicles.

TABLE II

Recovery of lipids from vesicles

Vesicles were re-extracted into $CHCl_3/CH_3OH$ (1:1, v/v) after efflux experiments. Aliquots were separated on silica TLC in acetone/benzene/water (91:30:8, v/v) and charred with the phospholipid spray of Allen and Good [2]. 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 (±S.D.) of at least three separate preparations.

Mixture dispersed	% of recovered lipid				
	DGDG	MGDG	SQDG	PG	PC
DGDG	100	–	–	–	–
DGDG/MGDG	73.7	26.3	–	–	–
(3:1)	(±3.1)	(±3.1)			
DGDG/MGDG	54.3	45.6	–	–	–
(1:1)	(±3.9)	(±3.9)			
MGDG/PC	–	44.9	–	–	55.1
(1:1)		(±3.1)			(±3.1)
DGDG/PC	45.9	–	–	–	54.1
(1:1)	(±3.8)				(±3.8)
DGDG/MGDG/PG	33.6	48.0	–	18.3	–
(1:2:1)	(±4.2)	(±3.5)		(±4.9)	
DGDG/MGDG/SQDG	30.5	51.4	18.0	–	–
(1:2:1)	(±2.9)	(±4.9)	(±3.8)		
DGDG/MGDG/SQDG/PG	29.8	49.1	8.9	12.2	–
(1:2:0.5:0.5)	(±0.3)	(±3.5)	(±1.5)	(±2.6)	
DGDG/MGDG/PC	29.6	49.2	–	–	21.1
(1:2:1)	(±2.5)	(±7.0)			(±9.4)
Soy PC	–	–	–	–	100

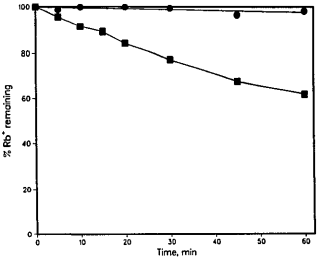

Fig. 1. Plot of % Rb^+ remaining in large unilamellar vesicles of DGDG (■) and soy PC (●)vs. time at 30 °C. Vesicles were made with 0.110 mM RbCl inside and containing 1–5 μCi $^{86}Rb^+$. External solution was changed to an equal concentration of NaCl by gel filtration on Sephadex G-50. Aliquots of vesicles were removed at various times and external tracer removed by another passage over Sephadex G-50. Labelled $^{86}Rb^+$ co-eluting with the vesicles was assayed by liquid scintillation counting.

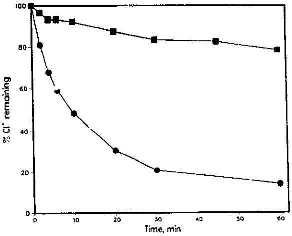

Fig. 2. Plot of % Cl^- remaining in large unilamellar vesicles of DGDG (■) and soy PC (●) vs. time at 30 °C. Vesicles were made in 9.30 mM NaCl containing 5 μCi $^{36}Cl^-$. External solution was changed to 9.19 mM NaCl, 0.11 mM sodium acetate by gel filtration on Sephadex G-50. Aliquots of vesicles were removed at various times and external tracer removed by another passage over Sephadex G-50. Labelled $^{36}Cl^-$ co-eluting with the vesicles was assayed by liquid scintillation counting.

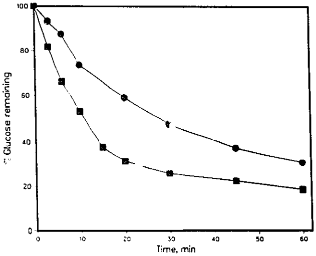

Fig. 3. Plot of % glucose remaining in large unilamellar vesicles of DGDG (■) and soy PC (●) vs. time at 30 °C. Vesicles were made in 50 mM D-glucose, 5 mM Tricine (pH 7.5) containing 5 μCi D-[2-^{3}H]glucose. External solution was changed to 50 mM mannitol, 5 mM Tricine (pH 7.5) by gel filtration on Sephadex G-50. Aliquots of vesicles were removed at various times and external tracer removed by another passage over Sephadex G-50. Labelled [^{3}H]glucose co-eluting with the vesicles was assayed by liquid scintillation counting.

Efflux of solutes from DGDG and PC vesicles

The efflux of $^{86}Rb^+$ from large unilamellar vesicles of pure DGDG and pure soy PC is shown in Fig. 1. Tracer flux occurred at a significantly greater rate from DGDG vesicles than from PC. In order to specifically measure Rb^+ flux rates it was necessary to ensure that the counter-flow of Na^+ into the vesicles, and/or the co-transport of Cl^- out of the vesicles occurred at rates greater than or equal to that of Rb^+. The efflux of $^{86}Rb^+$ from DGDG vesicles was similar with or without (not shown) the inclusion of the ionophore nystatin. This result argues that in DGDG vesicles without nystatin either the development of a Rb^+ electrical potential was too small to influence further Rb^+ efflux and/or that DGDG permeability to Na^+ or Cl^- is equal to, or greater than, that of Rb^+. It is possible that added nystatin did not function as an anion ionophore in these bilayers due to an apparent requirement for sterols [21]. Nevertheless, a $\Delta\psi$ inhibitory to Rb^+ efflux probably did not develop due to compensatory Na^+ influx or Cl^- efflux.

The permeability of DGDG and soy PC to $^{36}Cl^-$ is compared in Fig. 2. Phosphatidylcholine was significantly more permeable to Cl^- than was DGDG. In these experiments the development of a Cl^- diffusion potential was short-circuited by the addition of gramicidin D. Both DGDG and PC vesicles were relatively permeable to glucose, leaking 70–80% of trapped glucose within one hour (Fig. 3). Permeability coeffi-

TABLE III

Summary of permeability and fluidity characteristics of DGDG and soybean PC vesicles

The double bond index (DBI) was calculated from the fatty acid composition of the lipids (Table I), assuming no positional specificity, and indicates the average number of double bonds per lipid molecule. Electron spin resonance (ESR) spectra yielded the rotational correlation times, τ_0, and order parameters, S, respectively of I[1,14] and I[12,3] spin probes. Permeability coefficients were calculated from $P = J/(A \cdot \Delta C)$ or using $P = (kV/A) \cdot (C/\Delta C)$ as described in Methods and represent the means (± S.D.) of at least three independent preparations. The DGDG/PC row represents the ratio of data presented in the above two rows.

Lipid	DBI	ESR		P (cm·s^{-1})							
		τ_0 (ns)	S	$P = J(A \cdot \Delta C)$				$P = (kV.A) \cdot (C/\Delta C)$			
				Rb^+	Cl^-	glucose	Cl^-/Rb^+	Rb^+	Cl^-	glucose	Cl^-/Rb^+
DGDG	5.40	1.32	0.670	$3.0 \cdot 10^{-10}$ $(\pm 0.4 \cdot 10^{-10})$	$10 \cdot 10^{-10}$ $(\pm 8.6 \cdot 10^{-10})$	$3.2 \cdot 10^{-10}$ $(\pm 0.2 \cdot 10^{-10})$	3.4 [a]	$3.4 \cdot 10^{-10}$	$9.3 \cdot 10^{-10}$	$1.1 \cdot 10^{-10}$	2.7
Soy PC	3.43	–	0.668 [b]	$5.0 \cdot 10^{-12}$ $(\pm 3.8 \cdot 10^{-12})$	$7.8 \cdot 10^{-8}$ $(\pm 3.6 \cdot 10^{-8})$	$3.4 \cdot 10^{-10}$ $(\pm 1.4 \cdot 10^{-10})$	$1.5 \cdot 10^4$	$2.6 \cdot 10^{-12}$	$4.2 \cdot 10^{-8}$	$2.4 \cdot 10^{-10}$	$1.6 \cdot 10^4$
DGDG/PC	1.57	–	1.00	60.0	0.013	0.90 [a]	–	130	0.022	0.46 [a]	–

[a] Not significantly different.

[b] Calculated from Ref. 44 for soy PC dispersions.

cients were calculated using two different flux equations as described in the Methods (Table III). These data indicate that DGDG was two orders of magnitude more permeable to Rb^+ than was PC (permeability coefficients for these lipids were $3 \cdot 10^{-10}$ cm · s^{-1} for DGDG and about $4 \cdot 10^{-12}$ cm · s^{-1} for PC). This relationship did not hold for Cl^- with DGDG showing at least 50-fold lower permeability to Cl^- than PC ($1 \cdot 10^{-9}$ cm · s^{-1} and about $7.8 \cdot 10^{-8}$ cm · s^{-1}). In contrast, both kinds of vesicles had the same permeability to D-glucose ($3 \cdot 10^{-10}$ cm · s^{-1}).

For the flux of Rb^+ and glucose from both DGDG and PC vesicles the calculated permeability coefficients were very similar using either $P = J/(A \cdot \Delta C)$ or $P = (kV/A) \cdot (C/\Delta C)$. Identical permeability coefficients were also obtained using either equation for Rb^+ flux from vesicles composed of mixtures of thylakoid lipids (see below).

Measurement of DGDG vesicle fluidity

It has been reported [22] that differences in acyl chain unsaturation, and presumably in bilayer fluidity, can influence passive membrane permeability to solutes. It was of interest, therefore, to determine if DGDG vesicles were more fluid than those of PC and if such fluidity properties could account for the differences in the permeability properties between DGDG and PC liposomes. The electron spin resonance spectrum (not shown) of DGDG vesicles labelled with the spin probe I[12,3], which samples the hydrocarbon region near the polar head group, yielded an order parameter, S, of 0.670 (Table III). A very similar value of 0.668 has been calculated from spectra obtained from PC vesicles probed in this region of the hydrocarbon tails (Table III). When the spin probe I[1,14] was used the order parameter was not calculable as a result of the spectrum change to more rapid anisotropic motion of the doxyl group. Alternatively, the rotational correlation time, τ_0, the time required for the spin probe to rotate through an arc of one radian, has been determined. Vesicles of DGDG yielded a τ_0 of 1.32 ns while a similar value of 2.21 ns was calculated for the same probe in oriented multilayers of egg PC [23]. Calculation of the double bond index (DBI), the average number of acyl double bonds per lipid molecule, shows that DGDG was 1.5-fold higher in acyl unsaturation than soy PC (Table III).

These results clearly indicate that the fluidity of pure DGDG vesicles was similar to that of PC bilayers in both the upper and central areas of the hydrocarbon region. The higher double bond index (DBI, Table III) for DGDG compared to PC may explain the slightly faster rotational mobility of the I[1,14] probe deep in the hydrocarbon region (1.32 ns vs. 2.21 ns) but is clearly not directly related to acyl chain order near the polar head groups. Similar τ_0 values of 1.2 to 1.3 ns have been reported by Hiller and Raison [24] for vesicles of total lipid extracts from barley and spinach chloroplasts, and 1.65 ns in vesicles of total lipid extracts of maize chloroplasts [25]. These investigators also obtained order parameters close to those reported here. Hiller and Raison [24] measured S values of 0.66 to 0.67 in phospholipid and galactolipid mixtures from barley chloroplasts with the spin probe 6-doxylstearic acid. Nolan and Bishop [25] reported a $2A_{\parallel}$ value of 57.1 gauss for total chloroplast lipid liposomes measured with I[12,3] compared to the value of 53.5 gauss determined here for DGDG vesicles with the same probe. All these data suggest that differences in fluidity cannot account for the large differences in permeability characteristics between DGDG and soy PC vesicles.

TABLE IV

Calculated Rb^+ permeability coefficients for lipid mixtures

Permeability coefficients were calculated using $P = J/(A \cdot \Delta C)$ or using $P = (kV/A) \cdot (C/\Delta C)$ as described in Methods. Values are the mean (±S.D.) of at least three separate vesicle preparations.

Lipid	P (cm·s^{-1})	
	$P = J/(A \cdot \Delta C)$	$P = (kV/A) \cdot (C/\Delta C)$
DGDG/MGDG (3:1)	$3.0 \cdot 10^{-10}$ ($\pm 2.0 \cdot 10^{-10}$)	$3.2 \cdot 10^{-10}$
DGDG/MGDG (1:1)	$3.4 \cdot 10^{-10}$ ($\pm 1.6 \cdot 10^{-10}$)	$2.6 \cdot 10^{-10}$
MGDG/PC (1:1)	$2.0 \cdot 10^{-11}$ ($\pm 1.0 \cdot 10^{-11}$)	$1.6 \cdot 10^{-11}$
DGDG/PC (1:1)	$2.8 \cdot 10^{-11}$ ($\pm 0.8 \cdot 10^{-11}$)	$2.3 \cdot 10^{-11}$
DGDG/MGDG/PC (1:2:1)	$4.4 \cdot 10^{-11}$ ($\pm 2.0 \cdot 10^{-11}$)	$4.0 \cdot 10^{-11}$
DGDG/MGDG/PG (1:2:1)	$2.2 \cdot 10^{-10}$ ($\pm 0.2 \cdot 10^{-10}$)	$2.9 \cdot 10^{-10}$
DGDG/MGDG/SQDG (1:2:1)	$3.4 \cdot 10^{-9}$ ($\pm 2.0 \cdot 10^{-9}$)	$3.2 \cdot 10^{-9}$
DGDG/MGDG/SQDG/PG (1:2:0.5:0.5)	$1.4 \cdot 10^{-9}$ ($\pm 1.4 \cdot 10^{-10}$)	$2.5 \cdot 10^{-9}$

Effects of other lipids on DGDG permeability

Large unilamellar vesicles were made from mixtures of other purified thylakoid lipids with DGDG to assess their affect on vesicle permeability to Rb^+ (Table IV). Calculated permeability coefficients were very similar using either $P = (J/A \cdot \Delta C)$ or $P = (kV/A) \cdot (C/\Delta C)$; values discussed below are those obtained using $P = J/(A \cdot \Delta C)$. The addition of MGDG to DGDG at 25 mol% and 50 mol% neither increased nor decreased the permeability of vesicles to Rb^+, the permeability coefficients remaining at about $3.2 \cdot 10^{-10}$ cm · s^{-1}. Although MGDG is a hexagonal phase-forming species it can form bilayers when mixed with DGDG, and its strong tendency to form non-bilayer structures does not appear to influence bilayer permeability.

The equimolar addition of MGDG to PC caused a 4-fold increase in Rb^+ permeability. Similarly, the addition of equimolar DGDG to PC resulted in a 5–6-fold increase in Rb^+ permeability above values for pure PC. These vesicles were an order of magnitude less permeable to Rb^+ than pure DGDG vesicles (Table III). When PC was lowered to 25 mol% in the mixture DGDG/MGDG/PC (1 : 2 : 1), the permeability was even higher. However, these mixed lipid vesicles were an order of magnitude less permeable to Rb^+ than pure DGDG vesicles, i.e. they displayed behavior intermediate between pure DGDG and pure PC vesicles.

Other ternary and quaternary mixtures of thylakoid lipids were also tested for permeability to Rb^+ (Table IV). The permeability of DGDG/MGDG/PG (1 : 2 : 1) to Rb^+ was similar to that of DGDG/MGDG (1 : 1), both near $2.5 \cdot 10^{-10}$ cm · s^{-1}. The replacement of PG with another acidic lipid, SQDG, to give DGDG/MGDG/SQDG (1 : 2 : 1) caused a dramatic 10-fold increase in vesicle permeability up to the highest value measured here of $3.4 \cdot 10^{-9}$ cm · s^{-1}, Replacement of half of this SQDG with PG to give DGDG/MGDG/SQDG/PG (1 : 2 : 0.5 : 0.5) gave a permeability coefficient midway between DGDG/MGDG/PG (1 : 2 : 1) and PGDG/MGDG/SQDG (1 : 2 : 1). This mixture is of particular interest because it approximates the lipid composition of the thylakoid membrane. This mixture was an order of magnitude more permeable to Rb^+ than pure DGDG, and three orders of magnitude more permeable than pure soy PC. All ternary and quaternary mixtures of thylakoid lipids were significantly more permeable to Rb^+ than DGDG/MGDG/PC (1 : 2 : 1).

Discussion

Model membrane studies, primarily using phospholipids derived from animal systems, have indicated that the lipid bilayer is the main barrier to transmembrane solute flux and is sufficiently impermeable to maintain observed solute concentration gradients [9]. This paper reports results concerning the permeability of large unilamellar vesicles composed of lipids extracted from the thylakoids of spinach chloroplasts, to Rb^+, Cl^-, and glucose.

We have succeeded in producing large unilamellar vesicles of defined size and lipid composition, including vesicles with the same proportion of MGDG as in native thylakoid membranes (Table II). The latter variable is in contrast with the results of Sprague and Staehelin [18] who reported the preferential retention of MGDG on the sides of test tubes during the dispersal of mixtures containing 30–40 mol% MGDG. While dispersal was aided by the presence of SQDG or PG, presumably due to charge repulsion between hydrating bilayers, effective resuspension occurred even with binary glactolipid mixtures. These dispersions showed no sign of increased turbidity that would be indicative of vesicle aggregation [12] or aggregation followed by fusion. We were careful to keep the salt concentration lower than that which causes vesicle aggregation [12]. We conclude that vesicles used here were discrete, unfused vesicles of about 100 nm size. If inverted micelles formed by MGDG [18] were present in these bilayers, they apparently did not affect the permeability of these vesicles to Rb^+. This argues that the presence of MGDG at 50 mol% in the thylakoid membranes does not necessarily increase thylakoid membrane permeability to cations.

The permeability properties of control soybean PC vesicles measured here were in good agreement with those reported by other workers. Reported literature values for PC are 10^{-12} to 10^{-13} cm · s^{-1} for Na^+ and K^+ [9] and $3.3 \cdot 10^{-12}$ for Rb^+ [26]; and reported Cl^- values are several orders of magnitude higher than those of Na^+ [9]. Glucose permeability to 16 : 0/18 : 1-phosphatidylcholine vesicles of 100 nm diameter shows a *P* value equal to $1.5 \cdot 10^{-10}$ cm · s^{-1} (Wong, K., and Cullis, P.R., personal communication). In addition, permeability coefficients of 0.3 and $1.1 \cdot 10^{-10}$ cm · s^{-1} have been reported for planar bilayers and small unilamellar vesicles, respectively, of egg PC at 25°C [17]. We conclude, therefore, that the methods and calculations employed in this study were measuring permeability in a manner consistent with previous workers.

The permeability of large unilamellar vesicles of DGDG to various ionic solutes was significantly different from soybean PC vesicles. DGDG liposomes were two orders of magnitude more permeable to Rb^+ than PC vesicles, but at least 50-fold less permeable to Cl^-. However, DGDG vesicles were statistically identical to PC vesicles in terms of glucose permeability. Interestingly, calculated permeability coefficients for DGDG to both charged and uncharged solutes were all very similar, in the 10^{-10} range (Table III). These results are not in agreement with those reported recently by Foley et al. [10] who reported that multilamellar dispersions of DGDG and binary mixtures of DGDG and MGDG form bilayers as impermeable to KCl as PC. This discrepancy is probably due, in part, to their use of turbidity changes as a measure of osmotic swelling and, hence, permeability of vesicles [27] in the presence of concentrations of cations that have been shown to cause reversible vesicle aggregation [12]. This aggregation was measured by turbidity changes upon salt addition in the 1–20 mM concentration range, and was not related to osmotic swelling [12].

Our estimates of bilayer fluidity by ESR gave results very similar to those of other workers. Obtained values of τ_0 and *S* for reverse-phase DGDG vesicles in 150 mM KCl and 0.5 mM Tris-HCl (pH 8.0) were very close to those reported for thylakoid lipid extracts hydrated by conventional methods into 1 mM EDTA, 0.1 M

Tris-acetate (pH 7.2) [24] or into 0.33 M sorbitol, 1 mM $MgCl_2$, and 10 mM phosphate buffer (pH 7.4) [25]. Therefore, it is unlikely that the salt and buffer content of the aqueous phase, or the hydration method, has a significant impact on the bilayer fluidity.

The possibility that higher fluidity of DGDC vesicles, compared to PC vesicles, may explain the difference in permeability of these lipids was examined. It is clear from a comparison of τ_0 and S values in Table III that the fluidity of DGDG vesicles was not significantly higher than that of soy PC vesicles. Van de Ven and Levine [28] have reported similar motional properties of diphenylhexatriene in DGDG and soy PC bilayers. Related work with cholestane spin labels in DGDG bilayers led Koole et al. [29] to the conclusion that the introduction of additional double bonds does not increase the rate of hydrocarbon chain motion in DGDG. Data summarized in Table III demonstrates that fluidity differences in bilayers of DGDG cannot explain the differences observed between PC and DGDG in Rb^+ and Cl^- permeability. Furthermore, even if higher fluidity were able to explain higher Rb^+ permeability, similarly increased permeation did not occur for Cl^- and glucose as would be expected for a simple fluidity-based explanation to permeation.

Early work on the passage of ionic solutes through phospholipid bilayers suggested the importance of oriented surface dipoles in determining bilayer permeability properties [30]. The recent membrane potential profile model of Flewelling and Hubbell [31] also explains the large differences in cation and anion permeabilities observed in phospholipid bilayers as a result of oriented dipole moments on the lipid molecule. In particular the model minimizes the contributions of oriented water dipoles and the phosphate dipole and emphasizes a large contribution of ester dipoles to a resultant membrane electrical potential of several hundred millivolts with the membrane interior positive. Such a potential is expected to promote anion permeation and develop an energy barrier opposing cation flux. This would explain the several orders of magnitude higher permeability of anions compared to cations in phospholipids (Table III, Cl^-/Rb^+).

The data reported here are not consistent with the identity of the ester groups as the major contributor to the overall bilayer dipole moment [31]. The DGDG molecule should contain both ester dipole moment vectors present on diacylphospholipids but have no phosphate dipole contribution. In addition, the galactose moieties of DGDG may have the same orientation as the galactose on galactocerebrosides [32], generating a small dipole potential, positive outside [33], similar in direction to that expected for the phosphate dipole [31]. If ester dipoles are the major contributors to permeability discrimination against cations, then DGDG vesicles should show the same difference in cation and anion permeation as observed in PC vesicles. We have observed, however, that DGDG vesicles show a small, statistically insignificant, higher permeability to Cl^- than Rb^+ (Table III). It seems likely, therefore, that the contribution of head group and water dipoles to ion permeation through phospholipid bilayers has been underestimated and/or the contribution due to ester dipoles overestimated [31]. For DGDG vesicles the similar permeability coefficients for Cl^- and Rb^+ (Table III), ions of very similar radii [34] and identical charge quantity, suggests ion permeation occurring primarily under the influence of Born energy considerations with smaller contributions from charged groups and dipole potentials. Alternatively, solutes may permeate galactolipid bilayers by a mechanism completely distinct from that in PC bilayers. Such mechanisms may include passage through transient defects in bilayer integrity and/or through so-called 'statistical pores' [35]. Permeation by such mechanisms could account for the similarity of observed permeability coefficients for Rb^+, Cl^-, and glucose in DGDG vesicles.

The addition of other lipids to DGDG vesicles had very different effects on vesicle permeability to Rb^+. The equimolar addition of MGDG (Table IV) did not significantly increase the permeability. However, the same addition of PC to DGDG lowered the permeability by 10-fold. This result suggests that MGDG has the same, or similar, permeability properties discussed above with reference to DGDG. The further addition of PG to give DGDG/MGDG/PG (1 : 2 : 1) caused a small, but not statistically significant, decrease in Rb^+ leakage. The addition of the other anionic lipid, SQDG, has the opposite effect of causing an order of magnitude increase in Rb^+ permeability. This shows that surface charge alone does not regulate ionic permeability in these vesicles. The mixture resembling the in vivo mixtures of thylakoid lipids. DGDG/MGDG/SQDG/PG (1 : 2 : 0.5 : 0.5) had a Rb^+ permeability coefficient of $2.0 \cdot 10^{-9}$ cm · s^{-1}.

These results are consistent with measurements of the permeability of thylakoids to cations and anions. Rottenberg et al. [36] have reported rapid, and similar, rates of passive efflux for $^{86}Rb^+$, $^{36}Cl^-$, and [^{14}C]sorbitol from lettuce chloroplasts and report a low activation energy for these fluxes. Barber [37] has calculated a permeability coefficient for Cl^- in spinach thylakoids of $2.1 \cdot 10^{-10}$ cm · s^{-1}. This value is close to that observed here for DGDG vesicles of $9 \cdot 10^{-10}$ cm · s^{-1} (Table III). Furthermore, a similar permeability coefficient of $5.1 \cdot 10^{-10}$ cm · s^{-1} can be calculated from reported [38] NaCl and KCl fluxes of $2.3 \cdot 10^{-13}$ mol · cm^{-2} · s^{-1} into *Beta vulgaris* thylakoids exposed to 450 mM solute concentration gradients. These results suggest that the permeability of thylakoids is determined primarily by the thylakoid lipids, and is not a result of the high surface/volume ratio of thylakoids [37,38].

Finally, it should be noted that the incorporation of membrane proteins into liposomes generally increases passive bilayer permeability [39–41]. Indeed, while phospholipid vesicle permeability coefficients are about 10^{-12} cm · s^{-1} for K^+ [9], those of many biological membranes are around 10^{-7} to 10^{-8} cm · s^{-1} [42]. The similarity of solute permeabilities of thylakoids, summarized above, and of thylakoid lipid vesicles suggests that the same phenomena may not occur in chloroplasts. This may indicate that the thylakoid lipids effectively 'seal' the lipid/protein interface in intact thylakoid membranes. Alternatively, if thylakoid membrane proteins also increase the permeability of thylakoid lipid vesicles to cations, then the permeability properties reported here could significantly contribute to the rapid light-induced redistribution of Mg^{2+} and K^+ in thylakoids [43].

In summary we have demonstrated that large unilamellar vesicles of the thylakoid lipid digalactosyldiacylglycerol, as well as binary, ternary, and quaternary mixture of thylakoid lipids show permeability properties significantly different from those of phosphatidylcholine. Furthermore, it is argued that these vesicles serve as good models for studying the permeability of thylakoids to ionic and non-ionic solutes. We propose that these permeability properties, primarily those of the galactolipids, may make a significant contribution to the function of the intact thylakoid.

Acknowledgements

The authors wish to express their thanks to Dr. Don McRae for help with the electron spin resonance measurements and to Dr. Kim Wong for helpful discussions on the permeability calculations. We would also like to thank Drs. Edith Camm, Pieter Cullis, Anthony Glass, and Colin Tilcock for critically reading the manuscript. This work was supported by a Natural Sciences and Engineering Research Council of Canada grant to B.R.G.

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