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POLYUNSATURATED FATTY ACYL RESIDUES OF GALACTOLIPIDS ARE INVOLVED IN THE CONTROL OF BILAYER/NON-BILAYER LIPID TRANSITIONS IN HIGHER PLANT CHLOROPLASTS

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Total polar lipid extracts of chloroplasts isolated from broad beans (*Vicia faba*) tend to form non-bilayer structures when dispersed in dilute salt solutions. Monoglactosyldiacylglycerol is shown to play a dominant role in this process. The tendency of this lipid to form non-bilayer structures when dispersed alone in water was found to depend upon the degree of unsaturation of its associated fatty acyl chains. Highly unsaturated lipids (average number of double bonds per lipid molecule greater than about 5.0) form inverted hexagonal (Hex_{II}) structures in water at 20°C, whilst more saturated lipids (average number of double bonds per lipid molecule less than about 4.5) form lamellar sheets. Wide-angle X-ray diffraction and differential scanning calorimetry measurements indicate that these lamellae consist of gel-phase lipid that can adopt either of two structures depending on the thermal history of the sample. Freeze-fracture studies performed on total polar lipid extracts that have been hydrogenated using Adams' catalyst, and reconstituted extracts in which monogalactosyldiacylglycerol has been selectively hydrogenated, show that the degree of unsaturation of this lipid is a key factor in determining whether or not non-bilayer structures are formed in such extracts. Increasing the extent of saturation of the acyl residues of monogalactosyldiacylglycerol reduces the tendency to form non-bilayer structures. Similar effects are observed on lowering the temperature of the dispersions. Fluorescence polarisation measurements using 1,6-diphenyl-1,3,5-hexatriene indicate that the disappearance of non-bilayer structures is accompanied by a marked decrease in the fluidity of the lipid matrix. The possible significance of these observations is discussed in terms of the thermal adaptation and chilling sensitivity of plant membranes.

Introduction

The membrane lipids of higher plant chloroplasts, particularly the galactolipid fraction, are characterised by the presence of unusually high proportions of polyunsaturated fatty acyl chains [1]. Studies on the properties of pure phospholipids dispersed in aqueous media have established that the insertion of a single *cis* double bond into fatty acid chains of lipids such as the phosphatidylcholines leads to a marked reduction of many tens of degrees in their gel-to-liquid crystal phase-transition temperatures [2]. At the same time, their fluidity, as reflected in the mobility of fluorescent probes such as 1,6-diphenyl-1,3,5-hexatriene

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Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

(DPH), is greatly increased [3]. Considerations such as these have led to the idea that the polyunsaturated lipids of the chloroplast are required in order to maintain membrane fluidity at low temperatures.

Chloroplast membranes, however, also contain high proportions (approx. 50%) of monogalactosyldiacylglycerol, which forms inverted hexagonal (Hex_{II}) phases in water [1]. The phase properties of 'non-bilayer forming' lipids of this type are also known to show a marked dependence on acyl chain saturation. For example, cardiolipin isolated from bovine heart (containing 88% linoleoyl fatty acid residues) exists in an Hex_{II} phase in the presence of Ca^{2+} at room temperature and forms lamellar phases only at temperatures well below 0°C [5,6]. The dipalmitoyl derivative, in contrast, undergoes a gel-to-liquid crystal phase transition at 88°C under these conditions [4], whilst fully saturated lipids containing bulky branch-chain fatty acids undergo a lamellar-to-Hex_{II} transition at about 50°C [5]. Similar trends in the temperatures of lamellar-to-Hex_{II} phase transitions in response to changes in hydrocarbon chain length and saturation [7] and the introduction of methyl branched chains [8] has also been reported for phosphatidylethanolamine.

Many studies of mixed lipid systems have been undertaken in an attempt to establish the role of non-lamellar forming lipids in biological membranes. Non-lamellar structures have been identified in mixtures of phospholipids [9–16], glucolipids from *Acholeplasma laidlawii* [17–19] and glycolipids from chloroplasts [20–23]. Although non-bilayer structures are readily formed in total polar lipid extracts of chloroplast membranes [24] they are not normally found in native membranes. They can, however, be induced in such membranes by short exposures to elevated temperatures [25], suggesting that the preservation of a balance between the tendency of membrane lipids to assume bilayer and non-bilayer structures may be an important structural feature governing membrane stability. In view of the established importance of the fatty acyl residues in influencing the tendency of phospholipids to assume non-bilayer structures, one of the functions of the polyunsaturated fatty acids that characterise the polar lipids of the chloroplast membrane may, therefore, be to preserve a particular balance between the bilayer and non-bilayer forming tendencies of monogalactosyldiacylglycerol rather than to provide an appropriate membrane fluidity as is so often assumed.

In a recent paper [26], we reported that whilst dispersions of the dilinolenoyl derivative of monogalactosyldiacylglycerol form Hex_{II} structures when dispersed in water at room temperature, the distearoyl derivative forms lamellar structures. In this study, we report a systematic investigation of the part played by changes in fatty acyl saturation of this lipid in determining the structures formed in aqueous dispersions of total polar lipid extracts of broad-bean (*Vicia faba*) chloroplasts. The overall structure of the dispersions has been examined using freeze-fracture electron microscopy. These data have been correlated with measurements of membrane fluidity (as reflected in the mobility of intercalated DPH), fatty acyl chain packing as revealed by wide-angle X-ray diffraction measurements and lipid phase behaviour as reflected in thermal measurements. The relevance of these observations is discussed in terms of the changes in lipid composition and/or saturation that are reported to occur in plants and micro-organisms adapted to growth at different temperatures [27–29].

Materials and Methods

Chloroplast and lipid isolation. Chloroplasts were isolated from fresh leaf tissue of 4–5-week post-emergent broad beans (*Vicia faba*; var. Express) by the method of Stokes and Walker [30]. Total lipid extracts of the freshly isolated chloroplasts were prepared according to the procedure of Bligh and Dyer [31]. Neutral lipid and pigments were removed from the extracts by column chromatography on silicic acid [32]. The polar lipid fraction was dried using a rotary evaporator and stored in chloroform under nitrogen.

Monogalactosyldiacylglycerol isolation. This lipid class was isolated by passing the total polar lipid extract through an acidified Florisil column sequentially eluted with chloroform, chloroform/acetone (1 : 1, v/v), chloroform/methanol (1 : 1, v/v), and methanol. Monogalactosyldiacylglycerol was collected with the chloroform/acetone fraction, while the remaining polar lipids were col-

lected in the chloroform/methanol and methanol fractions. The purity of these fractions was verified by TLC on silica gel plates against standard lipid preparations.

Lipid hydrogenation. Lipid dissolved in chloroform/methanol (2 : 1, v/v) was placed in a stoppered tube and dried under N_2. Approx. 10 ml of N_2-saturated benzene was added and the resulting solution was bubbled for 15–20 min with dry N_2. Adams' catalyst (Johnson-Matthey Chemicals, Ltd., U.K.) was added in an amount equal by weight to that of the original lipid. The mixture was bubbled with N_2 for a further 5 min and then with H_2. Samples of hydrogenated lipid were removed at designated time intervals and the catalyst precipitated by centrifugation. The extents of saturation of the fatty acyl residues of the different samples were determined by gas chromatography of their methyl ester derivatives [33] and expressed in terms of the average number of double bonds per lipid molecule defined as Σ (fatty acid %) $\times$ (number of double bonds in fatty acid) $\times$ 0.02.

Lipid reconstitution. Samples of hydrogenated monogalactosyldiacylglycerol and of non-hydrogenated samples of the total polar lipid extract from which the monogalactosyl lipid had been removed were dissolved in chloroform/methanol (2 : 1, v/v) and then mixed in stoppered tubes in the same proportion as that existing in the original extract (i.e., approximately equal molar ratios of monogalactosyldiacylglycerol to other polar lipids). The solvent was removed under a stream of oxygen-free nitrogen leaving a thin film of lipid on the sides of the tubes. The tubes were then stored overnight under vacuum to remove any remaining solvent. Water was added to the lipid samples and the resulting suspension was dispersed by sonication for 5 min in an ultrasonic bath. Samples containing non-hydrogenated monogalactosyldiacylglycerol together with hydrogenated samples of the remainder of the extract were prepared similarly.

Fluorescence polarization measurements. The fluorescence probe, DPH, was added to the lipid preparation in organic solvents in a ratio of 1 probe molecule per 500 lipid molecules. The sample was dried under N_2 and the mixture was dispersed in 4 ml of water or salt solution by ultrasonic irradiation in sealed tubes under nitrogen at a temperature above the lipid phase transition. Fluorescence measurements were made using a Perkin Elmer MPF-44A fluorescence spectrometer. The value of the fluorescence polarization, P, was calculated using the equation:

$$P = \frac{(I_{\parallel\parallel} I_{\perp\perp}) - (I_{\parallel\perp} I_{\perp\parallel})}{(I_{\parallel\parallel} I_{\perp\perp}) + (I_{\parallel\perp} I_{\perp\parallel})}$$

where I is the intensity of fluorescence and the subscripts refer to the orientations of the polarizer and the analyzer, respectively. A lipid dispersion lacking fluorescence probe was used to correct for light-scatter in the preparations.

Electron microscopy. The lipids were mixed in the desired molar ratio as described above. The dry lipid mixture was then dispersed in nitrogen-saturated water or 10 mM $MgCl_2$, as indicated in the figure legends, by ultrasonic irradiation. The dispersed lipids were equilibrated at the required quench temperature for at least 15 min prior to freezing in a slurry of nitrogen. The frozen samples were fractured at -115°C in a Polaron freeze-fracture device and shadowed by platinum-carbon immediately after fracture. The replicas were washed with chloroform/methanol (2 : 1, v/v) and examined in a Philips EM 301 electron microscope.

X-ray diffraction. Wide-angle X-ray diffraction patterns were obtained using a powder camera. Freeze-dried lipids were dispersed in excess water saturated with N_2 and sealed in thin-wall (0.01 mm) glass capillaries (1 mm diameter). X-rays were produced using a Philips Generator fitted with a fine-focus stationary anode tube. Exposure times were approx. 1 h.

Differential scanning calorimetry. Lyophilized preparations of monogalactosyldiacylglycerols were hydrated with a 10-fold excess by weight of aqueous ethylene glycol (30%, w/w) and sealed in aluminium pans. Heating and cooling thermograms in the temperature range -30 to 90°C were recorded with a reference pan of aqueous ethylene glycol and a scan rate of 10 $K \cdot min^{-1}$ and, unless indicated otherwise, sensitivity of 1 $mcal \cdot s^{-1}$. Calorimetry was performed in a Perkin Elmer DSC-2 instrument. Enthalpy values were determined by estimation of peak areas from the recorder tracings of thermograms using an Apple

computer supplied with a graphics tablet. The lipid content of the pans were determined by gas chromatographic analyses using an internal standard of pentadecanoyl methyl ester.

Results

Monogalactosyldiacylglycerol dispersions.
The dilinolenoyl derivative of monogalactosyldiacylglycerol is known to form typical inverted hexagonal (Hex_{II}) structures when dispersed in water at temperatures about 20°C, whilst the fully saturated distearoyl derivative forms bilayers under these conditions [26]. The influence of the degree of unsaturation of the acyl chains on the structures formed by this lipid on dispersion in water was investigated by examining a series of samples of monogalactosyldiacylglycerol extracted from chloroplasts and subsequently hydrogenated to different extents using Adams' catalyst. The changes in pattern of fatty acyl residues of monogalactosyldiacylglycerol subjected to increasing times of incubation under hydrogenating conditions are summarised in Table I. As expected, there is a progressive conversion of trienoic to di- and monoenoic acids and ultimately to the fully saturated form. The extent of deacylation was found to the extremely low. No attempt was made to establish the positions of the remaining double bonds along the fatty acyl chains. It is, however, unlikely that this type of heterogeneous catalytic hydrogenation introduces any particular positional specificities with respect to the location of the unsaturated bond in the fatty acyl chain [29].

TABLE I

FATTY ACID COMPOSITION OF MONOGALACTOSYLDIACYLGLYCEROL FROM BROAD BEAN CHLOROPLASTS HYDROGENATED IN THE PRESENCE OF ADAMS' CATALYST

Reaction time (min)	Fatty acid compositon (mol%)					Average number of double bonds per molecule
	16:0	18:0	18:1	18:2	18:3	
0	1.6	0.5	2.1	17.2	78.6	5.44
7	1.8	9.9	14.6	21.0	52.7	4.30
12	1.7	22.1	31.8	22.9	21.5	2.84
17	1.8	26.9	37.3	19.9	14.1	2.39
22	1.8	49.6	26.8	13.1	8.7	1.36

Representative electron micrographs of freeze-fracture replicas prepared from a series of such samples are shown in Fig. 1. The native lipid (average number of double bonds per lipid molecule, 5.44) shows a characteristic Hex_{II} organisation (Fig. 1a), whilst hydrogenated samples with average numbers of double bonds per molecule below about 4.5 show lamellar structures (Fig. 1c) of the type seen in the fully saturated distearoyl derivatives [26]. Samples with intermediate numbers of double bonds per molecule contained regions of both lamellar and Hex_{II} structures. The direct juxtaposition of bilayer and non-bilayer structures in the replica shown in Fig. 1b emphasises the dynamic equilibrium that exists between such structures in this particular lipid. It is noteworthy that the lamellae formed by the monogalactosyl lipid are invariably in the form of sheets rather than liposomes of the type observed for phospholipids such as phosphatidylcholine, and indeed even digalactosyldiacylglycerols [26]. These structural differences were reflected in difficulties in dispersing the lipids in water. Even samples dispersed at temperatures above the gel-to-liquid crystalline phase transitions of the lipids tended to precipitate rapidly on standing.

The electromicrographs shown in Figs. 1a–1c were all obtained for samples thermally quenched from 20°C. As the distearoyl derivative of monogalactosyldiacylglycerol undergoes a gel-to-liquid crystal phase transition at 82°C [34], it is likely that some of the more saturated samples were in the gel phase. Replicas obtained from preparations quenched from higher temperatures (above 50°C) invariably showed a complete transformation back to the Hex_{II} structure, supporting this view. Shipley et al. [5] have reported that highly unsaturated monogalactosyldiacylglycerols isolated from plant leaves undergo a lamellar-to-Hex_{II} transition at about −30°C. In agreement with this finding, replicas of the native monogalactosyl lipid prepared from dispersions equilibrated at −28°C prior to thermal quenching showed only lamellar structures (Fig. 1d).

In a more direct attempt to check whether the hydrogenated lipid samples were in a gel or liquid crystalline phase at room temperature, we examined the wide-angle X-ray diffraction patterns of a series of samples with average numbers of

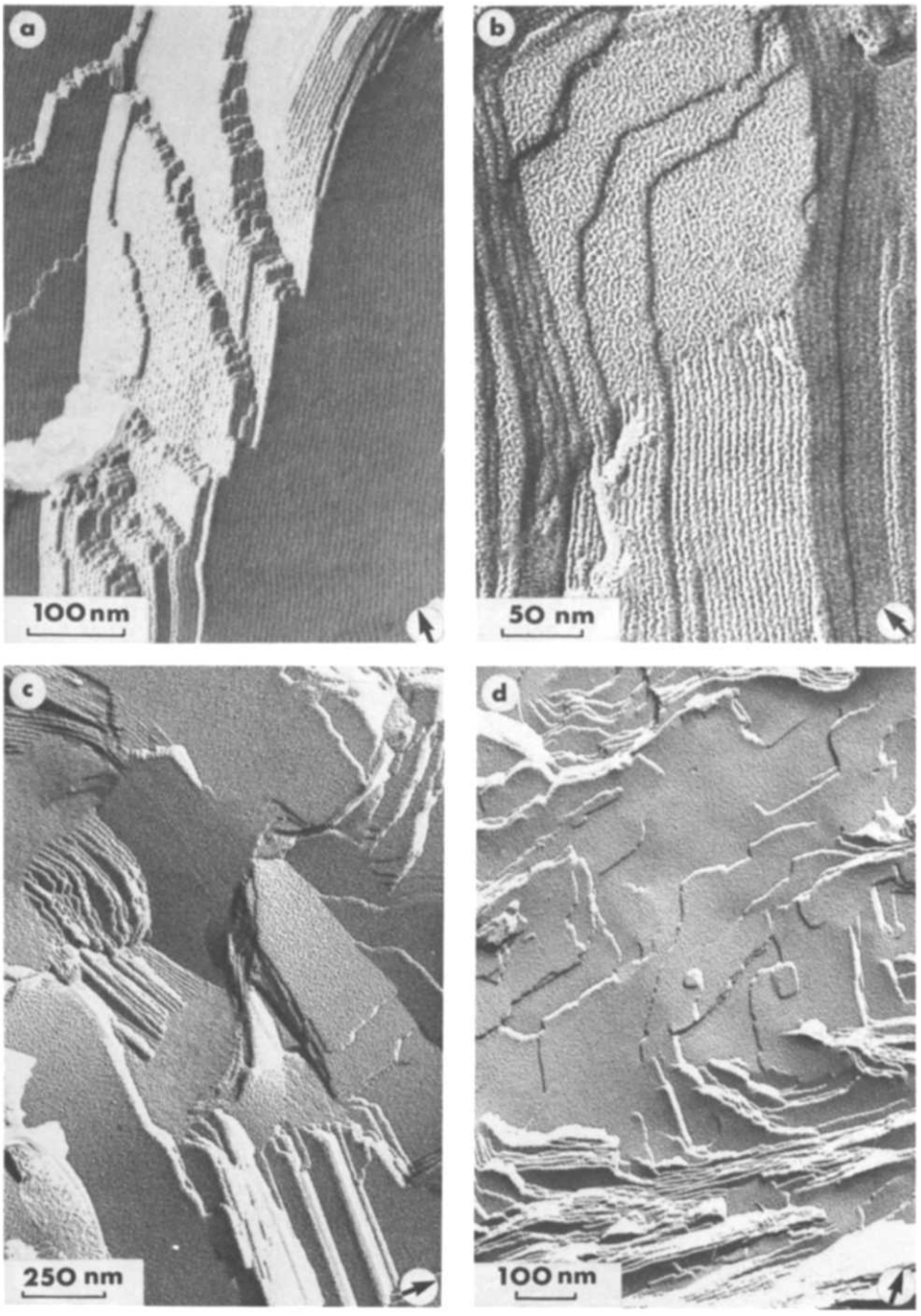

Fig. 1. Electromicrographs of freeze-fracture replicas of the native monogalactosyldiacylglycerol thermally quenched from (a) 20°C and (d) −28°C and from hydrogenated samples quenched from 20°C (b) and (c). The average number of double bonds per molecule of the different samples are (a) and (d) 5.44, (b) 5.10 and (c) 3.88.

double bonds per lipid molecule ranging from 5.40 to 1.32. Lipids with more than 5.0 double bonds per molecule showed a diffuse diffraction maxima at 0.46 nm (Fig. 2a), indicating that they were in a

liquid-crystalline state. Samples with less than about 4.5 double bonds per lipid molecule showed, depending on their thermal history, either a single sharp diffraction maximum at 0.42 nm (Fig. 2b) or a pair of intense diffraction maxima at 0.44 nm and 0.40 nm together with a weaker maximum at 0.48 nm (Fig. 2c). Lipid samples that had been recently heated tended to yield diffraction patterns of the type shown in Fig. 2b, whilst samples that had been equilibrated at about 20°C for several hours showed patterns of the type shown in Fig. 2c. In either case, the sharpness of the maxima indicates that the lipids were in the gel state. The existence of two lamellar forms of the gel-phase lipid is typical of saturated monogalactosyldiacylglycerols. As we have explained elsewhere [34], it reflects the formation of a metastable state in which the hydrocarbon chains of the lipids are packed on a regular hexagonal lattice and a more stable crystalline form in which this packing is quasi-hexagonal.

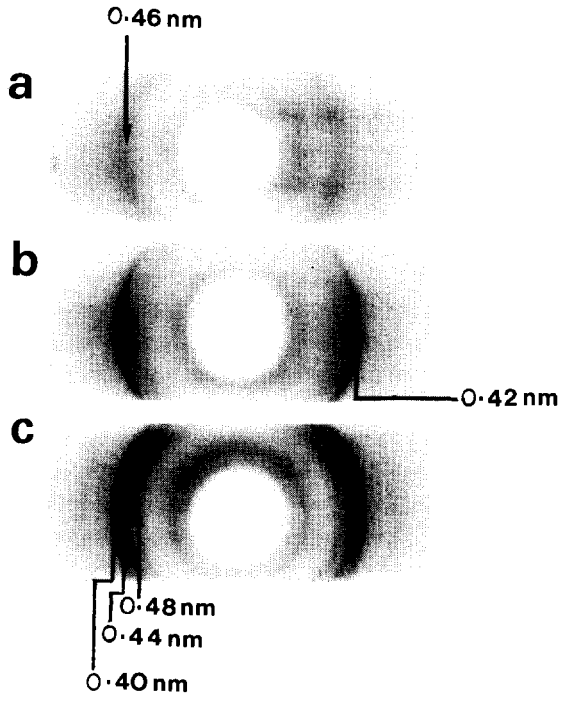

Fig. 2. Wide-angle X-ray diffraction patterns obtained for aqueous dispersions of samples of monogalactosyldiacylglycerol. The pattern shown in (a) was obtained for a lipid sample with an average number of double bonds per lipid molecule of 5.40 and those in (b) and (c) for a sample with an average number of double bonds per lipid molecule of 1.32. The pattern shown in (b) was obtained for a sample heated to approx. 80°C shortly prior to measurement whilst that shown in (c) was obtained for a sample that had been stored at 20°C for 2 days. All measurements were performed at 20°C.

The phase properties of these lipid preparations were further investigated by differential scanning calorimetry. Despite the fact that freeze-fracture electron microscopy provides clear evidence that the native lipid exists in a lamellar phase at −28°C (Fig. 1d) but in an Hex_{II} phase at 20°C (Fig. 1a), calorimetric studies revealed no significant thermal events over the temperature range −30 to 90°C. It thus appears either that the enthalpy of the lamellar-Hex_{II} transition is too small to detect or that it is a non-cooperative transition taking place over a wide temperature range. However, when the monogalactosyldiacylglycerol is hydrogenated, even to a relatively small extent (average number of double bonds per lipid molecule reduced from 5.45 to 4.30) prominent thermal transitions can be detected in the dispersions. This is illustrated in Fig. 3, which shows thermograms from the preparations of the native lipid, hydrogenated for 7, 12, 17 and 28 min, respectively, referred to in Table I.

In order to investigate the properties of the stable and metastable forms of the hydrogenated lipids revealed by the X-ray diffraction studies, the lipid dispersions were allowed to equilibrate to 20°C for 3 days prior to measurement. The presence of different molecular species makes precise estimates of the transition temperatures for the different dispersions difficult. However, all four samples showed broadly similar thermal behaviour. The initial heating thermograms were, in all cases, dominated by an endotherm between about 55°C and 80°C, corresponding to the melting of the more stable crystalline form. On cooling and subsequent reheating, these were largely replaced by endotherms at around 40°C corresponding to the melting of the metastable form. The thermal behaviour of these partially hydrogenated lipids is thus very similar to that of the fully-saturated distearoyl derivatives of monogalactosyldiacylglycerol [34]. The one exception was the sample hydrogenated for only 7 min (Fig. 3A(c)) which showed an exotherm in the second heating. Sphingolipids characterised by two gel-phase forms analagous to those seen in the monogalactosyldia-

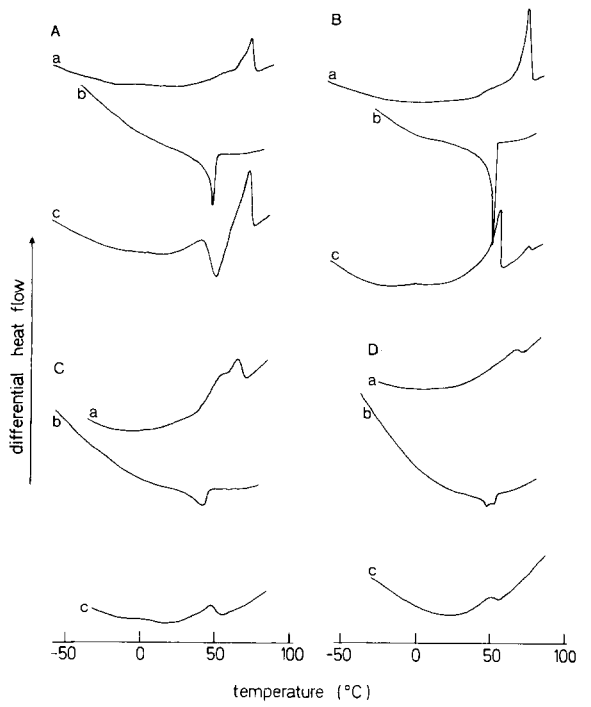

Fig. 3. Differential scanning calorimetric curves of partially hydrogenated monogalactosyldiacylglycerols dispersed in aqueous ethylene glycol. The average number of double bonds per molecule for the different samples were (A) 4.30, (B) 2.84, (C) 2.39, (D) 1.36. Initial heating scans (a) were performed on dispersions thermally equilibrated for 3 days at 20°C prior to cooling to −70°C at the commencement of the run. Cooling scans (b) were then recorded followed immediately by a second heating scan (c). All thermograms were recorded at a scan rate of 10 $K \cdot min^{-1}$ and a sensitivity of 1 $mcal \cdot s^{-1}$ except A(a) and B(b) (2 $mcal \cdot s^{-1}$) and C(b)(c) and D(a)(c) (0.5 $mcal \cdot s^{-1}$).

cylglycerols [36,37] exhibit similar behaviour. As in these systems, this heating exotherm can be eliminated, and the lower-temperature endotherm restored by performing the heating scan at faster rates, indicating that the exotherm arises from a transition between the metastable and stable forms occurring during the course of the measurement. In general, only a single exotherm is observed in cooling thermograms and this occurs at a temperature intermediate between the two heating endotherms. in some more extensively hydrogenated preparations (see Fig. 3D(b)), two exotherms were present, but repeated cycles of heating and cooling caused the elimination of the higher-temperature even and its replacement by a single exothermic transition at the lower temperature.

The transition temperatures and ethalpy values associated with the various endotherms and exotherms of the thermograms shown in Fig. 3 are listed in Table II. In agreement with earlier studies performed on the distearoyl derivative of monogalactosyldiacylglycerol, the heats associated with the melting of the stable crystalline form are more than twice those associated with the metastable form. It is also noteworthy that, whilst the enthalpy value of each individual sample increases with hydrogenation, the corresponding transition temperatures remain fairly constant.

Total polar dispersions

Role of ions. Total polar lipid extracts of broad-bean chloroplasts tend to form small (30–40 nm diameter), single-shell liposomes when dispersed by ultrasonication in distilled water. The addition of monovalent or divalent cations to yield final ionic concentrations in the range 2–10 mM causes a rapid fusion of these liposmes and the formation of non-bilayer structures. These effects, which are associated with a reduction of charge-re-

TABLE II

TRANSITION TEMPERATURES AND ENTHALPIES OF AQUEOUS DISPERSION OF MONOGALACTOSYLDIACYLGLYCEROLS HYDROGENATED TO VARYING EXTENTS IN THE PRESENCE OF ADAMS' CATALYST

Transition temperatures were estimated from intercept of leading edge of the endotherm, or exotherm, and base-line. ΔH is the total enthalpy of combined endotherms or exotherms.

Average number of double bonds per molecule	Thermal history	Transition temperature (°C)	ΔH ($kJ \cdot mol^{-1}$)
4.30	1st heating	42; 61	48.0
	cooling	51	21.2
	2nd heating	34; 66	16.8
2.84	1st heating	42; 73	54.9
	cooling	58	24.9
	2nd heating	49; 75	25.4
2.39	1st heating	41; 59	76.8
	cooling	48	28.2
	2nd heating	32	28.3
1.36	1st heating	46; 57	–
	cooling	55	–
	2nd heating	36	–

pulsion between the acidic lipids present in the dispersions, have been detailed elsewhere [24]. In order to avoid complications arising from these electrostatic factors, we routinely dispersed the total lipid extracts used in this investigation in 10 mM $MgCl_2$.

Role of lipid saturation. Total polar lipid extracts of chloroplast membranes were hydrogenated to different extents using Adams' catalyst (Table III). Electronmicrographs of freeze-fracture replicas prepared from these samples are set out in Fig. 4. An electronmicrograph of a replica prepared from the native dispersion (average number of double bonds per lipid molecule 4.65) is presented in Fig. 4a. It shows a number of different lamellar and non-lamellar structures. These include areas of normal bilayer (A) and spherical and cylindrical inverted micelles sandwiched within bilayers (B and C, respectively). Reduction of the average number of double bonds per lipid molecule of the sample from 4.65 to 4.16 leads to an elimination of tubular micelles. Under these conditions, the sample is much more homogeneous in appearance, consisting predominantly of spherical micelles sandwiched within a bilayer phase (Fig. 4b). Further hydrogenation, resulting in lipid containing 3.08 double bonds per molecule, leads to the elimination of all inverted micelles from the lamellar phase (Fig. 4c). The lamellae formed under these conditions, it should be noted, are very similar to those observed for saturated monogalactosyldiacylglycerols (Fig. 1c), forming sheets rather than closed vesicles. No further changes in structure were observed for more extensively hydrogenated samples.

TABLE III

FATTY ACYL COMPOSITION OF CHLOROPLAST POLAR LIPID EXTRACTS AFTER HYDROGENATION IN THE PRESENCE OF ADAMS' CATALYST

Reaction time (min)	Fatty acid composition (mol%)					Average number of double bonds per molecule
	16:0	18:0	18:1	18:2	18:3	
0	9.7	2.5	6.6	14.8	66.5	4.65
5	10.7	10.1	7.1	15.2	56.8	4.16
10	10.9	27.3	9.2	13.6	39.0	3.08
20	11.4	39.9	8.2	11.9	28.6	2.35
30	11.6	43.4	8.0	11.8	26.2	2.16
60	10.1	63.8	5.	6.0	14.8	1.24

The formation of non-bilayer lipid structures in dispersions of this type is believed to depend on the ability of the monogalactosyldiacylglycerol to form Hex_{II} structures. In order to test the specific role of monogalactosyldicylglycerol hydrogenation in the changes described above, the monogalactosyl lipid was separated from the other lipids, selectively hydrogenated to different extents, and then co-dispersed with the remainder of the polar lipid extract in the same proportions as originally present. A typical electronmicrograph of a replica prepared from such a dispersion is shown in Fig. 4d. The average number of double bonds per lipid molecule in the hydrogenated monogalactosyldiacylglycerol fraction and of the final reconstituted sample were 3.94 and 4.40, respectively. Despite the fact that this latter value is well within the range expected to give rise to non-bilayer structures, the sample consisted only the lamellar structures. Spherical and cylindrical inverted micelles are not normally observed if the average number of double bonds per lipid molecule in the reconstituted dispersion is 4.40 or less.

The apparent displacement of non-bilayer structure formation to higher numbers of double bonds per molecule when monogalactosyldiacylglycerol is selectively hydrogenated indicates that it is the level of unsaturation of this particular lipid rather than that of the sample as a whole that determines whether or not such structures are formed. If, instead of selectively hydrogenating the monogalactosyl lipid, the other lipids are hydrogenated and then redispersed together with the native monogalactosyldiacylglycerol, the structures observed are essentially identical in appearance to those found in the native extract, again emphasising the fact that formation of non-bilayer structure is determined primarily by the degree of saturation of the monogalactosyl lipid.

The pivotal role of this lipid is even more strikingly demonstrated by measurements of the fluorescence polarisation of DPH intercalated into the dispersions. Plots showing the variation of fluorescence polarisation of DPH as a function of the average number of double bonds per lipid molecule for dispersions of the native extract hydrogenated as a whole, and reconstituted samples

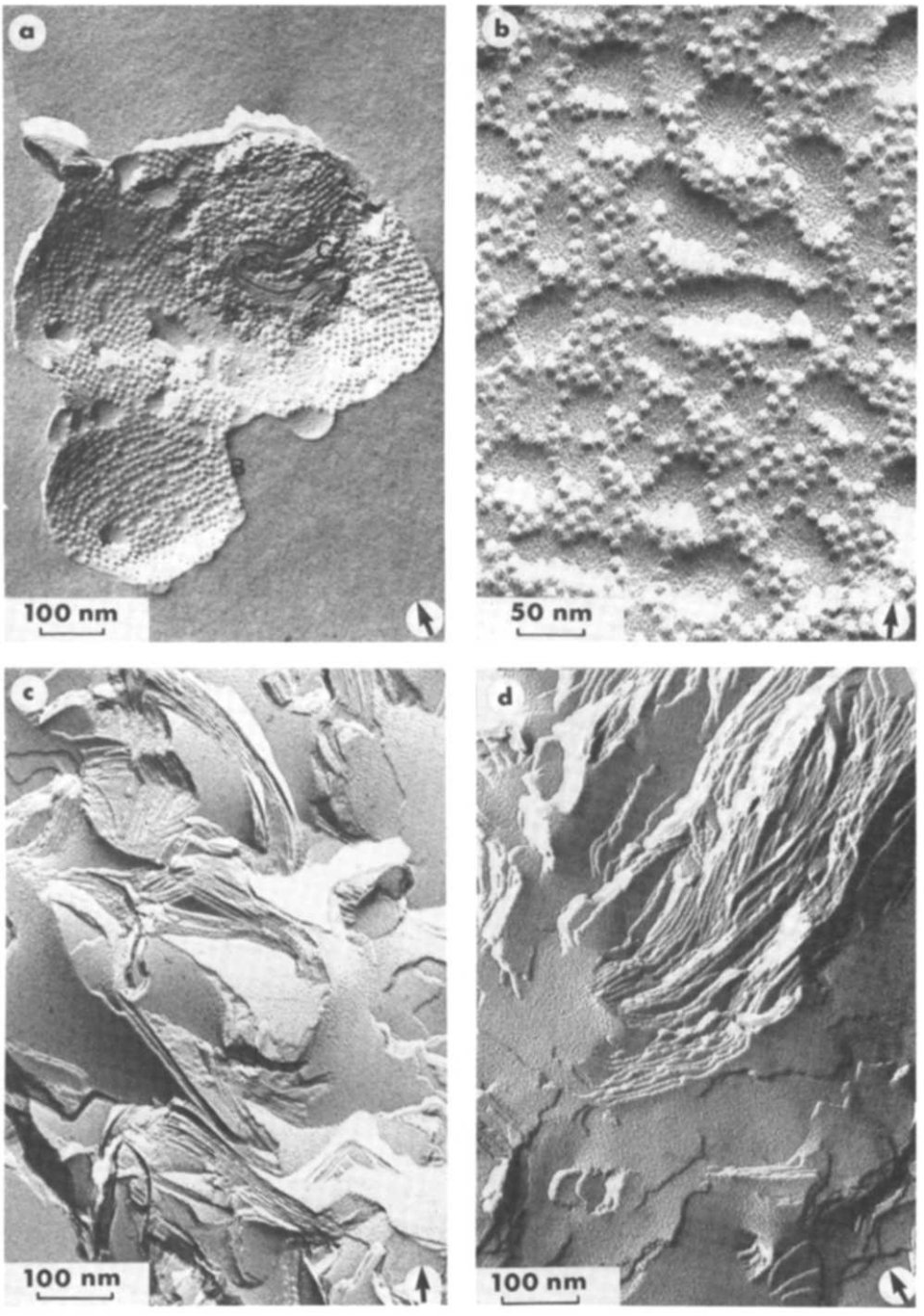

Fig. 4. Electronmicrographs of freeze-fracture replicas prepared from sonicated dispersions of (a) the native polar lipid extract, (b) and (c) hydrogenated samples of such extracts and (d) a reconstituted dispersion in which the monogalactosyldiacylglycerol has first been isolated from the other lipid components, hydrogenated and then recombined in its original proportion with the other non-hydrogenated components. The final average number of double bonds per lipid molecule in the different samples were (a) 4.65, (b) 4.16, (c) 3.08 and (d) 4.40. All samples were dispersed in 10 mM $MgCl_2$ and thermally quenched from 20°C. Note the presence in the native extract of areas of normal bilayer (A), spherical inverted micelles (B) and cylindrical inverted micelles (C).

containing hydrogenated monogalactosyldiacylglycerol, are presented in Fig. 5. The general structure of the samples, as revealed by freeze-fracture studies, is indicated alongside the plots for comparison. Two important features emerge from these measurements. Firstly, the fluorescence polarisation values are invariably low for samples containing large proportions of unsaturated monogalactosyldiacylglycerols, but increase steeply as this lipid becomes more saturated. This effect, which is particularly noticeable for those samples in which the monogalactosyl lipid has been specifically hydrogenated, clearly indicates that the probe mobility in the lipid matrix of these dispersions, like their tendency to form non-bilayer structures, is primarily determined by the degree of unsaturation of the monogalactosyldiacylglycerol fraction. Secondly, the sharp increase in DPH polarisation appears to coincide with the onset of the formation of the sheet-like lamellar structures. The high fluorescence polarisation values observed for samples that contain these structures suggests that they greatly restrict the mobility of the probe.

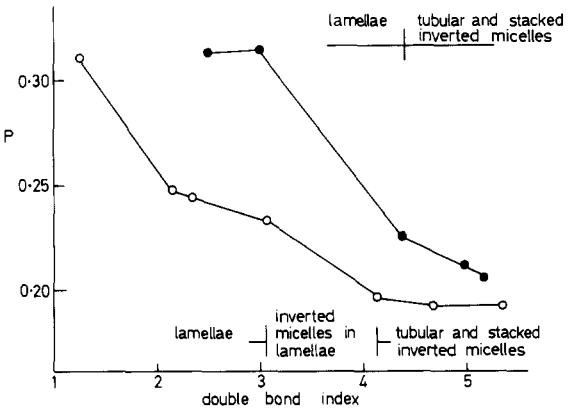

Fig. 5. Fluorescence polarisation (P) of DPH intercalated into sonicated dispersions of total polar lipid extracts of broad-bean chloroplasts subjected to hydrogenation for different periods of time (○———○) and reconstituted dispersions in which the monogalactosyldiacylglycerol has been selectively hydrogenated (●———●). All samples were dispersed in 10 mM $MgCl_2$ and the measurements were made at 20°C. The results are plotted as a function of the final double-bond index value of the samples. The range over which different bilayer and non-bilayer structures were observed in freeze-fracture replicas prepared from the different dispersions is indicated. The structural information at the top and the bottom of the figure is related to curves designated ● and ○, respectively.

Wide-angle X-ray diffraction measurements, however, revealed no evidence of sharp diffraction maxima associated with gel-phase lipid, suggesting that the constraints on probe mobility in these samples cannot be attributed to a simple ordering of the hydrocarbon chains of the lipids forming these lamellae.

Effect of temperature. Changes similar to those brought about by hydrogenation are observed if the temperature of the dispersions formed from the native extract is reduced. Fluorescence polarisation data, together with a summary of the structural data obtained from freeze-fracture studies on dispersions thermally quenched from low temperatures, are presented in Fig. 6. The resemblance of these results to those obtained for the hydrogenated lipid is quite striking. Again, as with the hydrogenated samples, wide-angle X-ray diffraction measurements revealed only a broad diffuse maximum at 0.46 nm.

It should be emphasised that the formation of sheet-like lamellae of the type seen in Figs. 4c and 4d, and the accompanying increases in DPH fluorescence polarisation, occur only in samples containing added cations. Samples dispersed in distilled water show no such structures and are made up almost exclusively of small single-shell vesicles.

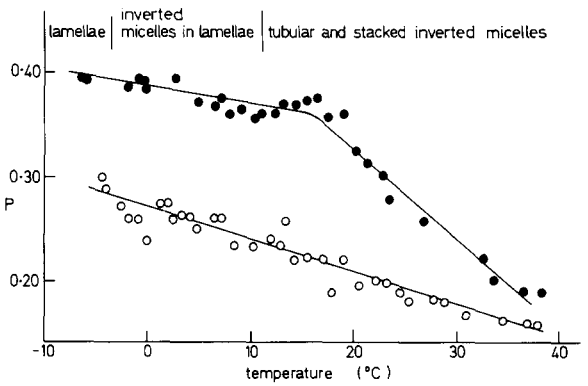

Fig. 6. Fluorescence polarisation (P) of DPH intercalated into sonicated dispersions of total polar lipid extracts of broad-bean chloroplasts as a function of temperature. Samples were measured in the presence (●) and absence (○) of 10 mM $MgCl_2$. The temperature ranges over which different bilayer and non-bilayer structures were observed in freeze-fracture replicas is indicated for samples prepared in the presence of 10 mM $MgCl_2$. Micelles in lamellae are also observed at temperatures greater than 10°C.

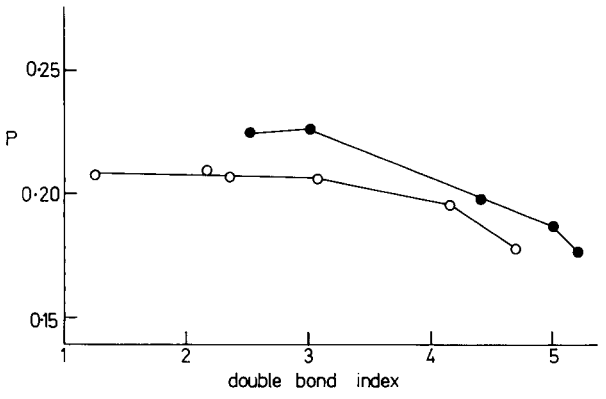

Fig. 7. Fluorescence polarisation (P) of DPH intercalated into aqueous dispersions of hydrogenated (○) total polar lipid extracts of broad bean chloroplast and preparations reconstituted with hydrogenated monogalactosyldiacylglycerol (●). The dispersions were prepared by sonication in distilled water and contained no added cations. Measurements were performed at 20°C.

Neither do they show the marked increase in DPH fluorescence polarisation associated with the formation of such structures (Figs. 6 and 7). This again underlines the compex interaction that takes place between the various factors determining the formation of bilayer and non-bilayer structures in this system.

Discussion

The occurrence of changes in phase behaviour of monogalactosyldiacylglycerol following hydrogenation is not unexpected. Low-angle X-ray diffraction studies [35] have shown that whilst aqueous dispersions of polyunsaturated derivatives of this lipid forms Hex_{II} structures at temperatures at least as low as -15°C, the distearoyl derivative exists as a lamellar phase at 25°C [26]. Differential scanning calorimetry studies cited by Shipley et al. [35] suggest that the polyunsaturated lipid undergoes a gel-to-liquid crystalline phase transition at about -30°C, whilst the corresponding transition for the fully saturated lipid occurs at about 82°C [34]. What is surprising, however, is the relatively small changes in fatty acyl composition required to bring about these changes in phase behaviour. Whereas monogalactosyldiacylglycerol samples containing an average of 5.0–5.5 double bonds per molecule tend to form Hex_{II} phases at room temperature (20°C), those with 4.0–4.5 double bonds or less tend to form lamellar structures (Fig. 1).

Sen et al. [34] have reported values for the enthalpy changes (ΔH) associated with the gel-to-liquid crystal phase transitions of the stable and metastable forms of distearoylmonogalactosylglycerol of about 67 and 31 $kJ \cdot mol^{-1}$, respectively. If the partially saturated molecular species of this lipid present in the hydrogenated samples are assumed to have similar values, the enthalpy values listed in Table II indicate that the percentage of lipids in the stable form, as estimated from the initial heating endotherm, varies from about 70% in the least saturated sample to approaching 100% in the two most saturated samples. This conclusion, which is consistent with the freeze-fracture and X-ray diffraction data shown in Figs. 1 and 2, strongly underlines both the sensitivity of the phase behaviour of this particular lipid to changes in acyl chain composition and the importance of polyunsaturated residues to such behaviour.

The formation of non-bilayer structures in aqueous dispersions of lipid mixtures is dependent on the presence of at least one component that forms Hex_{II} structures when dispersed alone in water, a role which in chloroplast lipid extracts is normally filled by monogalactosyldiacylglycerol. The changes in phase behaviour of this lipid following the hydrogenation of samples of chloroplast lipid extracts thus readily accounts for the loss of non-bilayer structures in such samples (Fig. 3a–c). This loss, as might be anticipated, is greater if the monogalactosyldiacylglycerol is selectively hydrogenated (Fig. 3d).

Khan et al. [38] have reported related variations in the structures formed by mixtures of monoglucosyl and diglucosyl lipids isolated from *Acholeplasma laidlawaii*. In their experiments the proportion of monoglucosyl to diglucosyl lipid was varied without changing the level of unsaturation of the lipids. Increases in the proportion of the non-bilayer-forming monoglucosyl lipid led to the formation of a reversed cubic phase. Cubic phases of this type can also be induced in binary mixtures of monogalactosyl and digalactosyldiacyglycerol by the addition of high concentrations of cryoprotectants such as glycerol and dimethyl sulphoxide [23,39], emphasising the fact that the manipulation

of either of the relative proportions of non-bilayer forming components, or the relative propensity of the individual components to form non-bilayer structures, leads to similar results.

Wide-angle X-ray diffraction measurements (Fig. 2) indicate that the lamellar structures formed in partially hydrogenated samples of monogalactosyldiacylglycerol readily undergo transitions between a gel phase characterised by a single intense diffraction maximum at 0.42 nm, corresponding to a packing of lipid chains on an hexagonal lattice, to a more crystalline phase characterised by intense diffraction maxima at 0.40 nm and 0.44 nm. The ready precipitation of these lipids and their open-sheet appearance in freeze-fracture electronmicrographs (Fig. 1c) presumably reflect the formation of this latter phase. Related structures are visible in electronmicrographs or hydrogenated chloroplast lipid extracts (Fig. 4c, d). Measurements of fluorescence polarisation of DPH molecules intercalated in such extracts indicate that the motional freedom of the probe is severely restricted as non-bilayer structures are replaced by lamellar structures. The value of the fluorescence polarisation increases sharply from $P \approx 0.2$ to $P \approx 0.3$ during this process (Fig. 5). Despite the fact that these latter values closely approach those reported for gel-phase lipids [40], wide-angle X-ray diffraction measurements show little or no indication of the formation of ordered phases in these mixtures, indicating that the motion of DPH is subject to very different constraints in these lipid extracts to those associated with the formation of more conventional bilayer structures of the liposome type.

The sensitivity of the lipid phase-separations that are involved in the formation of non-bilayer structures to the extent of fatty acid unsaturation has considerable implications with regard to membrane stability and the adaptation of organisms to growth at different temperatures. Thermotolerant organisms, for example, often undergo adaptive processes that involve alterations in the fatty acyl composition of membrane lipids (see reviews, Refs. 27–29). In general, the fatty acyl chains of photosynthetic micro-organisms induced to grow at low temperatures show more unsaturation than normal. The changes observed in higher plants are usually more complex, but similar alterations in fatty acid saturation have been reported in, for example, *Atriplex lentiformis* [41]. Comparable differences in membrane lipid saturation amongst species native to hot deserts and cool maritime climates have also been noted [42].

It has been suggested that these changes reflect the operation of a homoviscous adaptation process necessary to maintain membrane fluidity in a state consistent with the physiological and biochemical processes that the membranes perform [43,44]. This explanation fails, however, to account for the fact that thermal adaptation, in higher plants at least, is usually limited to minor changes in the levels of polyunsaturated fatty acids. Such changes would, in the absence of bilayer/non-bilayer transformations of the type discussed above, be expected to lead to minimal changes in membrane fluidity. An alternative explanation suggested by our results is that these changes in fatty acid saturation are required to preserve an appropriate balance of bilayer and non-bilayer forming lipids in the membrane. We have argued elsewhere [45] that the presence, in chloroplast membranes, of lipids able to take up non-bilayer configurations is an essential factor in the incorporation of large pigment-protein complexes into the lipid matrix. Further support for this view has emerged from recent experiments in our laboratory [25] indicating that losses of chloroplast membrane stability and function in response to temperature stress are associated with irreversible changes in the lipid matrix leading to the formation of non-bilayer structures in the native membrane, and from reconstitution studies [46] showing that the monogalactosyldiacylglycerol is required for the linkage of chlorophyll *a*/*b* light-harvesting protein complexes to the core particles of the Photosystem II light-harvesting apparatus.

Many reports have appeared in the literature suggesting that chilling-sensitive plants are distinguished from chilling-resistant plants by the fact that the membrane lipids of the former plants undergo characteristic fluidity changes in the temperature range 5–15°C. These changes were initially ascribed to gel-to-liquid crystal phase transitions [47]. In the absence of supporting evidence from thermal and X-ray diffraction studies, however, this view has been largely discounted and, indeed, the very existence of such a correlation has

been challenged [28]. Nevertheless, there are many outstanding reports of discontinuities in the temperature profiles of spin-label and fluorescence-probe mobility in plant lipid extracts over this temperature range and it is likely that some, at least, of these are reflections of the formation of lamellar phases of the type we describe here. If so, as in thermal adaptation, it is probably the occurrence of bilayer/non-bilayer lipid transformations and their influence on the packaging of proteins rather than fluidity changes that are of primary importance in the chilling-sensitivity phenomenon.

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