Biochimica et Biophysica Acta 939 (1988) 430–440
Elsevier

BBA 73961

Permeability of liposomes composed of binary mixtures of monogalactosyldiacylglycerol and digalactosyldiacylglycerol

Adrian A. Foley [a], Anthony P.R. Brain [b], Peter J. Quinn [b] and John L. Harwood [a]

[a] *Department of Biochemistry, University College, Cardiff,* and [b] *Department of Biochemistry and Electron Microscope Unit, King's College London, Campden Hill, London (U.K.)*

(Received 10 November 1987)

Key words: Galactolipid; Chloroplast lipid; Liposome permeability; Freeze-fracture; Electron microscopy

The permeability of liposomes, formed from binary mixtures of monogalactosyldiacylglycerol and digalactosyldiacylglycerol (the major lipids of chloroplast membranes) towards potassium chloride and protons was measured. The results showed that mixtures of the two galactolipids containing up to 40 mol% of monogalactosyldiacylglycerol were capable of maintaining a potassium chloride gradient. At greater percentages, no reversible osmotic shrinkage could be observed. In addition, measurements of the trapped aqueous space, using a carboxyfluorescein dye marker, showed a correlation between trapped volume and the osmotically dependent changes in light scattering. These results agreed well with electron microscopic data which showed that mixtures containing high proportions (60 mol%) of digalactosyldiacylglycerol were capable of forming bilayer structures and multilamellar liposomes. In contrast, dispersions of galactolipids containing 50 mol% or more of monogalactosyldiacylglycerol showed many areas of non-bilayer structures which clearly could not form permeability barriers to small solutes and ions. Further experiments with liposomes made from digalactosyldiacylglycerol showed that these formed as effective a permeability barrier towards protons as phosphatidylcholine liposomes. The data are discussed with regard to the properties of the glycosyldiacylglycerols and their function in chloroplast membranes.

Introduction

Galactosyldiacylglycerols represent more than 80% (w/w) of the thylakoid acyl lipids of higher plants and algae [1]. However, the chloroplast acyl lipids are not directly involved in photosynthesis in so far as they do not undergo known chemical or photochemical reactions during light-driven NADP reduction or ATP synthesis. For this reason research on photosynthesis almost totally neglects the possible importance of individual acyl lipids to photosynthesis or for photosynthetic investigations. Yet the highly conserved nature of the thylakoid acyl lipids, their ubiquity in O_2-evolving photosynthetic organisms and other unusual features of their molecular structure (see Ref. 2), coupled with the principles of cell economy and evolution, suggest a vital role(s) in photosynthesis.

One way of probing possible functions of these lipids in photosynthesis is by reconstitution experiments (see Ref. 2) where the acyl lipids are extracted from whole tissue and studied in isolation or in various combinations. It has been shown that galactosyldiacylglycerols exhibit mesomorphic phase behaviour in aqueous systems and form lamellar, hexagonal-II or other non-lamellar phases

Correspondence: J.L. Harwood, Department of Biochemistry, University College, Cardiff, CF1 1XL, U.K.

(see Ref. 3). There have been several X-ray [4,5] and electron microscopic studies [6–9] of the structures adopted by monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol and mixtures thereof. The general consensus based on these studies with isolated naturally-ocurring lipids, which tend to be highly unsaturated, is that monogalactosyldiacylglycerol, the major thylakoid acyl lipid, paradoxically tends to form an hexagonal H_{II} structure rather than bilayers in excess water. The other thylakoid lipids, digalactosyldiacylglycerol, sulphoquinovosyldiacylglycerol and phosphatidylglycerol all form bilayer structures. When monogalactosyldiacylglycerol is codispersed in aqueous systems with digalactosyldiacylglycerol [10,11] and other lipids extracted from thylakoid membranes [12] phase separations between the monogalactosyldiacylglycerol and the bilayer forming lipids are observed. In these instances, intermediate phases such as inverted micelles sandwiched between leaflets of a bilayer structure may arise. There have also been some reports of the interaction of chloroplast lipids with other thylakoid components including chlorophyll [13], proteins such as CF_1-CF_0 ATPase [14] and the Photosystem-II complex [15,16].

One of the primary functions with which these lipids must be involved is in the creation of a permeability barrier to the movement of ions and other solutes. Whilst aqueous dispersions of phosphodiacylglycerols have been widely studied with regard to their permeability to protons and inorganic ions [17] little work has been done in this respect with galactosyldiacylglycerols. In this study we have examined the structure and ion permeability of liposomes formed from galactosyldiacylglycerols. This had the two-fold purpose of gaining a closer understanding of the structure-function relationship of galactosyldiacylglycerols and also to form a more realistic basis for the reconstitution of photosynthetic processes into artificial membranes.

Materials and Methods

Materials. All chemicals were of analytical grade and were obtained from B.D.H. Chemicals Limited, Poole, Dorset, U.K. unless otherwise stated. Monensin was a gift from Professor D. Lloyd, Department of Microbiology, University College, Cardiff. Pentadecanoic acid, tripentadecanoylglycerol and egg yolk phosphatidylcholine were purchased from Sigma Company Ltd. (London). Unbleached wheat flour was kindly supplied by Dr. T. Galliard, RHM Research, High Wycombe, Bucks, U.K.

Isolation of wheat flour galactosyldiacylglycerols. The isolation procedure was adapted from that of O'Brien and Benson [18]. Quantification and fatty acid analysis of the galactosylglycerides was performed by GLC using an internal standard of methylpentadecanoate in 15% by weight ethylene glycol succinate silicone (EGSS-X) on 80/100 mesh Chromosorb Q (Supelco, Bellefonte, PA, U.S.A.) packed in a 2 m × 5 mm (internal diameter) glass column. Isothermal runs were usually performed at 185 °C and routine identifications were by co-chromatography with authentic standards. However, all the fatty acids had been fully identified previously (cf. Ref. 19). Molar ratios were calculated from the fatty acid quantitation after correction for the masses of individual acyl groups. Mol% refers to moles/100 moles of lipid.

Microscopy. Aqueous lipid dispersions were routinely examined with a Reichert-Biovar phase contrast light microscope. Aqueous galactosyldiacylglycerol dispersions for negative staining were formed either in 2% (w/v) aqueous ammonium molybdate or made 2% (w/v) with respect to ammonium molybdate after swelling [20]. Wheat flour galactosyldiacylglycerols (1 μmol) hydrated for 16 h in 250 μl of distilled water (under nitrogen) were examined by freeze-fracture methods. The sample was thermally quenched from 15 °C in a slurry of nitrogen and fractured at −115 °C in a Polaron freeze-fracture device. Platinum-carbon replicas were examined in a Philips EM 301G electron microscope or a Philips EM 400 electron microscope.

Permeability experiments with aqueous lipid dispersions. Solutions of galactosylacylglycerols or egg-yolk phosphatidylcholine in chloroform/methanol (2:1), v/v) were rotary-evaporated to dryness under N_2. The aqueous solvent was added and swelling allowed to proceed at 25 °C for 2 h with agitation for 30 s at 30 and 60 min. This ensured complete removal of lipid from the sides

of the container (see Ref. 9).

For permeability studies it was necessary to separate the aqueous lipid dispersion from the original swelling solution. Gel filtration on Sephadex G-50 columns was performed for phosphatidylcholine liposomes [21]. This method could not be used for the galactosyldiacylglycerol dispersions since the lipid could not be eluted quantitatively from the column. The galactosyldiacylglycerol suspensions were sedimented at $2000 \times g$ for 5 min and resuspended in appropriate solutions. The centrifugations and resuspensions were repeated twice. The lipid composition of the final galactosyldiacylglycerol suspension was identical to the original mixture, showing that both galactolipids had been effectively hydrated and removed from the sides of the container [9].

Light-scattering experiments. These were carried out essentially as described by Bangham [20]. Osmotic shrinkage was followed by an increase in light scattering at 450 nm after galactosyldiacylglycerols swollen in distilled water were added to KCl solutions of increasing concentrations. Reversible osmotic swelling of galactosyldiacylglycerol mixtures was begun by adding 200 μl of the lipid dispersion formed in 18 mM KCl to 2.7 ml of distilled water and the absorbance was observed for 15 min. The suspension was gently stirred as the galactosyldiacylglycerol suspensions were found to settle at a slow but detectable rate; after stirring, light scattering returned to its initial value. The addition of 100 μl of 0.504 M KCl returned the lipid dispersion to isotonic conditions and the new light-scattering reading was recorded and observed for 5 min to ensure stability. Controls contained 200 μl of the lipid dispersion added to 2.8 ml 18 mM KCl.

Experiments with carboxyfluorescein. The fluorescent dye carboxyfluorescein ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm) has been developed as an aqueous space marker for liposomes by Weinstein et al. [21]. Fluorescence was recorded on a Hitachi MP/Perkin Elmer fluorescence spectrometer. Before use carboxyfluorescein (Eastman Kodak Company, Rochester, NY 140) was recrystallised from hot ethanol/water (1 : 2, v/v) after treatment with activated charcoal.

The permeability of vesicles formed from wheat flour digalactosyldiacylglycerol was found by swelling in 20 mM carboxyfluorescein (K^+ salt) (pH 7.4); and exchanging with 17 mM KCl, 2 mM KH_2PO_4/K_2HPO_4 (pH 7.4). Leakage of carboxyfluorescein was found by diluting a 200 μl sample of the lipid dispersion in the exchange buffer, centrifuging and measuring fluorescence directly. Rates were expressed as a percentage of total carboxyfluorescein released against time.

The trapped volume (aqueous space) of galactosyldiacylglycerol dispersions containing an increasing percentage of monogalactosyldiacylglycerol in digalactosyldiacylglycerol was found by swelling 2 μmol of the lipid mixtures in 20 μM carboxyfluorescein (K^+ salt) (pH 7.4). After exchange with 17 mM KCl, 2 mM KH_2PO_4/K_2HPO_4 (pH 7.4); the lipid was resuspended in 5 ml of buffer containing 100 μl Triton X-100 to destroy any permeability barrier. After centrifugation at $200 \times g$ for 5 min to clarify the solutions, fluorescence was measured as before and the internal volume of the lipid dispersion found from the volume of assayed solution after making allowance for dilution. Controls contained the same concentration of Triton X-100 added after centrifugal removal of liposomes.

Proton permeabilities. After swelling in a buffer of high acidity the liposomes were resuspended in 0.1–1.5 ml lightly buffered isotonic solution at high pH. These suspensions were checked for lipid composition to exclude the possibility of selective enrichment by digalactosyldiacylglycerol (see permeability experiments above). The liposomal suspension was then placed in a 4 cm deep, water-jacketed, perspex well, fitted over a magnetic motor which rotated a small flea to stir the suspension. The pH microelectrode was inserted into the well with a perspex jacket that fitted tightly into the well to prevent exchange of carbon dioxide. The jacket had a 1 mm wide groove parallel to the length of the well for injection of ionophores through a microsyringe. Initial rates of change of pH were measured after the pH microelectrode had adjusted to the new buffer and were expressed as a percentage of the total pH change found after the rapid collapse of the pH gradient with ionophores. Any pH gradient was routinely collapsed by addition of 0.5 μg of monensin in 1 μl of ethanol (this quantity of ethanol was found to have no effect on pH equilibration rates). Back-

ground rates of pH change were found to be negligible and could, therefore, be ignored in calculating initial rates. The electrode, jacket and well were always rinsed with ethanol and buffer before each experiments. Detailed experimental conditions are given in the legends to figures.

Results

The fatty acid content of galactosyldiacylglycerols

Linoleate was the predominant fatty acyl group in both wheat flour monogalactosyldiacylglycerol and digalactosyldiacylglycerol (Table I). These lipids had double bond indices (number of double bonds per molecule) of 3.6 and 3.5, respectively, compared to values of about 5.4 and 5.1 for the same compounds derived from leaves [1].

Aqueous dispersions of galactosyldiacylglycerols

Dry films of the two galactosyldiacylglycerols, and mixtures thereof, were swollen in the presence of water or aqueous solutions. Observation of the dispersions by phase-contrast microscopy revealed that digalactosyldiacylglycerol and mixtures of the galactosyldiacylglycerols containing up to 30 mol% of monogalactosyldiacylglycerol formed highly birefringent vesicles. As the concentration of monogalactosylglycerol was increased the birefringent structures became less frequent until at 50 mol% monogalactosyldiacylglycerol they were completely replaced by poorly birefringent lipidic particles (data not shown).

Electron microscopy of aqueous dispersions of galactosyldiacylglycerols

Negative staining of samples of galactosyldiacylglycerols confirmed that digalactosyldiacylglycerol was able to form vesicles, including multilamellar structures, when dispersed in aqueous solution. In contrast, monogalactosyldiacylglycerol at 50 mol% or 75 mol% in mixtures of the galactosyldiacylglycerols showed regions of extensive hexagonal H_{II} structures (data not shown).

Freeze-fracture samples of wheat flour digalactosyldiacylglycerol consisted almost entirely of lamellar sheets or multiple bilayers in concentric rings resembling the classic multi-lamellar liposome (Fig. 1a). Addition of only 20 mol% monogalactosyldiacylglycerol in digalactosyldiacylglycerol resulted in considerable heterogeneity in the structures observed (Fig. 1b) with areas of bilayers interrupted by vesicle-like structures. An unusual feature was a series of stud-like particles or pits, apparently regularly spaced and of about 25–45 nm which occurred on the rim of raised lamellar structures.

When the monogalactosyldiacylglycerol content was increased to 50 mol% the lipid mixture became more heterogeneous (Fig. 1c) with hexagonal H_{II} phases present as well as vesicles. This suggested that lipid phase separation had taken place during swelling. Pure monogalactosyldiacylglycerol in excess water appeared homogeneous and consisted entirely of extensive bundles of cylinders of hexagonal H_{II} phase (Fig. 1d).

Permeability of galactosyldiacylglycerol liposomes to potassium

Having established the morphology of structures in aqueous dispersions of galactosyldiacylglycerol mixtures we then conducted several types of permeability experiments. These had the twofold aim of determining, firstly, whether galactosyldiacylglycerols themselves could form a suffi-

TABLE I

THE FATTY ACID COMPOSITION OF LIPIDS USED

16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic; n.d., none detected. Means ± S.D., $n = 5$. PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol ; DGDG, digalactosyldiacylglycerol.

Lipid	Fatty acids (% total mass)						
	16:0	16:1	18:0	18:1	18:2	18:3	Others
Egg-yolk PC	32.10 ± 0.53	1.54 ± 0.41	12.68 ± 0.51	31.90 ± 0.68	16.45 ± 0.78	n.d.	5.65 ± 0.76
Wheat-flour MGDG	8.69 ± 0.54	0.14 ± 0.04	0.42 ± 0.07	7.87 ± 0.32	77.07 ± 0.16	5.78 ± 0.27	n.d.
Wheat-flour DGDG	12.62 ± 0.18	0.27 ± 0.11	0.65 ± 0.38	5.32 ± 0.41	74.33 ± 0.40	6.83 ± 0.26	n.d.

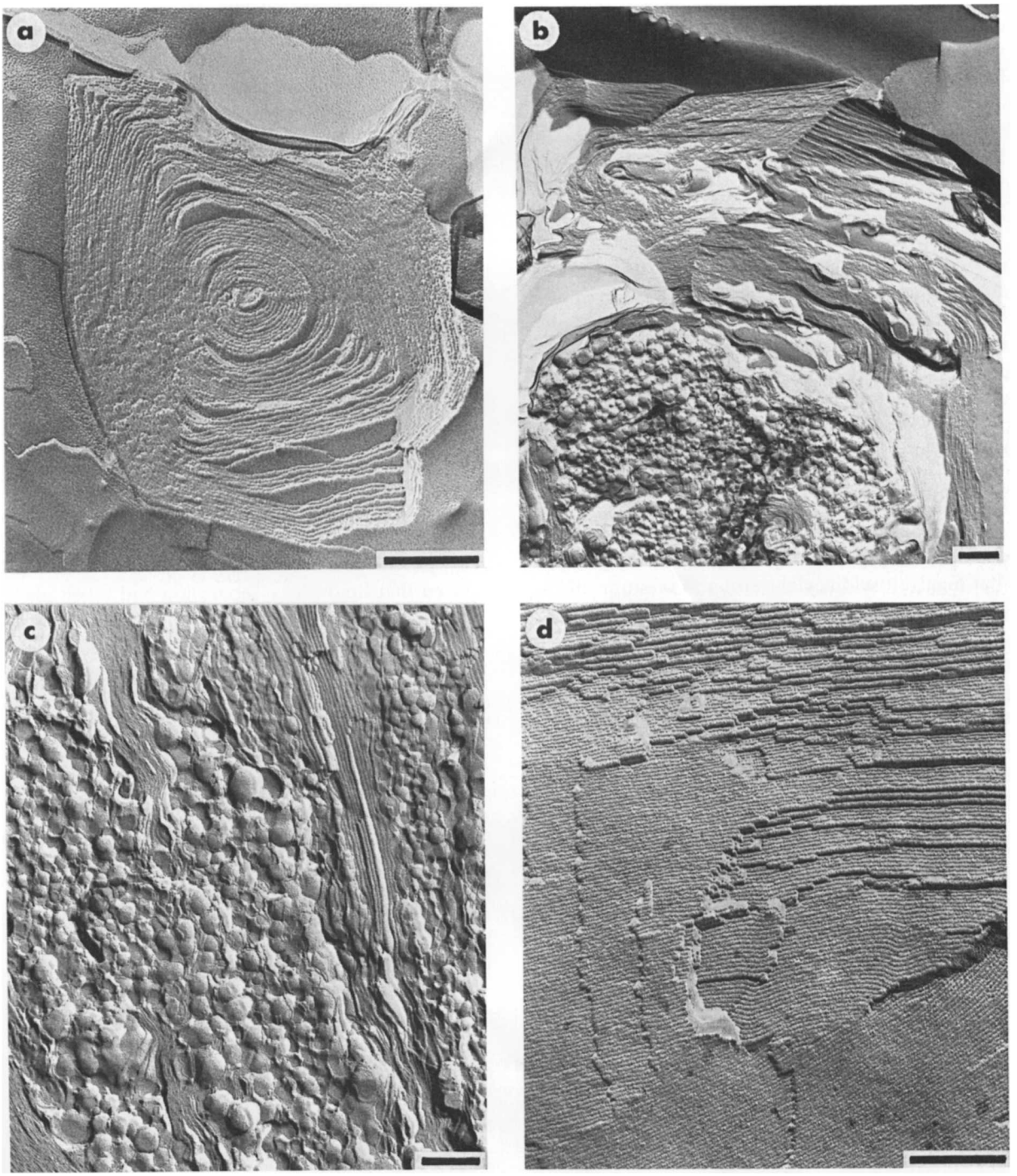

Fig. 1. Electron micrographs of freeze-fracture replicas of wheat-flour galactosyldiacylglycerols swollen in distilled water. (a) Digalactosyldiacylglycerol; (b) digalactosyldiacylglycerol/monogalactosyldiacylglycerol, 4:1 molar ratio; (c) digalactosyldiacylglycerol/monogalactosyldiacylglycerol, 1:1 molar ratio; (d) monogalactosyldiacylglycerol (bars = 150 nm).

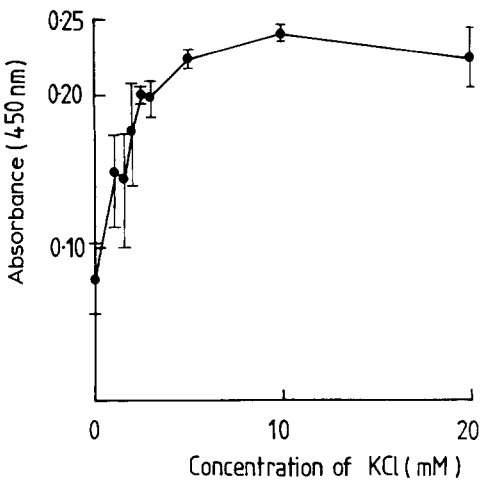

Fig. 2. Light-scattering changes in dispersions of wheat-flour digalactosyldiacylglycerol swollen in water upon addition of KCl. 1 mg of digalactosyldiacylglycerol was swollen with 2 ml distilled water as described in Materials and Methods and 200 μl aliquots were added to 2.8 ml KCl solution. Light scattering was recorded at 450 nm after 10 min. Results are means ± S.D. for three experiments.

cient permeability barrier to support chloroplast functions and, secondly, whether changes in physical structure observed by microscopy could be parallelled by changes in permeability.

Bangham [20] correlated volume changes in liposomes with changes in absorbance reading at 450 nm. Thus in hypotonic solution, the liposomes became swollen and light scattering decreased. The technique could, therefore, be used in experiments to determine whether a permeability barrier existed to the movement of the particular solutes.

Digalactosyldiacylglycerol liposomes formed in distilled water had a greater light scattering at 450 nm as the external concentration of potassium chloride was increased (Fig. 2). This indicated that increasing osmotic shrinkage of the liposomes takes place as the potassium chloride concentration increased. Control experiments where the liposomes were swollen in potassium chloride solutions and then diluted in solutions at the same molarity did not show any increase in light scattering, thus confirming that the change in light scattering was due to osmotic effects and not due to the dilution process per se. Therefore, the liposomes maintained a diffusion barrier to the movement of potassium chloride during the time-course of the experiment.

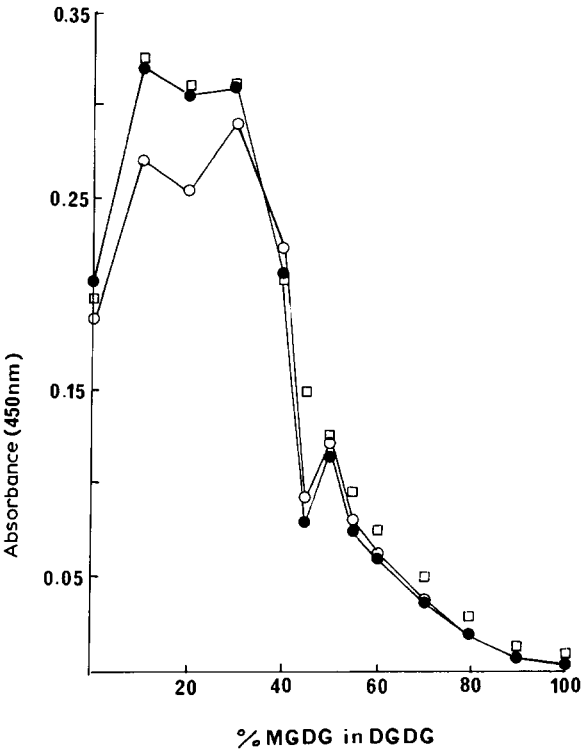

Fig. 3. The light scattering and reversible osmotic swelling of wheat-flour digalactosyldiacylglycerol containing an increasing molar proportion of wheat-flour monogalactosyldiacylglycerol. (●) Galactosyldiacylglycerol liposomes formed and diluted in 18 mM KCl; (○) galactosyldiacylglycerol liposomes formed in 18 mM KCl and diluted in distilled water; (□) galactosyldiacylglycerol liposomes formed in 18 mM KCl, diluted in distilled water and restored to 18 mM KCl. For experimental details see Materials and Methods.

When mixed lipid dispersions of wheat-flour digalactosyldiacylglycerol and monogalactosyldiacylglycerol were prepared in 18 mM potassium chloride solution and diluted in distilled water, osmotic swelling of the lipid dispersion was observed in mixtures containing up to 40 mol% of monogalactosyldiacylglycerol. With increased proportions of monogalactosyldiacylglycerol in the mixture a reversible osmotic swelling of the lipid dispersion was not detected and light scattering decreased rapidly as the monogalactosyldiacylglycerol concentration was increased (Fig. 3). Therefore, above 40 mol% monogalactosyldiacylglycerol in digalactosyldiacylglycerol the lipid dispersion did not act as a permeability barrier to diffusion of potassium chloride. The aqueous space

TABLE II

TRAPPED VOLUMES OF GALACTOSYLDIACYLGLYCEROL DISPERSIONS DETERMINED BY CARBOXYFLUORESCEIN DYE RETENTION

MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

MGDG/DGDG (mol%)	Volume trapped (μl per μmol of lipid)			
	Expt. 1 ($n = 3$)	Expt. 2 ($n = 3$)	Expt. 3 ($n = 3$)	Mean ($n = 9$)
0	1.5±0.2	1.6±0.3	1.9±0.1	1.7±0.3
10	1.2±0.2	1.6±0.3	2.2±0.2	1.6±0.5
20	1.2±0.3	1.0±0.3	0.8±0.6	1.0±0.4
30	1.2±0.1	0.4±0;2	0.6±0.1	0.7±0.4
35	0.1±0.1	0.2±0.1	0.7±0.2	0.6±0.4
40	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1
45	trace	trace	n.d.	trace
50	n.d.	n.d.	n.d.	n.d.

volume of galactosyldiacylglycerol dispersion was measured using a carboxyfluorescein dye method. In the absence of ionophore, the digalactosyldiacylglycerol liposomes exhibited an average rate of carboxyfluorescein leakage of $0.061\% \cdot min^{-1}$ ($n = 3$). The results showed a correlation between the presence of a trapped aqueous volume and osmotically dependent changes in light scattering for different mixtures of galactosyldiacylglycerols (see Discussion and Table II). This further evidence for a permeability barrier at up to 40 mol% monogalactosyldiacylglycerol in digalactosyldiacylglycerol and thus the experiments with carboxyfluorescein corroborated the light scattering experiments. No dye entrapment could be detected when the mol% of monogalactosyldiacylglycerol in digalactosyldiacylglycerol exceeded the proportion where osmotically dependent changes in light scattering had ceased (i.e. 40 mol%). It was also noteworthy that light scattering decreased shortly after the trapped volume had decreased to zero, probably indicating some aggregation of the lipid structures.

Permeability of galactosyldiacylglycerol liposomes to protons

Liposomes of both wheat-flour digalactosyldiacylglycerol and egg-yolk phosphatidylcholine were able to maintain a pH gradient which was only slowly dissipated under the experimental conditions used (Table III; Figs. 4 and 5). In all experiments the initial rates of pH change were of approximately the same order. The use of ionophores demonstrated that the rate of pH equilibration was limited by a diffusion potential.

It was of primary importance to measure the initial rates of pH equilibration to determine how efficiently the thylakoid galactosyldiacylglycerols were at maintaining a pH gradient; the use of ionophores was of secondary interest. Monensin was used to rapidly equilibrate the pH at the end of each experiment in order to calculate the total, final pH change attributable to titration of the

TABLE III

RATE OF pH EQUILIBRATION FOR EGG-YOLK PHOSPHATIDYLCHOLINE OR WHEAT-FLOUR DIGALACTOSYLDIACYLGLYCEROL LIPOSOMES

pH measurements were made by the pH electrode method (see Materials and Methods). Valinomycin was added (0.4 $\mu g \cdot ml^{-1}$ final concentration). Results are expressed as means (±S.D.) with the number of experiments in parenthesis. PC, egg-yolk phosphatidylcholine; DGDG, wheat-flour digalactosyldiacylglycerol; n.m., not measured.

Lipid	Internal buffer		External buffer		pH equilibration (% min^{-1})	
					initial rate	after valinomycin addition
PC	100 mM KH_2PO_4;	pH 5	66 mm K_2SO_4, 0.5 mM K_2HPO_4;	pH 7.4	1.7±0.3 (3)	9.6 (1)
PC	50 mM EPPS;	pH 5.7	33 mM K_2SO_4, 0.5 mM EPPS;	pH 8.2	1.8±0.7 (3)	n.m.
DGDG	100 mM EPPS;	pH 5.7	33 mM K_2SO_4, 0.5 mM EPPS;	pH 8.4	1.5 (2)	11.7 (2)
DGDG	50 mM KH_2PO_4;	pH 5	33 mM K_2SO_4, 0.5 mM K_2HPO_4;	pH 7.4	1.8±0.2 (3)	50.5 (2)
DGDG	100 mM KH_2PO_4;	pH 5	66 mM K_2SO_4, 0.5 mM K_2HPO_4;	pH 7.4	2.7±0.9 (8)	15.6±6.3 (6)
DGDG	100 mM NaH_2PO_4;	pH 5	66 mM Na_2SO_4, 0.5 mM Na_2HPO_4	pH 7.4	2.2±1.0 (13)	4.0±2.4 (12)

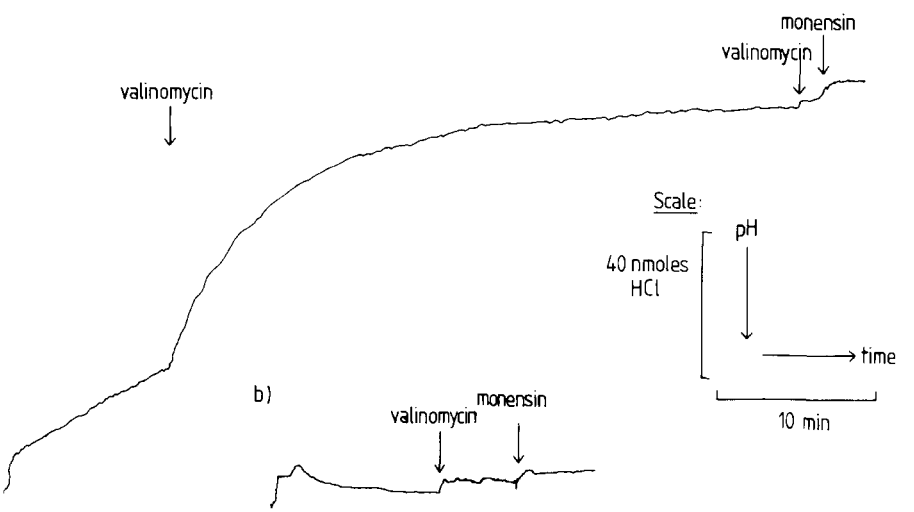

Fig. 4. The rate of dissipation of a pH gradient across bilayers of wheat-flour digalactosyldiacylglycerol: the effect of valinomycin and monensin in the presence of K^+. Liposomes were prepared with 5 mg of digalactosyldiacylglycerol in 100 mM KH_2PO_4; pH 5 and exchanged with 66 mM K_2SO_4, 0.5 mM KH_2PO_4/K_2HPO_4; pH 7.4 (final volume = 1.5 ml). The pH electrode procedure is given in Materials and Methods. Temperature 20 ° C. (a) The rate of pH equilibration of digalactosyldiacylglycerol liposomes and the effect of addition of valinomycin in the presence of potassium ions. (b) Control, buffer only (1.5 ml 66 mK K_2SO_4, 0.5 mM KH_2PO_4/K_2HPO_4; pH 7.4). Valinomycin (0.4 $\mu g \cdot \mu l^{-1}$) and monensin (0.5 $\mu g \cdot \mu l^{-1}$) were added in ethanol (1 μl) as indicated. The electrode was calibrated by the addition of 4 μl 0.01 M HCl.

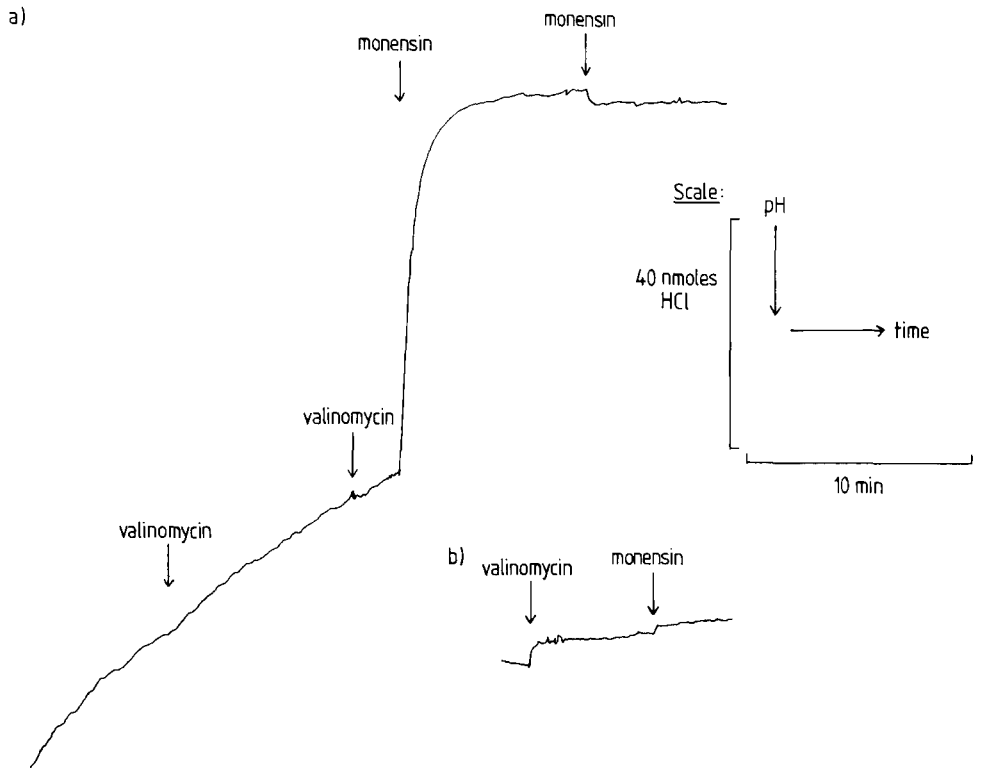

Fig. 5. The rate of dissipation of a pH gradient across bilayers of wheat-flour digalactosyldiacylglycerol: the effect of valinomycin and monensin in the presence of Na^+. Liposomes were prepared with 5 mg of digalactosyldiacylglycerol in 100 mM NaH_2PO_4; pH 5 and exchanged with 66 mM Na_2SO_4, 0.5 mM NaH_2PO_4/Na_2HPO_4; pH 7.4 (final volume 1.5 ml) (a) The rate of equilibration of digalactosyldiacylglycerol liposomes and the effect of valinomycin and monensin addition in the presence of sodium ions. (b) Control, buffer only (1.5 ml 66 mM Na_2SO_4, 0.5 mM NaH_2PO_4/Na_2HPO_4; pH 7.4). Valinomycin (0.4 $\mu g \cdot \mu l^{-1}$) and monensin (0.5 $\mu g \cdot \mu l^{-1}$) were added in ethanol (1 μl) as indicated. The electrode was calibrated by the addition of 4 μl 0.01 M HCl.

external liposomal buffer by the internal liposomal buffer. Monensin, which acts as an ionophore for H^+, Na^+ and K^+, was found to equilibrate the pH between the internal and external compartments almost instantaneously at the concentrations used, the initial rate being approx. $24 \cdot 10^{11}$ H^+/s. Additional monensin did not result in any further pH change (Fig. 5).

Valinomycin was utilized to check whether a diffusion potential was limiting proton movement. In Figs. 4 and 5 the rate of pH equilibration was at $7 \cdot 10^{11}$ H^+/s. However, the addition of valinomycin resulted in a 3-fold increase of this rate. Further addition of valinomycin and, shortly afterwards, of monensin, after pH equilibration resulted in no further pH change (Fig. 4). However, when potassium was replaced by sodium as the major cation there was no significant increase in the rate of pH equilibration after valinomycin addition (Fig. 5). The addition of monensin resulted in an almost instantaneous equilibration of the pH, as evidenced by the failure of a further addition of monensin to affect the external pH.

Discussion

The preference of wheat-flour monogalactosyldiacylglycerol to form an hexagonal H_{II} phase in excess aqueous medium, as shown by freeze-fracture electron microscopy (Fig. 1d), is in agreement with the X-ray diffraction data for monogalactosyldiacylglycerol from wheat-flour [4] and *Pelargonium* leaves [5] and with freeze-fracture electron microscopy of this lipid from the *Vicia faba* [22]. Although monogalactosyldiacylglycerol readily forms lipid monolayers at the air-water interface [23] it adopts an hexagonal H_{II} phase in bulk aqueous solutions.

Gounaris et al. [8] reported that reduction, by hydrogenation, of as little as 0.5 double bonds per molecule of monogalactosyldiacylglycerol isolated from *Vicia faba* was sufficient to cause substantial conversion from hexagonal H_{II} structure to a lamellar phase. The double bond index after partial hydrogenation was 4.0–4.5 compared to 3.6 for the wheat-flour monogalactosyldiacylglycerol used in the present work. However, the positions of the reductions were not determined and it is known that the position of the double bonds has a considerable effect on chain disorder and hydrocarbon volume (see also Ref. 24). Moreover, it has been reported that dilinoleoylmonogalactosyldiacylglycerol (as in wheat flour) has a very similar packed volume to dilinoleoylmonogalactosyldiacylglycerol (as in leaves) [23]. Hydrogenation also produces extensive amounts of *trans* double bonds [24] which behave more like saturated than *cis*-unsaturated fatty acids. Thus, the failure of Gounaris et al. [8] to observe hexagonal H_{II} phases with monogalactosyldiacylglycerol of an average double bond index as high as 4.0–4.5 is not necessarily at variance with the present data.

The observation by three different microscopic methods that wheat-flour digalactosyldiacylglycerol forms bilayer structures (liposomes and lamellar sheets) was in agreement with the X-ray diffraction data of Larsson and Puang-Ngern [4] for wheat-flour lipid and the freeze-fracture results for *Vicia faba* digalactosyldiacylglycerol of Sen et al. [10,11].

For binary mixtures of monogalactosyldiacylglycerol and digalactosyldiacylglycerol the lamellar structures showed evidence of phase separations of non-lamellar structures formed with 50 : 50 molar proportions of the two galactosyldiacylglycerols (Fig. 1c). The structures observed by various methods agreed with the excess water regions of ternary phase diagrams for wheat-flour galactosyldiacylglycerols obtained by Larsson and Puang-Ngern [4] using X-ray diffraction. However, the freeze-fracture experiments reported here were able to differentiate between various structures in the broad transition region between lamellar and hexagonal phases. A surprising feature of the results presented here and of the X-ray diffraction data of Larsson and Puang-Ngern [4] was evidence for the co-existence of liposomes, hexagonal-liquid crystals and water; there being no critical molar ratio where lamellar phase was suddenly transformed into hexagonal phase. In the freeze-fracture electron micrographs at 20 and 50 mol% monogalactosyldiacylglycerol in digalactosyldiacylglycerol the former had various lamellar-related structures and particles whilst in the latter, hexagonal H_{II} phase lay side by side with vesicles (Figs. 1b and 1c). This suggests that lipid phase separation may occur during swelling of the lipids. The presence of particles, ridges, pits and depres-

sions for 20 and 50 mol% monogalactosyldiacylglycerol in digalactosyldiacylglycerol were also reported by Sen et al. [10,11] for *Vicia faba* galactosylacylglycerol mixtures, and interpreted as regions of deformation in the lamellar structures.

The large quantities of the hexagonal H_{II} phase-forming lipid monogalactosyldiacylglycerol in chloroplasts may be needed for packaging intrinsic membrane proteins (see Ref. 2) or for stabilising the curved regions of thylakoids [28,29]. The slight enrichment of the lipid in granal relative to stromal thylakoids (see also Refs. 2 and 30) would support the latter suggestion but enrichment in the outer leaflet would not [2,31]. Nevertheless, Figs. 1a and 1b show that 20 mol% monogalactosyldiacylglycerol in galactosylacylglycerol mixtures has the ability to increase the curvature of vesicles.

Sprague and Staehelin [9] found that the swelling procedure used here and by Sen et al. [10,11] could possibly leave monogalactosyldiacylglycerol adhering to the vessel surface during swelling. However, in the present work, vortexing ensured complete uptake of monogalactosyldiacylglycerol (see Materials and Methods). Sprague and Staehelin [9] reported that without sonication 30–40 mol% monogalactosyldiacylglycerol could be accommodated into digalactosyldiacylglycerol liposomes (Fig. 3), in agreement with the results reported here.

The changes in light scattering for digalactosyldiacylglycerol liposomes containing up to 30 mol% monogalactosyldiacylglycerol showed that these lipids were able to maintain a potassium gradient during the time-course of the experiment (Fig. 3). The inability of galactosyldiacylglycerol liposomes containing up to 60–70 mol% monogalactosyldiacylglycerol (the ratio found in chloroplasts [1]) may be due to the presence of inverted micelles within the lamellar phase which would allow free ion exchange with the aqueous medium. Therefore, a critical function of the thylakoid membrane, namely to maintain chemiosmotic solute gradients, cannot be due to the galactosylacylglycerol complement only but also depends on the stabilising effect of other lipids (see Ref. 2) as well as thylakoid proteins. In this context it is noteworthy that a membrane such as the outer envelope of the chloroplast that contains a predominance of typical bilayer-forming lipids such as digalactosyldiacylglycerols and phosphatidylcholines is relatively permeable.

The light-scattering values for galactosyldiacylglycerol dispersions in isotonic medium probably reflected the size and number of the lipidic particles. Digalactosyldiacylglycerol liposomes, would be large with relatively large trapped volume; this was confirmed by direct assay of liposome trapped volume (Table II). The addition of monogalactosyldiacylglycerol would have led to more curved bilayers and thus a greater number of smaller vesicles. This was consistent with microscopic data (Fig.s 1b and c). The decrease in light scattering values in dispersions containing greater than 40 mol% of monogalactosyldiacylglycerol in digalactosyldiacylglycerol was because of increased lipid cohesion with the production of the hexagonal H_{II} phase which tends to form extensive structures in aqueous media.

The acceleration of pH equilibrium by valinomycin in the presence of potassium ions, but not sodium (Table III), is consistent with the 10 000 : 1 preference of valinomycin for K^+ over Na^+. It is also clear that digalactosyldiacylglycerol is as effective as phosphatidylcholine in forming a permeability barrier to liposomes. It was apparent from the results that an opposing gradient of Na^+ or K^+ was sufficient to maintain a pH gradient by the generation of a diffusion potential. The collapse of this diffusion potential by addition of valinomycin resulted in a much faster collapse of the pH gradient (Fig. 4). This is also true in vivo where valinomycin is known to act as an uncoupler of oxidative phosphorylation [32] presumably by dissipating a K^+ gradient opposing the proton gradient and thus allowing proton conductance across the bilayer.

The advantage of a potassium and magnesium ion gradient in an opposite direction to the pH gradient across a thylakoid membrane is to prevent a prohibitive membrane potential from opposing the build-up of a proton gradient [33], the membrane potential of a photosynthesising thylakoid being only about 10 mV. In addition, the results reported here for digalactosyldiacylglycerol and egg-yolk phosphatidylcholine show that the counter movement of K^+ and Mg^{2+} prevents the dissipation of a pH gradient by any back

conductance. This rapid proton conductance is thus opposed by a diffusion potential. Thus the H^+ gradient can be easily maintained during steady-state photosynthesis. In the experiments reported above it is clear that, provided the galactosyldiacylglycerols are capable of adopting the lamellar phase, then these lipids can maintain K^+ and H^+ gradients as effectively as the typical mammalian membrane lipid, phosphatidylcholine.

Acknowledgments

This study was supported by the SERC (studentship to A.A.F.). The authors are grateful to Dr. A.D. Bangham for his help and advice during the initial permeability measurements and also for his generosity in making laboratory facilities available to A.A.F.

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