Biochimica et Biophysica Acta, 728 (1983) 129–139
Elsevier Biomedical Press

BBA 71535

THE FORMATION OF NON-BILAYER STRUCTURES IN TOTAL POLAR LIPID EXTRACTS OF CHLOROPLAST MEMBRANES

KLEONIKI GOUNARIS [a], ARINDAM SEN [a], ANTHONY P.R. BRAIN [b], PETER J. QUINN [a] and W. PATRICK WILLIAMS [c]

[a] *Biochemistry and* [c] *Biophysics Departments and* [b] *Electron Microscopy Unit, Chelsea College, University of London, London SW3 6LX (U.K.)*

(Received October 4th, 1982)

Key words: Chloroplast membrane; Lipid vesicle; Non-bilayer; pH dependence; Cation effect; Freeze-fracture; (V. faba)

The structural organisation of aqueous dispersions of total membrane lipid extracts of broad bean (*Vicia faba*) chloroplasts is dependent on pH and the presence of cations. In the absence of inorganic salts, sonicated dispersions of lipid extract in distilled water form smooth, single-shell vesicles approximately 30–50 nm in diameter. Reducing the pH of the dispersions, to neutralise the acidic lipids present in the extract, or the addition of low concentrations of metal cations, leads to the fusion of the vesicles and a partial phase-separation of the non-bilayer forming lipid monogalactosyldiacylglycerol to form spherical inverted micelles similar to those previously reported for binary mixtures of monogalactosyl and digalactosyldiacylglycerol (Biochim. Biophys. Acta 685, 297–306). Increasing concentrations of polyvalent, but not monovalent, cations lead to further structural rearrangements involving the formation of para-crystalline arrays of tubular and spherical inverted micelles. The factors determining the formation of these different structures, and their possible relevance to the structural organisation of the native chloroplast membrane, are discussed.

Introduction

Two classes of membrane lipids, monogalactosyl and digalactosyldiacylglycerol, dominate the lipid composition of the thylakoid membranes of higher plant chloroplasts. Together they account for approx. 75% of the polar lipid content of these membranes [1]. Despite their similarity in chemical structure the physical properties of these two classes of lipid are very different. Monogalactosyldiacylglycerol when dispersed alone in water normally takes up an inverted hexagonal (H_{II}) structure whilst the digalactosyl derivative forms conventional lipid bilayers [2,3]. Freeze-fracture electron microscopy studies performed in this laboratory have shown that aqueous dispersions of binary mixtures of these two lipid classes are characterised by the presence of large numbers of 10–15 nm diameter particles that correspond to inverted lipid micelles sandwiched between the leaflets of lipid bilayers [4–6].

These observations are of particular importance in view of the fact that freeze-fracture replicas prepared from isolated thylakoid membranes are also characterized by the presence of intramembranous particles in this general size range [7]. These particles are normally attributed to the presence of intrinsic membrane proteins associated with the light-harvesting apparatus of the chloroplasts [8,9]. This assignment, however, is based entirely on evidence from electron microscopy studies. No direct biochemical evidence relating to the composition of the particles is available. It is thus clearly of great interest to ascertain whether non-bilayer structures observed in simple binary mixtures of monogalactosyl and digalactosyldiacylglycerols are also formed in total lipid extracts of thylakoid membranes or whether other mem-

brane lipids present in such extracts suppress their formation.

In this paper we have used freeze-fracture electron microscopy and light-scattering measurements to examine the structures formed by polar lipid extracts of chloroplast thylakoid membranes when dispersed in aqueous media. In particular we have characterised the role of acidic lipids in modifying the structure of the binary galactolipid mixtures. This has been done by examining the effects of manipulating the pH or adding different concentrations of inorganic salts to the dispersion media. Finally, we have attempted to draw some conclusions regarding the possible significance of these measurements in terms of the structural properties of the native chloroplast membranes.

Matrials and Methods

Chloroplast and lipid isolation. Chloroplasts were isolated from fresh leaf tissue of 4–5 week post-emergent broad beans (*Vicia faba*; var. Aquadulce) by the method of Stokes and Walker [10]. Total lipid extracts of the freshly isolated chloroplast were prepared according to the procedure of Bligh and Dyer [11]. Neutral lipid and pigments were removed from the extracts by column chromatography on silicic acid [12]. The polar lipid fraction eluted from the column was dried using a rotary evaporator and stored in chloroform.

Lipid analyses. The different lipid classes present in the polar lipid extracts were separated by two-dimensional chromatography on Silica gel G. Plates were developed in solvent consisting of chloroform/methanol/water (65 : 25 : 4, by vol.) in the first direction and then in a solvent of chloroform/methanol/acetic acid/water (85 : 15 : 10 : 3, by vol.) in the second direction. The lipid classes were made visible under ultraviolet light by spraying the plate with aqueous rhodamine 6G (0.01%). The individual lipid spots were scraped off the plates and extracted with chloroform/methanol (1 : 1, v/v). The fatty acid compositions of the lipids were determined by gas chromatography[13].

Turbidity measurements. Samples of the polar lipid extract in chloroform were evaporated to dryness under N_2 and stored under vacuum overnight to remove any residual solvent. The dry lipid samples were dispersed in deoxygenated water at 0°C by ultrasonic irradiation for a total of 2 min (10–15 s bursts separated by similar intervals for cooling) using an Artek 300 sonic dismembrator. Turbidity measurements were made in a 1 cm cell at 400 nm using a Pye Unicam SP1800 spectrophotometer. Small aliquots of 1 M solutions of Analar grade salts containing different monovalent, divalent and trivalent cations were added using a microsyringe. The dispersions were thoroughly mixed and the turbidity measured. Half-times ($t_{1/2}$) were determined by monitoring turbidity changes at different intervals following the addition of salts.

Electron microscopy. Dried samples of polar lipid extracts of chloroplast membranes were dispersed in 30% (w/w) glycerol as described above. They were thermally quenched in a nitrogen slurry and replicas prepared using a Polaron freeze-fracture device. The replicas were cleaned with bleach, washed and dried. All replicas were examined in a Philips EM 301 electron microscope. Estimates of fracture-face sizes were made from measurements of perimeter lengths made on electron micrographs of randomly selected areas of replicas using an Apple II microcomputer with a graphics tablet.

Results

Lipid composition

The composition of a typical total polar lipid extract of broken, broad-bean chloroplast membranes of the type used in this investigation is set out in Table I. The major components are monogalactosyl and digalactosyldiacylglycerol which between them account for 75–80% of the polar lipids present in chloroplast membranes. The other noteworthy feature of these uncharged galactolipids in their relatively high content of polyunsaturated fatty acids, particularly linolenic acid. In addition to these two lipids, the extracts contain the acidic lipids sulphoquinovosyldiacylglycerol and phosphatidylglycerol, accounting for 15–20% of the total lipid, and a small amount ($< 5\%$) of phosphatidylcholine.

Structural studies

A typical electron micrograph of a replica prepared from a polar lipid extract of broad-bean chloroplasts sonicated in distilled water is shown

TABLE I

LIPID COMPOSITION OF TOTAL POLAR LIPID EXTRACTS OF BROAD BEAN CHLOROPLASTS

Lipid class	Mol % total lipid	Fatty acid composition (mol%)				
		16:0	18:0	18:1	18:2	18:3
Monogalactosyldiacylglycerol	50.7	4.1	1.1	3.3	10.7	80.7
Digalactosyldiacylglycerol	27.4	6.9	2.0	2.2	7.2	81.7
Phosphatidylglycerol	4.1	26.8	5.3	15.0	22.5	30.3
Sulphoquinovosyldiacylglycerol	14.4	29.7	7.1	17.6	7.3	38.4
Phosphatidylcholine	3.4	36.3	8.0	17.0	18.6	20.0

in Fig. 1a. These dispersions consist entirely of very small (30–50 nm diameter) single-shell vesicles. The fracture faces of some of the larger of these vesicles occasionally contain one or two intramembranous particles but the faces of the smaller vesicles are invariably smooth. Extensive arrays of inverted micelles and para-crystalline aggregates of inverted lipid micelles associated with binary mixtures of monogalactosyl and digalactosyldiacylglycerol (see Fig. 1b) are not observed.

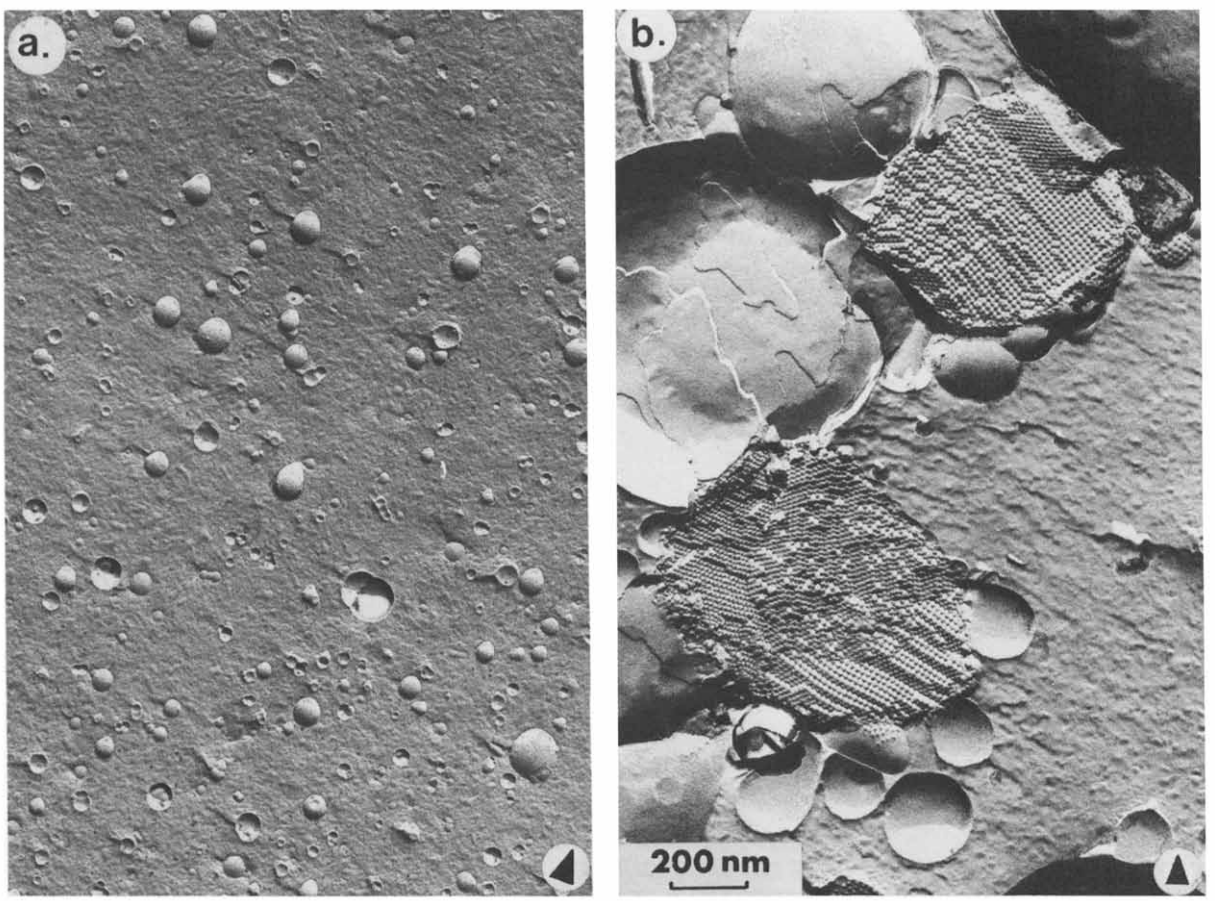

Fig. 1. Electron micrographs of freeze-fracture replicas prepared from sonicated dispersions of (a) a total lipid extract of broken broad bean chloroplasts; and (b) a 2:1 molar ratio mixture of chloroplast monogalactosyl and digalactosyldiacylglycerols. Suspending medium distilled water.

We attribute these very marked differences in structure between dispersions of total polar lipid extracts of chloroplast membranes and dispersions of binary mixtures of the neutral galactolipids to the presence in the extracts of charged acidic lipids. In order to test this hypothesis we investigated the effect of changing the pH of the dispersions and of adding cations capable of neutralising the negatively charged lipids. The presence of cryoprotectants, used in freeze-fracture studies to prevent ice-crystal formation, also influences the formation of non-bilayer structure in galactolipid dispersions [6]. This effect was, therefore, also investigated.

pH-induced changes. The apparent pK_a value of phosphatidylglycerol is thought to be pH 3.0–3.5 [14]. The corresponding value for sulphoquinovosyldiacylglycerol has not been established but sulphonate groups of the type found in this lipid usually show pK_a values at pH < 4.0 [15]. The final pH of dispersions of the lipid extracts in distilled water was normally about pH 5.5. Under these conditions the acidic lipids present in the extracts are almost completely ionised. The very different appearance of the dispersions formed by the lipid extracts and the binary mixtures of neutral galactolipids probably reflects the ability of the acidic lipids to stabilise the small vesicles seen in the former dispersions by charge repulsion effects. The view is supported by the fact that reducing the pH of the samples, leading to a neutralisation of these charged groups, results in a fusion of the single-shell vesicles to form larger lipid aggregates that contain extensive arrays of

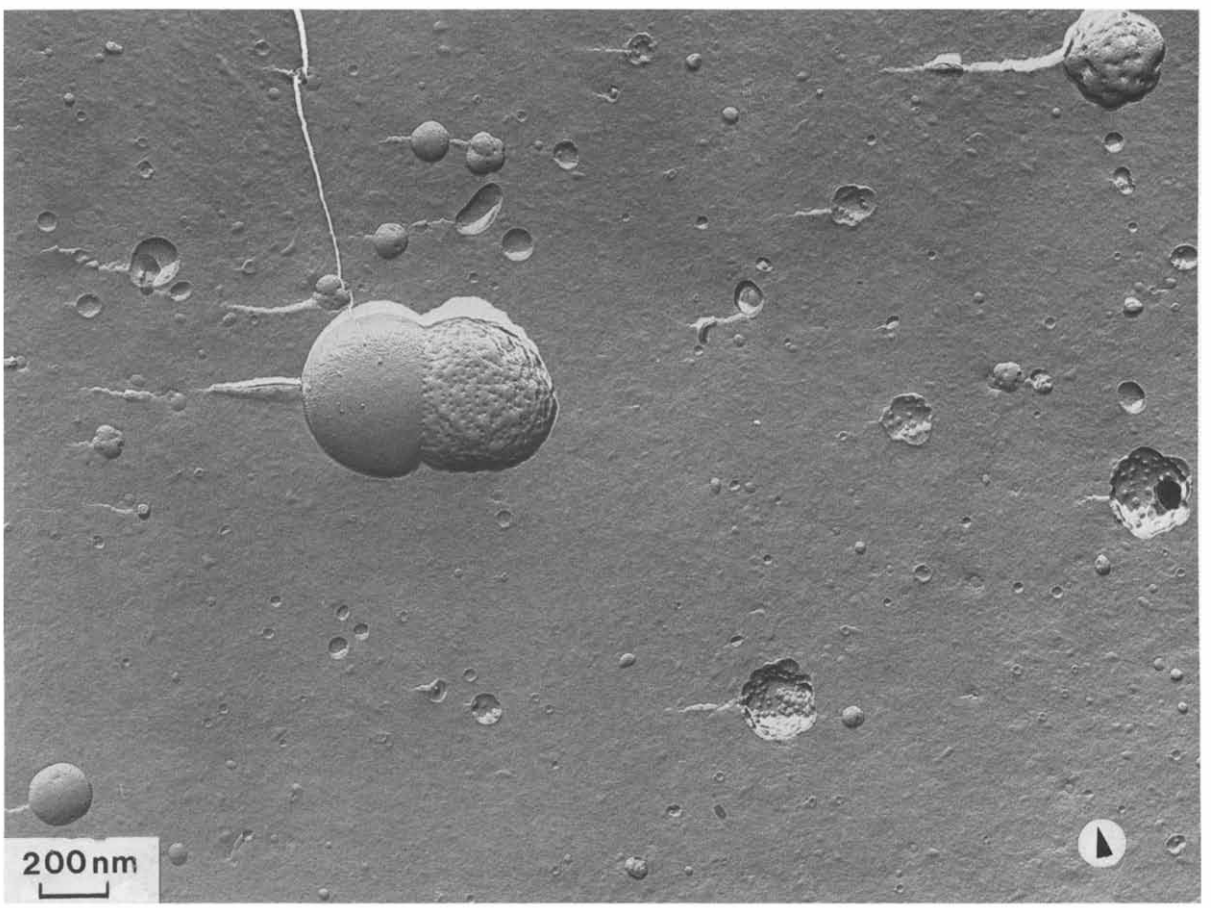

Fig. 2. Electron micrograph of a freeze-fracture replica prepared from a sonicated dispersion of a total polar lipid extract of broken broad bean chloroplasts (pH 3).

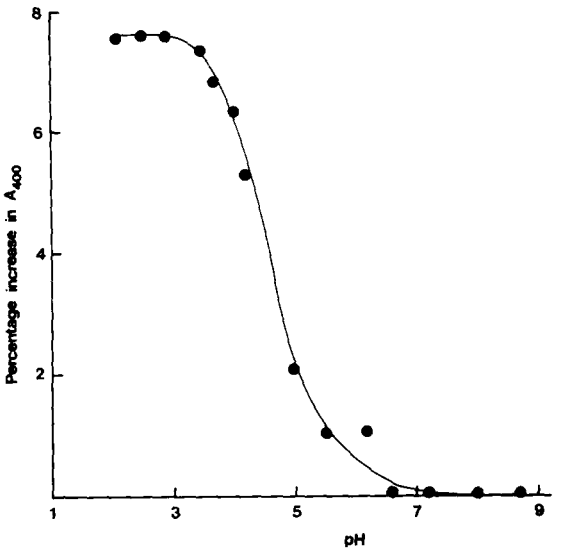

Fig. 3. The pH dependence of light-scattering changes associated with the neutralisation of the acidic lipids of sonicated dispersions of total polar lipid extracts (1 mg·ml^{-1}) of broad bean chloroplasts.

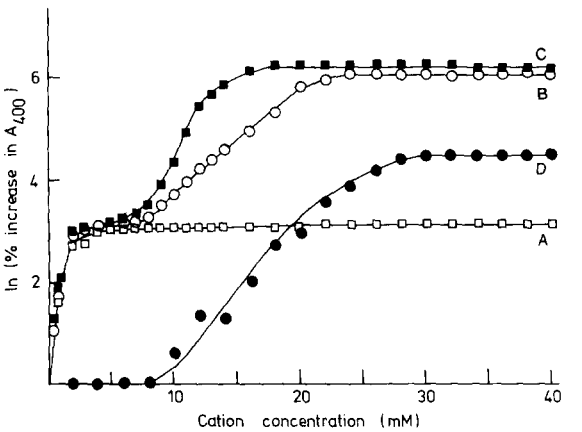

Fig. 4. Light-scattering changes (A_{400}) associated with the addition of cations to sonicated dispersions of total polar lipid extracts of broad bean chloroplasts in distilled water. The effect of monovalent cations, Na^+ (□, A); divalent cations, Mg^{2+} (○, B) and Ca^{2+} (■, C) are shown together with the effect of Mg^{2+} additions on dispersions prepared in the presence of 2 mM NaCl (●, D).

inverted micelles (Fig. 2). Changes in light-scattering accompanying aggregation were followed by measuring the apparent absorbance of the dispersions at 400 nm (A_{400}). A plot of the pH dependence of these changes is shown in Fig. 3. The results suggest that aggregation depends on the neutralisation of a group, or groups, with pK_a values in the range pH 3.5–5.0.

Salt-induced changes. Similar changes in aggregation are observed following the addition of cations to the dispersions. The light-scattering changes associated with cation-induced aggregation are summarised in Fig. 4. Two distinct cation-induced effects can be distinguished. At low concentrations (< 2 mM) addition of monovalent or divalent cations lead to light-scattering changes similar in extent to those observed on decreasing the pH of the dispersions. A further, much larger, increase is observed in the range 10–20 mM for divalent ions but not monovalent ions. Addition of 2 mM Na^+ to the samples, abolishes the light-scattering changes induced by concentrations of divalent cations less than 2 mM but does not affect the changes induced by these cations in the 10–20 mM range (Fig. 4, curve D). This indicates that monovalent cations can substitute for divalent cations in the aggregation process that takes place in dilute salt solutions but not in those changes associated with more concentrated divalent cation solutions. The trivalent cation tris(ethylene diamine)cobaltic chloride leads to similar light-scattering changes to those observed for divalent cations (Fig. 5). The apparent absorption at 650 nm (A_{650}) was used to monitor light scattering in the case of the trivalent cation as its absorbs strongly at 400 nm.

A closer examination of the concentration de-

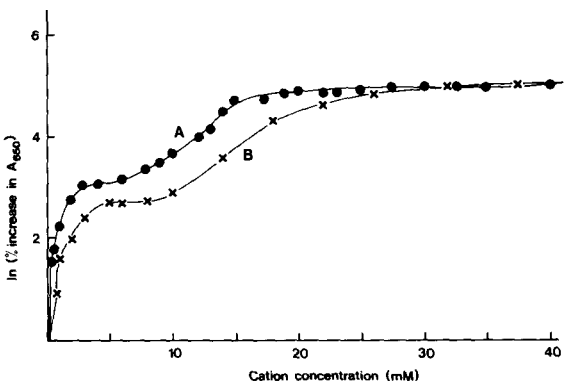

Fig. 5. Light-scattering changes associated with the addition of divalent, Mg^{2+} (x) or trivalent, tris(ethylene diamine)cobaltic^{3+} chloride (●), to sonicated dispersions of broad bean chloroplasts in distilled water. Measurements were made at 650 nm to avoid specific absorption effects of the trivalent cation.

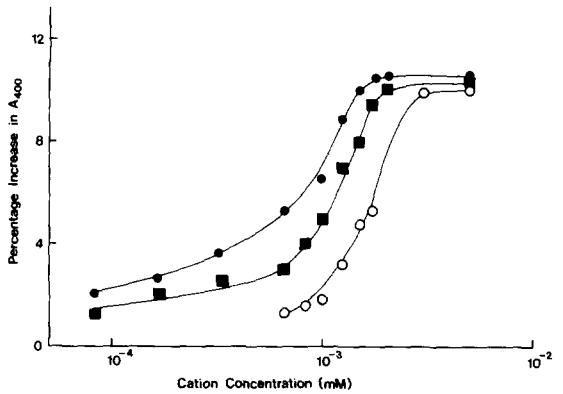

Fig. 6. Plot showing the valence dependence of the low concentration (< 2 mM) cation-induced light scattering changes. Tris(ethylene diamine) cobaltic^{3+} chloride (●); Mg^{2+} (■); Na^{+} (○).

pendence of the light-scattering changes observed in the concentration range below 2 mM indicate that they are dependent on the valence of the added cation (Fig. 6). The light-scattering changes in the concentration range above 10 mM showed no obvious valence dependence but considerable variations were noted in the efficiency of different divalent cations. The concentrations of different divalent cations required to trigger a half maximal response are plotted as a function of the radius of the non-hydrated ions in Fig. 7. The possible significance of these observations is discussed below.

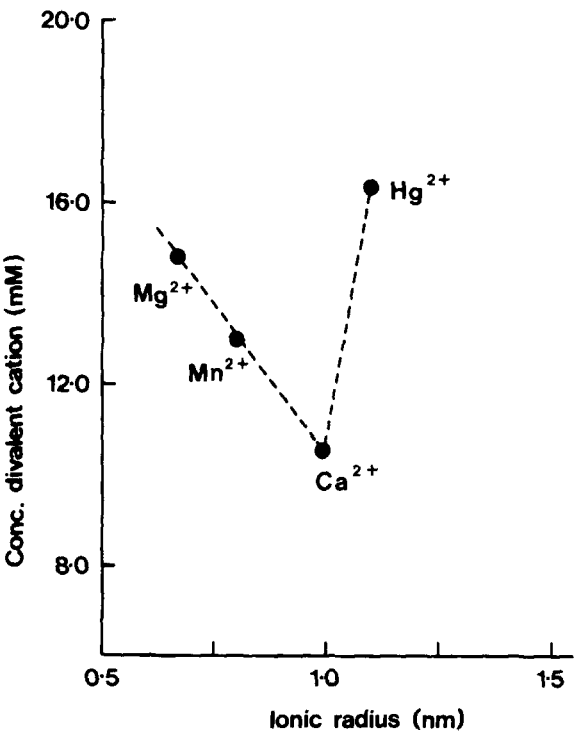

Fig. 7. Plot showing the variation of the efficiency of different divalent cations in inducing the high-concentration (10–20 mM) polyvalent-cation induced light-scattering changes as a function of the ionic radius of the non-hydrated cations.

The light-scattering changes shown in Figs. 3 and 4 were all relatively rapid ($t_{1/2} \approx 10$ s). In addition to these changes, longer-term aggregation involving the flocculation of the dispersions to form very large aggregates clearly visible to the eye occurred in samples containing high concentrations of cations. Dispersions containing more than approx. 100 mM monovalent, or approx. 10 mM divalent, cations tended to show appreciable flocculation if stored at room temperature for more than 2–3 h. No such flocculation was observed in dispersions containing low concentrations of ions even after storage for 24 h.

Electron micrographs showing typical areas of replicas prepared from dispersions containing 1.0 and 7.5 mM Mg^{2+} are presented in Fig. 8. As the Mg^{2+} concentration is increased the small (30–50 nm) diameter single-shell liposomes seen in salt-free dispersions (Fig. 1a) appear to fuse to form larger vesicles containing increasing numbers of 10–12 nm diameter freeze-fracture particles. Histograms showing the distribution of the perimeter lengths of the fracture-faces of the exposed vesicles for dispersions containing different concentrations of Mg^{2+} are presented in Fig. 9. The average diameter of these fracture-faces increases from about 42 nm in the salt-free dispersions to 47 nm in the presence of 1.0 mM Mg^{2+} and 139 nm in the presence of 7.5 mM Mg^{2+}. The small size of the freeze-fracture particles precludes an accurate statistical analysis of their numbers but it is quite clear that the numbers of such particles is greatly increased in the larger liposomes. Similar changes are observed if Na^{+} or K^{+} is substituted for Mg^{2+}.

On increasing the Mg^{2+} concentration to greater than 10–20 mM, much more extensive aggregation takes place. Large lipid aggregates containing extensive arrays of inverted micelles are formed. Structures of this type are not observed on the addition of higher concentrations of monova-

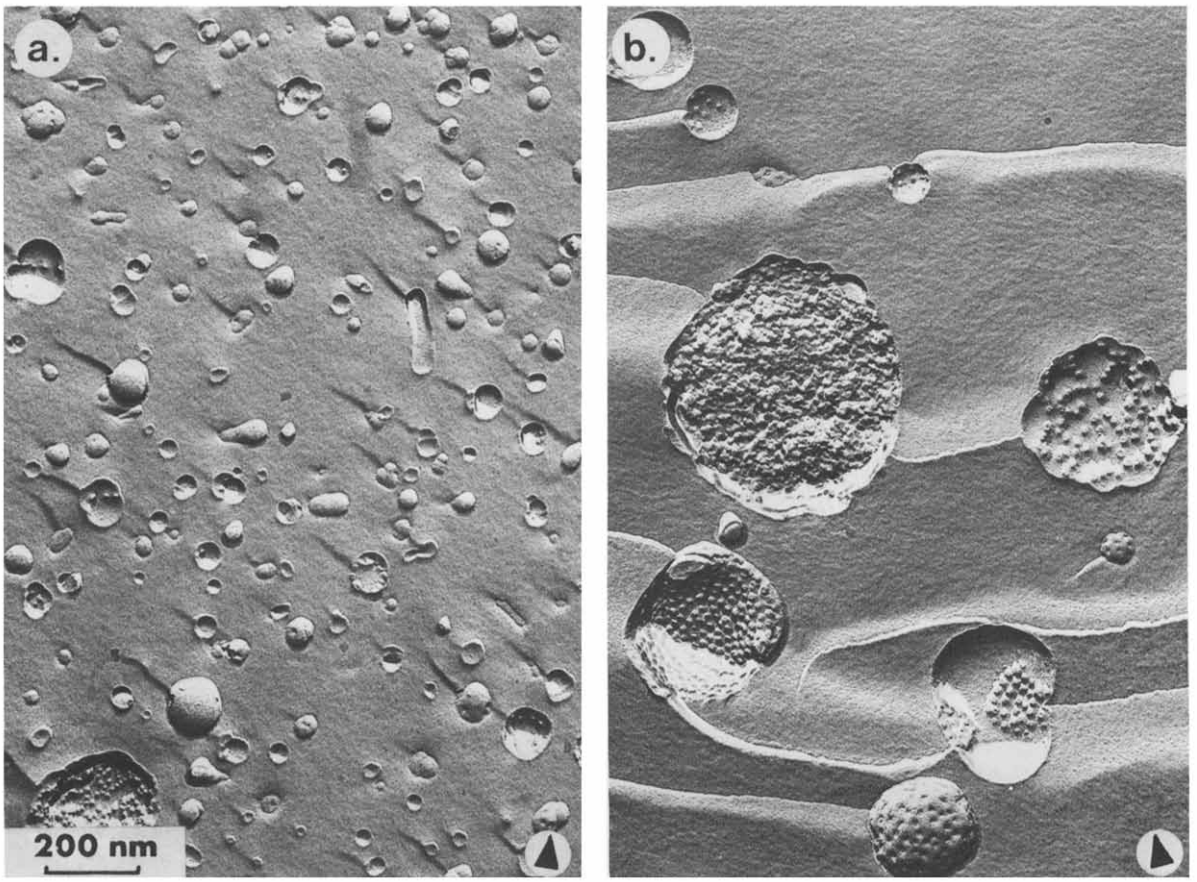

Fig. 8. Typical electron micrographs of freeze-fracture replicas prepared from dispersions of polar lipid extracts of broad bean chloroplasts suspended in (a) 1.0 mM $MgCl_2$; (b) 7.5 mM $MgCl_2$.

lent cations again suggesting that the initial increase in light-scattering observed in the presence of cation concentrations below 2 mM is associated with a vesicle fusion process whilst the second, divalent-specific increase, is associated with some other process.

Electron micrographs showing some of the main features of these larger aggregates are presented in Fig. 10. Whilst they show extended regions of non-bilayer lipid structures, they lack the large para-crystalline arrays of inverted lipid micelles normally seen in replicas prepared from binary mixtures of monogalactosyl and digalactosyldiacylglycerol. One of their most characteristic features, however, in the presence of cylindrical inverted-micellar arrangements (Fig. 10a) that bear a superficial resemblance to the H_{II} structures seen in replicas prepared from pure monogalactosyldiacylglycerols [2]. The diameter of the cylinders is, however, about 10–11 nm as opposed to 5–6 nm in the pure lipid. The cylindrical micelles seen in the polar lipid extracts appear to be sandwiched within individual lipid bilayers (Fig. 10b) and to pack on a cubic or orthorhombic, rather than hexagonal lattice. They are thus much more closely related to the inverted lipid micelle structures that we have previously reported for negatively stained preparations of mixed monogalactosyl and digalactosyldiacylglycerols [2] than conventional H_{II} structures. The occurrence of long parallel rows of freeze-fracture particles in close association with these cylindical structures (Fig. 10c) suggests that they correspond to spherical inverted lipid micelles formed by the budding-off

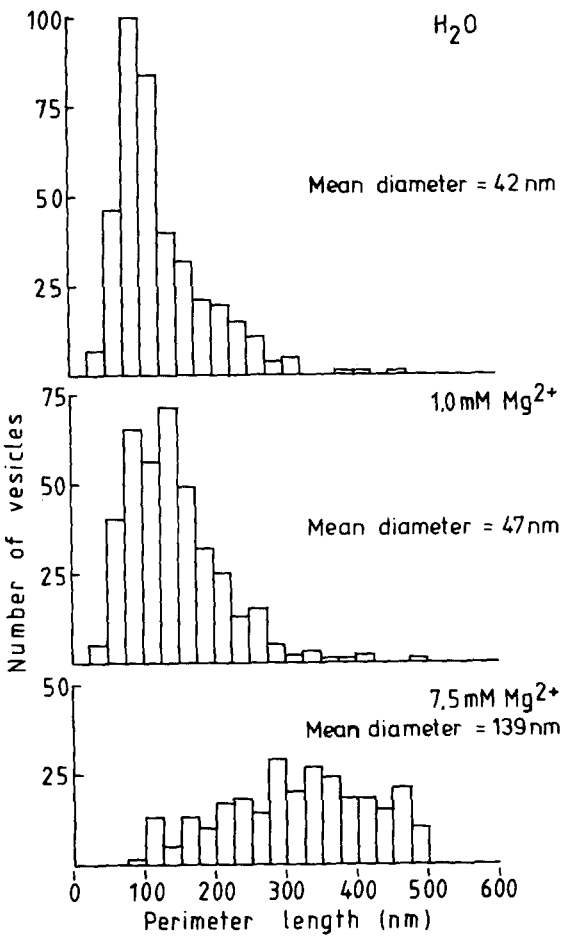

Fig. 9. Histograms showing the variation of the perimeter length of exposed fracture-faces in typical freeze-fracture replicas of total polar lipid extracts of broad bean chloroplasts for samples dispersed in (a) distilled water; (b) 1.0 mM $MgCl_2$; and (c) 7.5 mM $MgCl_2$. The average diameters of the exposed fracture-faces, calculated on the assumption that the faces are circular, are given in the figure. A total of 388 fracture-faces were analysed in each case.

of the tubular structures in a manner analagous to that reported by Van Venetie and Verkleij [16] for the formation of such micelles from H_{II}-related phases observed in phosholipid mixtures.

Cryoprotectant-induced changes. All the electron micrographs shown above are from preparations containing 30% (w/w) glycerol. Omission of glycerol does not alter the general organisation of the samples prepared in distilled water other than by the ususal distortion associated with the absence of cryoprotectant. In the case of the samples prepared in low salt (< 2 mM), the samples showed similar degrees of aggregation but the frequency of non-bilayer structures was lower and the diameter of the freeze-fracture particles somewhat reduced (an effect we have previously reported for binary mixtures of neutral galactolipids [4–6]). Replicas prepared from dispersions in more concentrated salt solutions (> 10 mM polyvalent cations) were essentially identical to those prepared in the presence of cryoprotectant apart from a similar reduction in the average diameter of the non-bilayer structures.

Discussion

The freeze-fracture particles seen in replicas prepared from total polar lipid extracts of chloroplast membranes dispersed in the presence of electrolytes are essentially identical to those we have previously reported for dispersions of monogalactosyl and digalactosyldiacylglycerol mixtures [4–6]. They correspond to inverted lipid micelles sandwiched within lipid bilayers. Similar particles are observed, under appropriate conditions, in phospholipid mixtures [16–21] and are a common feature of lipid mixtures in which one (or more) of the components takes up an H_{II} structure when dispersed alone in water whilst the other component(s) form bilayer structures under the same conditions.

We attribute the absence of non-bilayer structures in dispersions of total polar lipid extracts of chloroplast membranes sonicated in distilled water (Fig. 1a) to the fact that the small highly-strained vesicles formed in such dispersions are physically too small to allow their accommodation. Neutralisation of the acidic lipids that stabilise these vesicles, by lowering the pH of the dispersions (Fig. 2), or the addition of cations (Fig. 8), leads to their fusing to form larger liposomes in which these geometric constraints are removed and the non-bilayer forming lipid, monogalactosyldiacylglycerol, can partially phase-separate.

Two distinct cation-induced changes in lipid aggregation are observed (Fig. 4). The lower concentration (< 2 mM) effect is triggered with increasing efficiency by monovalent, divalent and trivalent cations. Valence dependencies of this type are often observed for processes involving the elec-

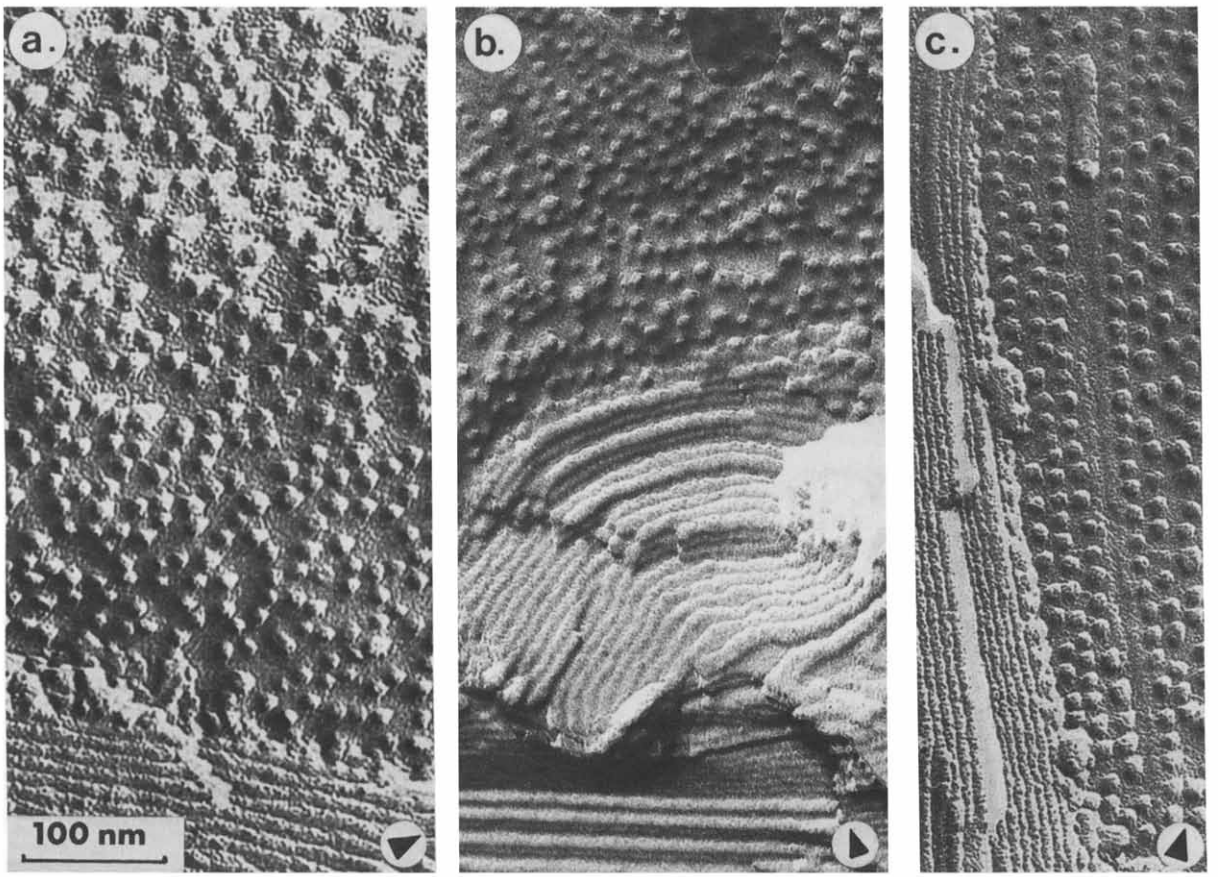

Fig. 10. Electron micrographs showing the main features of freeze-fracture replicas prepared from total polar lipid extracts of chloroplasts sonicated in 30 mM $MgCl_2$. See text for detailed explanations.

trostatic shielding of negatively charged surfaces in aqueous solutions [22,23]. However, the very low concentrations of ions required to bring about aggregation, together with their very limited valence dependence, suggest that the direct binding of cations to the acidic lipids is probably the dominant factor in this system. The aggregation induced by higher concentrations of polyvalent cations appears to reflect a different process. Its dependence on ionic radius (Fig. 7) is very similar to that reported by Rainier et al. [24] for the effectivity of different divalent cations in increasing the phase transition temperature of cardiolipin dispersions. These authors interpreted their results as a measure of the ability of the different ions to crossbridge the negatively charged headgroups of cardiolipin. Certainly, the involvement of cross-bridging between neighbouring lipid headgroups, or between bilayer surfaces, could account for the fact that the higher concentration effects are limited to polyvalent cations but considerably more data is required before this suggestion can be fully substantiated.

The lipid aggregation changes we report here bear many resemblances to the pH-dependent and divalent-cation induced changes that have been reported by earlier workers for dispersions of acidic phospholipids [25–30] and mixtures of acidic and neutral phospholipids [14–21,31–37]. Reducing the pH of such dispersions in order to neutralise the charged groups of the lipids is known to lead to minor changes in lipid organisation leading to small increases in the gel-to-liquid crystal phase transition temperature. Low concentrations of di-

valent cations can, in some cases, show similar effects [26]. Addition of higher concentrations of divalent cations usually leads to marked increases in the phase transition temperature of the dispersions and in mixed lipid systems often leads to extensive phase-segregation. High concentrations of monovalent ions, in contrast, tend to lead to an increased fluidity and a lowering of the phase transition temperature [25].

The main differences between these systems and the chloroplast lipid extracts studied here is that interest in the earlier studies has focussed on the phase behaviour of the acidic lipids. In the case of the chloroplast lipids, it is the phase behaviour of the neutral lipids that is of primary interest. Neutralisation of the acidic lipids appears to allow the formation of sufficiently large aggregates for partial phase-separation of the non-bilayer forming lipid monogalactosyldiacylglycerol to occur. Addition of excess polyvalent cations that are capable of phase-separating the acidic lipids in effect leaves a bulk lipid phase consisting almost exclusively of monogalactosyl and digalactosyldiacylglycerol. Under these conditions, extensive phase separation of these lipids can occur leading to the formation of non-bilayer structures related to those that we have previously described for binary mixtures of these lipids.

In conclusion, we can ask what significance do these findings have in terms of chloroplast structure. Chloroplasts are known to contain appreciable concentrations of both monovalent and divalent cations. Neutron activation analysis of intact pea chloroplasts indicate that they contain about 2.56 μmol per mg chlorophyll of K^+ and 1.72 μmol per mg chlorophyll of Mg^{2+} [38]. The apparent absence in chloroplast membranes of non-bilayer structures of the type seen in dispersions of total polar lipid extract of such membranes [39] strongly indicates that some component of the native membranes acts to suppress the formation of such stuctures. The presence of certain proteins is known to supress the formation of non-bilayer structures in model systems [40,41] and it appears likely that some related process is taking place in the chloroplast membrane.

Acknowledgements

This work was supported by a grant from the Agricultural Research Council. Equipment was also purchased from funds provided by the Central Research Fund of London University and the Royal Society.

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