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Review

Biochemical and biophysical properties of thylakoid acyl lipids

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Contents

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Abbreviations: CF_0-CF_1, ATP synthetase; DGDG, digalactosyldiacylglycerol; DOPC, dioleoyl-PC; DPPC, dipalmitoyl-PC; DPPG, dipalmitoyl-PG; DPH, diphenylhexatriene; DSC, differential scanning calorimetry; LHCII, light harvesting chlorophyll *a*/*b*-protein complex; MGDG, monogalactosyldiacylglycerol; MglcDG, monoglucosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PQ, plastoquinone; PS I, Photosystem I; PS II, Photosystem II; SQDG, sulfoquinovosyldiacylglycerol; 16:1*tr*, *trans*-Δ^3-hexadecenoic acid.

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I. Introduction

Oxygen-evolving photosynthesis is a process common to eukaryotic algae, higher plants, and one group of photosynthetic prokaryotes, the cyanobacteria (blue-green algae). Light harvesting and photosynthetic electron transport occur in a group of membranes known as the thylakoids. The thylakoids may be present as single unattached membrane sacs in the cytoplasm in prokaryotic algae, or be localized within a double membrane envelope in the eukaryotic algal and higher plant chloroplast. Irrespective of their location, the thylakoids are the predominant membranes in plant leaf cells, representing 60–80% of the total cellular membranes in higher plant mesophyll cells [1]. It follows, then, that the protein and lipid components of the thylakoid are amongst the most abundant organic compounds in the natural world.

The eukaryotic thylakoid membrane system consists of a single contiguous membrane that is laterally segregated into two major functional domains; the appressed, or granal, membranes and the non-appressed stromal membranes (Fig. 1) [2]. The thylakoid encloses a common lumenal space which accumulates protons during photosynthetic electron transport. A wide range of microscopic, biochemical fractionation and genetic studies have shown the separation of chlorophyll-protein and electron transport complexes into the two domains [2–5]. The appressed membranes are highly enriched in Photosystem II (PS II), and the major chlorophyll *a/b* light harvesting complex (LHCII). In contrast, the non-appressed membranes are enriched in Photosystem I (PS I), and the CF_0-CF_1 complex responsible for ATP formation. Some complexes appear to be equally distributed between both membrane fractions, particularly the cytochrome b_6/f complex [6]. In addition, there appears to be a reversibly phosphorylated subpopulation of LHCII, the so-called mobile-LHC, which in the phosphorylated form is dissociated from PS II and is free to diffuse into the non-appressed membranes [7–10] and facilitate energy transfer to PS I. It is clear, then, that some of the pigment-protein macrocomplexes are capable of extensive lateral mobility in the plane of the membrane. It is worth emphasizing that the granal and stromal membranes are continuous with each other and their lipid and protein components are capable, at least theoretically, of free diffusion between these domains.

The lipid composition of the thylakoid membrane is unique. The lipids of animal, bacterial and the non-chloroplastic compartments of plant cells are dominated by the phospholipids. However, the major lipids of both eukaryotic and prokaryotic thylakoid membranes are the two galactolipids, monogalactosyldiacylglyerol (MGDG) and digalactosyldiacylglycerol (DGDG). This is all the more surprising in that MGDG, although it comprises 50% of the total thylakoid acyl lipid, does not spontaneously form bilayers when dispersed in purified form. In addition, thylakoid membranes contain a unique sulfolipid, sulfoquinovosyldiacylglycerol (SQDG), as well as phosphatidylglycerol (PG) that is enriched in a 16:1 *trans* fatty acid. The unusual composition of this membrane raises questions about how these lipids might contribute to the properties of the thylakoid membrane system: Are they responsible for the morphology of the thylakoids, their appression in the granal regions, or the segregation of the two photosystems in connected, but functionally distinct, parts of the membrane? Are particular lipids required for the maintenance of a pH gradient of as much as 3 units across the membrane? Are they required for maintaining the integrity of intrinsic multiprotein macrocomplexes responsible for converting light energy to chemical energy? Might the lipids facilitate protein insertion into, and packing within, the thylakoid bilayer?

The intent of this review is to examine the biological and biophysical properties of the thylakoid acyl lipids and attempt to discern their roles(s), if any, in photosynthetic processes.

II. Structure and distribution of the thylakoid acyl lipids

Clues to the function of the thylakoid lipids might be found in their distribution, both taxonomically and cytologically, within photosynthetic organisms. In this section an overview of the chemical structures and distributions of the major thylakoid lipids is presented.

II-A. Thylakoid lipids

The predominant lipids of thylakoids (Fig. 2) are the uncharged galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These galactosylglycerols total about 75% of the total

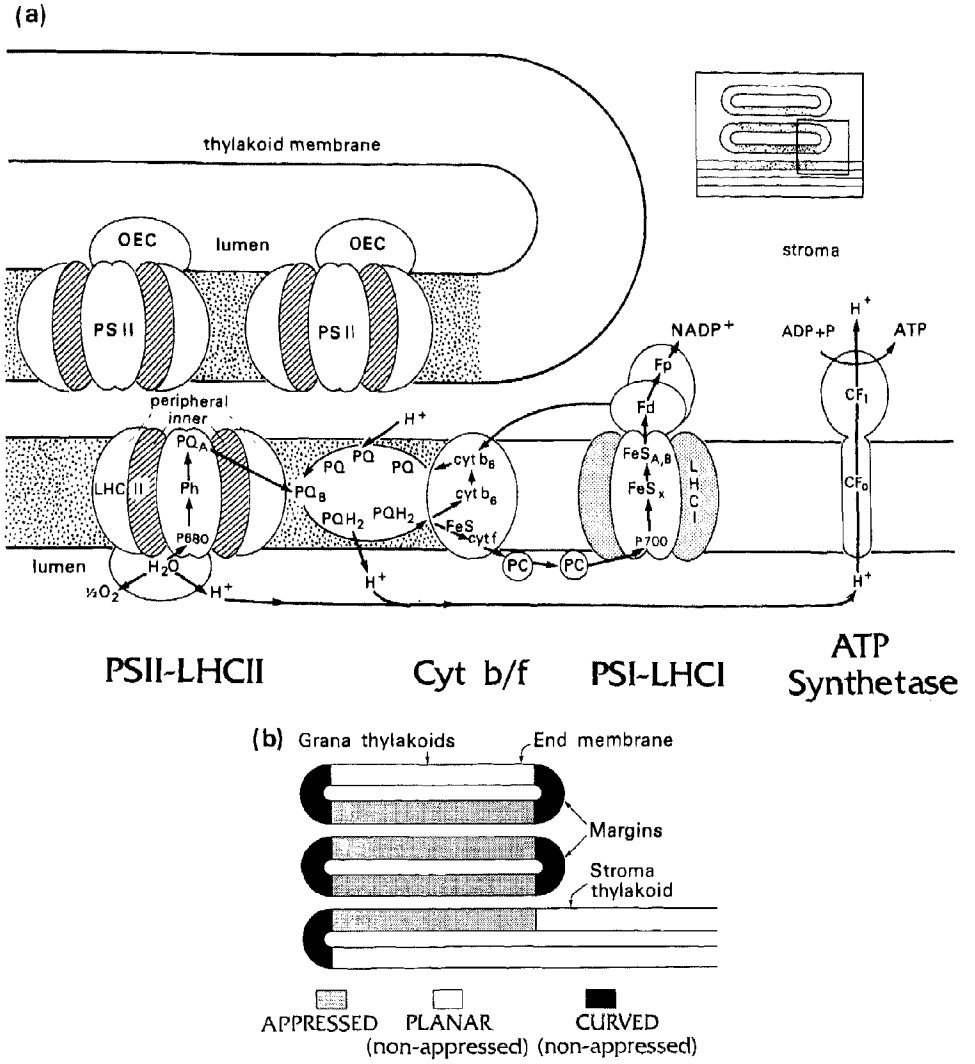

Fig. 1. Current conceptual model of thylakoid membrane system showing (a) lateral segregation of PS I and PS II, and highly curved nature of grana margins. (b) Definition of regions defined as appressed (grana thylakoids), non-appressed (stroma thylakoids) and margins. Courtesy of Dr. J.M. Anderson and Elsevier.

thylakoid lipid (50% and 25%, respectively). The remainder of the acyl lipid is comprised mostly of the anionic lipids sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) [11]. Thylakoid membranes do not contain sterols. A long-standing debate concerning whether phosphatidylcholine (PC) is an endogenous lipid in thylakoids or whether it is a contaminant present from the extra-chloroplastic membranes or chloroplast envelopes is still unresolved. Most workers routinely obtain a few mol% PC in 'purified' thylakoid preparations (Table I). Siegenthaler et al. [12] have demonstrated a constant PC/PG ratio during successive thylakoid purifications, and argued that this PC in the thylakoids must be endogenous. On the other hand, Dorne et al. [13] have recently shown that thylakoids purified from phospholipase-C-treated intact spinach chloroplasts contained no PC, whereas those purified from untreated intact chloroplasts did contain PC. This is strong evidence that the PC found in 'purified' thylakoids is a contaminant from the chloroplast envelopes. Several other galactolipids have also been detected in plant extracts including tri- and tetragalactosylglycerols as well as MGDG and DGDG acylated at the galactose group. These compounds may be artifacts of tissue homogenization and membrane isolation [14–17]. Methods for the purification and

analysis of the thylakoid lipids have been recently reviewed [18,19]. Both ^{13}C- and ^{1}H-NMR spectra have been described for the thylakoid lipids [20–23].

The fatty acids most commonly esterified to membrane lipids in higher plant thylakoids are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). In addition, photosynthetic tissues in plants possess significant quantities of the unusual fatty acid *trans*-Δ^3-hexadecenoic acid (16:1*tr*) esterified specifically to *sn*-2 of PG. Some species also contain high levels of palmitolenic acid (16:3) located almost exclusively on MGDG. As this discussion implies, fatty acids are specifically distributed between the various lipid classes and, in some cases, bound to specific sites within a given lipid. As a result, the four major classes of lipids (MGDG, DGDG, SQDG, and PG) represent dozens of different molecular species (specific head group and fatty acid combinations) of lipids.

It should be added that the thylakoids also contain a wide variety of lipophilic pigments, including the chlorophylls *a* and *b*, the xanthophylls, β-carotene, and the quinones. With the possible exception of the quinones, these pigments are non-covalently bound to the polypeptides of the pigment-protein complexes [24,25]. The chlorophyll-binding proteins involved in light absorption bind specific types and amounts of the chlorophylls and carotenoids [26,27]. Plastoquinone-9, the major thylakoid quinone, is present in a ratio of one molecule per 140 molecules of acyl lipid (0.7% of

TABLE I

Lipid compositions of thylakoid and subthylakoid preparations

Methods: mech., fragmentation with Yeda Press, followed by two-phase separation; Triton, solubilisation with Triton X-100; OG, solubilized with octylglucoside; cholate, solubilized with Na cholate: Triton, Mg, LHCII precipitated with 10-25 mM $MgCl_2$. OPL, other polar lipids; RC, reaction center.

Fraction	Method	MGDG	DGDG	PG	SQDG	PC	PE	OPL	Species	Ref.
Thylakoid	–	38	29	14	12	5	–	–	spinach	45
Thylakoid	–	48	24	14	8	2	–	3	spinach	44,48
Thylakoid	–	34	40	9	13	4	–	–	spinach	46
Thylakoid	–	50	25	13	8	–	–	–	spinach	49
Thylakoid	–	56	29	5	3	7	–	–	spinach	50
Thylakoid	–	52	26	2	13	4	1	2	tobacco	51
Thylakoid	–	40	25	12	13	6		4	pea	186
Grana	mech.	44	18	33	9	2	–	–	spinach	45
Grana	mech.	47	17	17	11	3	–	5	spinach	44
Stromal	mech.	36	31	12	6	4	–	10	spinach	44
Stromal	mech.	38	28	12	13	4	–	5	pea	186
PS II grana	Triton	31	6	24	14	13	–	11	spinach	48
PS II grana	Triton	44	39	9	5	3	–	–	spinach	46
PS II grana	Triton	50	37	6	4	3	–	–	spinach	47
PS II grana	Triton	59	27	10	3	1	–	–	spinach	50
PS II grana	Triton	53	22	3	15	9	–	–	tobacco	51
PS II grana	Triton	46	23	10	11	4	–	6	pea	186
PS II core	Triton	70	–	–	30	–	–	–	spinach	52
PS II core	OG	32	34	34	0	0	–	–	spinach	50
PS II core	OG	56	25	2	12	3.5	–	–	tobacco	51
PS II RC	Triton	91	0	9	0	0	–	–	spinach	50
CF_1-CF_0	cholate	–	10	–	90	–	–	–	spinach	49
LHCII	Triton, Mg	53	26	6	15	–	–	–	spinach	193
LHCII	Triton, Mg	28	25	33	6	7	2	–	rye	194
LHCII	SDS-PAGE	33	28	15	12	6	–	6	spinach	192
LRCII(II*)	SDS-PAGE	34	22	39	–	–	–	5	tobacco	195
LHCII(II)	SDS-PAGE	57	23	11	–	–	–	9	tobacco	
LHCII(II*)	SDS-PAGE	42	8	32	15	3	–	–	tobacco	196
LHCII(II)	SDS-PAGE	55	19	10	11	3	–	–	tobacco	
CPI	SDS-PAGE	–	–	–	72	–	–	28	spinach	192
CPI	SDS-PAGE	45	27	5	–	–	–	23	tobacco	195
CPI	SDS-PAGE	35	13	9	39	4	–	–	tobacco	196

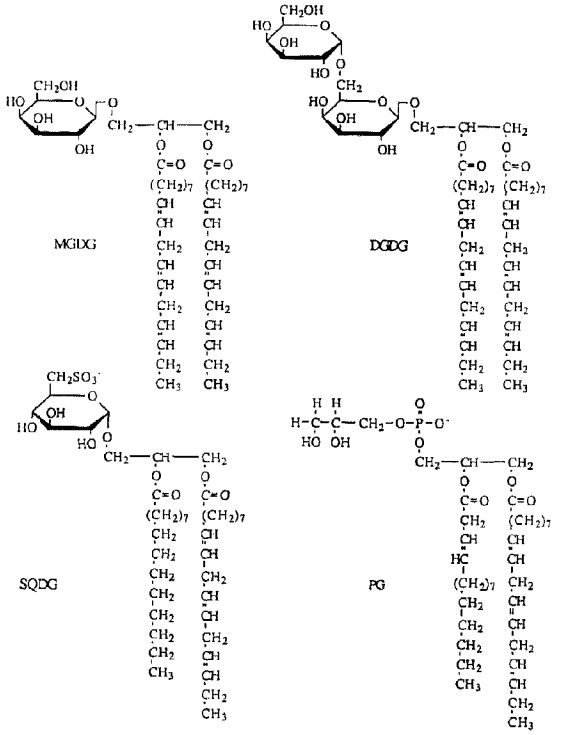

Fig. 2. Chemical structure of the major lipids of higher plant thylakoid membranes. Shown are (a) 18:3/18:3-MGDG, (b) 18:3/18:3-DGDG, (c) 18:3/16:0-SQDG, and (d) 18:3/16:1*tr*-PG.

acyl lipid phase) in both appressed and non-appressed regions [28]. Plastoquinone probably plays the role of long-distance electron carrier, which is required because of the lateral separation of PS II and PS I complexes. It is estimated that about 10% would be firmly bound to PS II reaction centers and the rest would be free to diffuse within the bilayer and form transient associations with PS II and the cytochrome complex [28]. Plastoquinone diffusion coefficients of $(1.3–3.5) \cdot 10^{-7}$ cm^2 s^{-1} have been reported in phospholipid vesicles [29] and ubiquinone diffusion coefficients as high as 10^{-6} cm^2 s^{-1} have been measured in phospholipid vesicles and mitochondrial membranes [30,31]. The influence of proteins on PQ diffusion has been estimated to reduce the diffusion coefficient by approx. 10-fold [32]. If this estimate is correct, PQ diffusion rates would still be high enough so that long-distance electron transport by PQ would not be the rate-limiting step of photosynthesis.

The exact location of the quinones along the bilayer normal is a point of some debate [33,34]. Protonation of the quinone head-group might lead to decreased head-group polarity and conformational changes that allow the entire molecule to bury deeply in the hydrophobic acyl region [35]. The function of quinones in photosynthesis has been reviewed [25,36,37], and will not be discussed further here.

II-B. Taxonomic distribution

The glycolipids of higher plant thylakoids are common to all oxygen-evolving photosynthetic organisms. These lipids have been detected in all higher plants, as well as in the red, green and brown algae, in the diatoms, in the dinoflagellates and in the cyanobacteria *Anabena* sp. and *Anacystis* sp. (reviewed by Douce and Joyard [38]). The galactolipids have also been found in a unique chlorophyll *b*-containing prokaryote, *Prochloron* sp., as has a closely related lipid monoglucosyldiacylglycerol (MglcDG) [39]. In some of the cyanobacteria, the ability to desaturate MGDG fatty acids beyond 16:1 and 18:1 is not present. Clearly, the glycolipids are highly conserved despite significant changes in thylakoid ultrastructure and light absorption strategies utilized by many of these 'lower' organisms [2]. In contrast, the lipids of the anaerobic photosynthetic purple bacteria, *Rhodospirillacea*, are predominantly those phospholipids common to other bacteria. This suggests that the presence of galactolipids is diagnostic of oxygenic, chlorophyll-*a*-containing photosynthetic membranes. Is this because the physical properties of the galactolipids make them particularly suited for this role, or does it simply reflect evolutionary conservation of biosynthetic and membrane assembly pathways from the first 'green' water-splitting prokaryote?

II-C. Cellular and subcellular distribution

It has been implied above that the galactolipids and SQDG occur exclusively in the thylakoid membranes. In fact, significant levels have also been found in the chloroplast envelopes of higher plants [40]. Consistent with an endosymbiotic origin of chloroplasts, the separation of the inner and outer membranes of the chloroplast envelope [41] has shown the inner membrane to be enriched in unsaturated glycolipids. Conversely, the outer membrane is composed predominantly of phospholipids similar to those in the cytoplasmic membranes and possesses lowered glycolipid levels and lower levels of acyl unsaturation.

The lateral segregation of protein macrocomplexes found in higher plant thylakoids (Fig. 1) suggests that such lateral heterogeneity might also exist for the membrane lipids. Granal and stromal membrane-enriched preparations can be made by mechanical disruption (e.g., in a Yeda or French press) followed by two-phase separation. Table I shows that there are small enrichments of 4–10 mol% for MGDG in granal membranes compared to stromal membranes or whole thylakoids of spinach; similar results were obtained

with wheat [42], but no differences were found with maize [43]. More variable results have been reported for the distributions of DGDG, PG, and SQDG [43–47]. Each value should be compared with the value for whole thylakoids in the same publication because of the differences in reported values even for the same species. Data for thylakoids of six species determined by the same laboratory are given in Ref. 53.

Overall, the lipid compositions of appressed versus non-appressed regions are not consistently different from those reported for whole thylakoids (Table I). Similar conclusions may be drawn from the reported fatty acid compositions of individual lipid classes in the same publications [43,45,46]. A possible exception to this observation may be made in the case of PG and 16 : 1*tr*, as is discussed in greater detail in subsection V-D. Of more likely significance, however, is the lateral heterogeneity in lipid/protein ratios of appressed and non-appressed membranes. The former fraction is highly depleted of lipid and enriched in protein, and the reverse is true of the latter fraction [44,45,54]. Plastoquinone has been shown to be evenly distributed between the thylakoid membrane domains [28], consistent with its role as a long-distance electron carrier.

A preparation consisting of granal (appressed) membranes missing their margins can be prepared by treatment with the detergent Triton X-100 ('PS II grana' in Table I). While these particles are relatively free of PS I and very active in oxygen evolution, they are also lipid-depleted compared to thylakoids [46,48,50,51]. There is no agreement on the fractional content of different lipid classes (Table I). This could be due to differences in relative solubilization as a result of small differences in detergent concentration and extraction conditions. Murata et al.[50] have pointed out that these preparations contain large amounts of residual Triton X-100 which interferes with standard analytical methods. When they removed the detergent before lipid analysis, the only difference between PS II membranes and thylakoids was that the former were enriched in PG (Table I).

Transmembrane specificity of thylakoid lipid distribution has also been examined by several groups (reviewed in Ref. 55,56). Three major experimental techniques have been used: (1) external or internal digestion of membranes by lipolytic enzymes assaying for the preferential destruction of certain lipids [12,57,58]; (2) external or internal labelling of lipids, usually the labelling of galactose with ^3H after galactose oxidase digestion [53,59]; (3) antibody binding experiments [60,61]. All of these methods suffer serious technical difficulties, not the least of which is the need for all lipids in a monolayer to be uniformly accessible to large external enzymes, antibodies, or reagents. In view of the high protein content of the membrane (60% by weight) this may be difficult to achieve. Further, lipolytic studies must be done with great care to avoid extensive membrane reorganization, particularly lipid transbilayer flip-flop, as a result of degradative lipid removal from one monolayer [12,62]. Finally, it is important that both the intact and the partially reacted membrane be impermeable to the digesting enzyme or labelling reagent. Other factors to be controlled are discussed in Ref. 53,58.

It is therefore not surprising that there is not complete agreement in the literature concerning the differential transbilayer orientation of these lipids. Several groups report an outside/inside ratio of about 60/40 for MGDG in several species, based on lipolytic and labelling methods [53,58,59]. This was qualitatively supported by immunogold labelling with a monoclonal antibody: thylakoids were much more densely labelled than inside-out vesicles [61]. MGDG's transbilayer distribution is apparently unaffected by dark/light transitions of the thylakoid [62]. Lipolytic and labelling studies gave an outside/inside ratio of 15/85 for DGDG [53,58,64], but another study using chemical labelling of inside-out and rightside-out vesicles found a 60/40 ratio [59]. Workers appear to agree, however, on a 70/30 outside/inside ratio for PG [60,64,65]. SQDG is very resistant to lipases [53] and largely inaccessible to antibodies [60]. It has been calculated from the distribution of the other lipids that up to 25% may be in the outer monolayer [53,58,59]. The effect of the thylakoid pH gradient on the transbilayer distribution of the charged lipids such as PG and SQDG could be a significant factor [66], but has not yet been investigated.

Some of the conflicting data on subcellular distribution in the literature may result from catalytic degradation of lipids during cell disruption and membrane purification. The very rapid destruction of thylakoid acyl lipids by enzymes released and/or activated during cellular rupture is well known [67], but has often been ignored by workers in this area. Both galactolipid-specific [68] and nonspecific [69] activities have been reported in a wide variety of species and tissues. These include potato tubers [69], leaves of *Phaseolus multiflorus* [61,68,70], and chloroplasts of *Phaseolus* sp. [71,72] and of *Triticum sativum* [73]. Indeed, rapid degradation of as much as 50% of thylakoid lipids occurred during the isolation of wheat chloroplasts [73] and barley thylakoids [74], as well as in granal and stromal vesicles made by mechanical disruption and aqueous two-phase partitioning [74]. Few investigators report measures taken to avoid lipolytic activity. Further, few workers report even the routine quantification of neutral lipid (free fatty acids and diacylglycerols) in such preparations, possibly because these compounds co-chromatograph with the pigments in many solvent systems. We note, though, that catalytic activity is probably species-specific: no

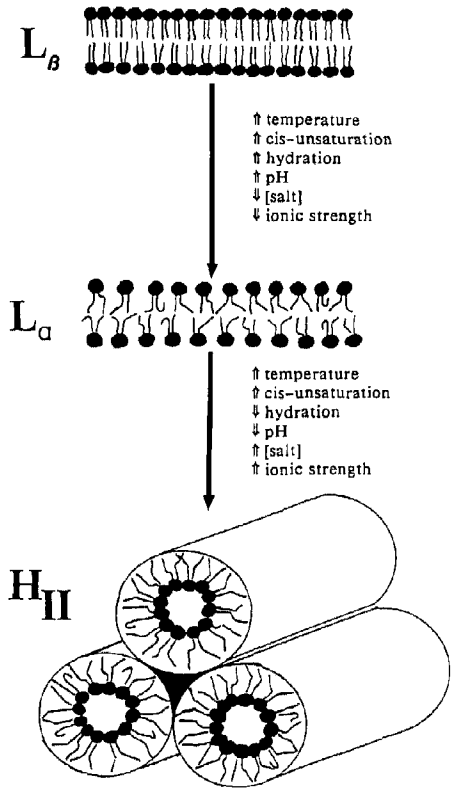

Fig. 3. Schematic representation of the structures of the major phases adopted by the thylakoid lipids and factors which contribute to phase transitions.

degradation of lipid is found in isolated pea thylakoids [18]. However, significant destruction of chloroplast lipids may occur even without cellular rupture [75,76]. It would be of interest to repeat some of this work with membranes isolated in the presence of lipase inhibitors such as *p*-chloromercuribenzoate and phenylmercuric acid [73,77].

III. Phase behavior of thylakoid acyl lipids

The ability of membrane lipids to modulate membrane behavior is determined by their physical characteristics. This section provides a review of the phase properties of thylakoid lipids as a prelude to a discussion of how these properties might affect photosynthesis. This subject has been previously reviewed by Quinn and Williams [78] and by Murphy [24,25]. The major phases that may be adopted by the thylakoid lipids are the gel (L_β) phase, the liquid-crystalline (L_α) phase characteristic of biological membranes, and the hexagonal-II (H_{II}) phase (Fig. 3). They can be interconverted by a number of factors as noted in the figure. While other phases also exist, including several metastable I_β phases and the cubic phase, their contribution to biologically relevant properties is unclear.

III-A. MGDG

The phase behavior of chloroplast galactolipids was first investigated by Rivas and Luzzati [79]. They reported that galactolipid mixtures from maize would adopt a variety of phases, including the hexagonal-II (H_{II}), cubic, and lamellar phases depending on the temperature and water content of the system. Kreutz [80] and Shipley et al. [81] showed that purified MGDG would adopt the H_{II} phase over a wide temperature range of -15 to 80°C at $>50\%$ lipid in water. The extent to which MGDG adopts the H_{II} phase is highly dependent on the degree of unsaturation of its acyl chains [82]. The formation of the H_{II} phase by MGDG at 20°C was significantly reduced by dropping the average number of double bonds per MGDG molecule by about one. More complete saturation promoted the formation of a laterally segregated lamellar gel L_β-H_{II} phase [83]. However, even monounsaturated MGDG can be forced into the H_{II} phase at 55°C [84]. Fully saturated MGDG yields a liquid-condensed monolayer [85] indicative of the L_β phase but showing the existence of several metastable gel states with complex phase behavior and thermal history dependence [84,86]. Reported temperatures for the L_β–L_α transition of distearoyl-MGDG are between 45 and 70°C as measured by differential scanning calorimetry (DSC) [82,86] and 70°C as measured by X-ray diffraction [87].

III-B. DGDG

The addition of a single galactose to MGDG to yield DGDG results in a lipid with completely different phase behavior. DGDG forms a lamellar phase under all known conditions of temperature and hydration. Polyunsaturated DGDG exists as a liquid-expanded lamellar (L_α) phase at 20° [85] and undergoes the L_α to L_β transition upon cooling below -50°C [81]. As with MGDG, saturated DGDG is in the gel phase, L_β, at room temperature. A series of metastable forms of the L_β phase have been identified [86]. The distearoyl-DGDG lipid shows the transition from L_β to L_α at 50–51°C [88,89].

III-C. PG and SQDG

The phase behavior of a wide variety of lipids, including PG, has been reviewed recently by Tilcock [90]. For unsaturated PG the L_α phase is expected under most physiological conditions. Dipalmitoyl-PG (DPPG) shows an L_β to L_α transition at 41°C in H_2O, but the transition temperature is raised to 54°C in the presence of Mg^{2+}, and to 86°C by Ca^{2+}. This probably

results from electrostatic screening of adjacent negatively charged phosphate groups [90] and/or ion binding and charge bridging. The addition of the *trans*-Δ^3 bond to the 16:0 at *sn*-2 position drops the temperature of the L_β–L_α transition by about 10°C below that of DPPG [91]. Thylakoids of some species may contain significant differences in quantities of 16:0/16:0 and 16:0/16:1 PG [92]. This may be relevant to the low-temperature sensitivity of some plant species. It has been proposed [92] that the high levels of high-melting-point (highly saturated) PG in the thylakoids of chilling-sensitive plants leads to the formation of L_β phase lipid in the membrane at killing temperatures (see subsection III-E).

The phase behavior of SQDG has not received as much attention as that of the galactolipids. SQDG purified from an algal source was in the L_α phase above 20°C, co-existing L_α and L_α phases from 20 to −10°C, and was exclusively in the L_β phase at below −10°C [81]. The fatty acid composition of this SQDG, however, was highly saturated compared to that of higher plants. The 18:3 content and molecular species composition of SQDG has been shown to be highly species-dependent [93]. The SQDG purified from spinach showed no evidence of L_β phase formation above 5°C, as detected by fluorescence polarization of *trans*-parinaric acid [93]. There are no published data concerning the possibility of an SQDG H_{II} phase at higher temperatures and high ionic strength.

III-D. Binary mixtures of MGDG and DGDG

The tendency of binary galactolipid mixtures and total thylakoid polar lipids to form the H_{II} phase or its inverted micellar intermediate [94,95] is due entirely to unsaturated MGDG [82]. Replacement of unsaturated MGDG with identical proportions of fully hydrogenated MGDG in thylakoid lipid extracts removed the ability of these preparations to form the H_{II} phase [82]. MGDG/DGDG mixtures show a complex interplay of L_α, H_{II}, cubic and water phases depending on both water content and temperature [79,96]. Promotion of the L_α to H_{II} transition in binary galactolipid mixtures has been accomplished by both increased temperature and by ethylene glycol addition. This has led Sen et al. [97] to conclude that the H_{II} transition requires both a large hydrophobic volume (from unsaturated fatty acids and/or thermal motion) and the disruption of lipid headgroup interaction with water.

It is important to recognize that the phase(s) adopted by binary galactolipid mixtures is strongly dependent on the reconstitution method used [98,99]. Sprague and

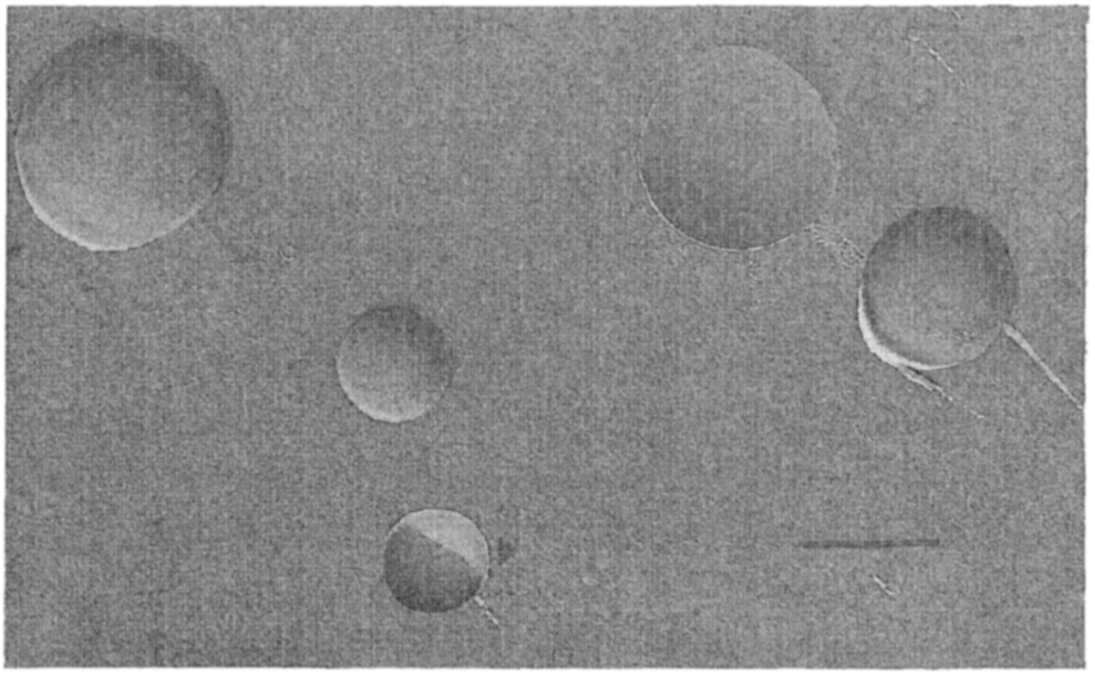

Fig. 4. Freeze-fracture electron micrograph of liposomes composed of MGDG/DGDG/SQDG/PG (50/25/12/12, mol/mol) dispersed in 5 mM $MgCl_2$ and 0.2 mM NaCl. The dispersion was extruded to 100 nm mean diameter and subsequently frozen and thawed on solid CO_2. Note the absence of both the H_{II} phase and inverted lipidic particles. In addition, the freeze-thaw step has resulted in vesicle fusion and a significant increase in mean liposome diameter. Bar represents 500 nm.

Staehelin found that a 1:1 mixture of MGDG and DGDG gave a mixture of lamellar and H_{II} phases, as detected by freeze-fracture electron microscopy, using a conventional dispersal method. They obtained larger proportions of lamellar phase lipid by using a reverse-phase evaporation method [98,99]. With slight modification to a conventional procedure [100], and ensuring that the dispersed vesicles are unilamellar, we have obtained liposomes with 50 mol% MGDG that completely lack the H_{II} phase and rarely possess inverted cylindrical micelles, as determined by freeze-fracture electron microscopy (Fig. 4). Significant changes in liposome size and phase behavior can be induced by a simple freeze-thaw step [23]. Furthermore, significant differences in vesicle size and internal solute content may arise from different reconstitution schemes and thus affect the lipid phase structure [101–103]. Since mixtures containing high proportions of MGDG are apparently in a region of the phase diagram where they are easily converted from the lamellar to H_{II} phases, this suggests that thylakoid bilayer structure must be significantly stabilized either by the presence of intrinsic membrane proteins and/or by the other lipid components.

III-E. Interactions between lipids

The stabilization of the lamellar phase in thylakoid lipid extracts by the presence of the anionic lipids SQDG and PG has been suggested based on the ability of these lipids to prevent vesicle fusion and H_{II} phase formation [104]. It could not be concluded from this work, however, whether stabilization resulted from direct lipid–lipid interactions or from electrostatic repulsion between adjacent vesicles. Such repulsion would inhibit close vesicle approach and subsequent vesicle fusion, and therefore prevent an increase in vesicle size. It could be argued that a lamellar phase for MGDG is promoted by smaller vesicle size as a result of more acceptable packing of the cone-shaped MGDG molecule at the inner monolayer of small vesicles. Such a transbilayer asymmetry based on packing considerations has been proposed for MGDG in thylakoid membranes, particularly at the inner surface of the highly curved end margins [105] (but see subsection III-G).

The possibility that small quantities of disaturated PG or PG esterified with one monosaturated and one *trans*-Δ^3-hexadecenoic acid might phase separate into an L_β phase has been considered in the context of chilling sensitivity of plant species [92]. Chlorophyll *a* fluorescence changes consistent with phase separation have been reported in semi-purified polar lipid extracts and chloroplasts from chilling sensitive species [106]. In addition, DSC-detected endotherms have been seen at 10–12°C in total thylakoid polar lipid extracts of sensitive plants [107,108]. However, contrary results have been reported by Low et al. [109], who observed no correlation between chilling-stress and endotherms in either thylakoids or their polar lipids from various plant species. Raison and Wright [107] reported that the addition of small quantities (< 5 mol%) of DPPG or DPPC to thylakoid polar lipids triggered the appearance of DSC endotherms in the 10°C range. We have found, however, that unilamellar galactolipid liposomes containing up to 12 mol% DPPG do not show endotherms unless the preparations have been subjected to a freeze-thaw step during their preparation (Webb, M.S., Lynch, D.V. and Green, B.R., unpublished results). As discussed in the previous section, it is likely that the phase behavior of a galactolipid-based dispersion is highly dependent on the specific dispersal method. Our conclusion that DPPG at 12 mol% or less in well-defined galactolipid liposomes does not phase-separate is consistent with the report that up to 50% DPPG in bilayers of dimannosyldiacylglycerol from *Micrococcus luteus* does not phase-separate at 20°C, even in the presence of Na^+ or Mg^{2+} [110]. These authors suggested that the formation of extensive hydrogen bonds between DPPG and the glycolipid caused the high miscibility of DPPG. Quinn and Williams [78] have also concluded that the evidence for the occurrence of gel-phase separation in thylakoids is unconvincing. An exception to this generalization is the well-characterized L_β phase separation observed in the prokaryote *Anacystis nidulans* as discussed in more detail in subsection IV-A.

Finally, no specific interactions have been observed between PC and either MGDG or DGDG [111]. Specific interactions have been reported in vitro between the galactolipids and a variety of sterols [112–114], as well as with the chlorophylls [115–121]. However, as pointed out earlier, the sterol content of the thylakoid is very low, and the chlorophylls and carotenoids are bound in pigment/protein complexes such that their free content in the bilayer is also expected to be very low. The results obtained from these studies will not be further discussed here.

III-F. The shape concept of lipid phase behavior

The phase preferences of membrane lipids may be qualitatively understood by the 'shape concept' of lipid polymorphism [121,122]. This model is closely related to the quantitative 'intrinsic curvature' model of Gruner [123] and the 'critical packing parameter' of Israelachvili et al. [124,125]. These models argue that the phase preference of a membrane lipid originates in the ratio of volumes occupied by hydrophobic acyl regions and hydrophilic head-group regions. Factors that increase the ratio of hydrophobic/hydrophilic volumes promote a time-averaged cone or wedge shape of the lipid. Lowest-energy packing of these lipids would be

an 'inside-out' phase with clustered interior head groups and external hydrophobic regions, i.e., the H_{II} phase (Fig. 3). Such factors include *cis*-unsaturation of acyl chains, high temperatures, and decreased head-group area caused by either decreased water content or electrostatic screening of charged groups.

It is obvious then that eukaryotic MGDG adopts the H_{II} phase because of both a very high degree of acyl unsaturation and the small swept area of the mono-galactosyl head-group. Conversely, the DGDG head group is significantly larger than that of MGDG, giving the molecule an overall cylindrical shape that is most easily accommodated by lamellar phase packing. Similarly, PG and SQDG show overall cylindrical shapes due to moderate unsaturation and large head-group areas due to inter-lipid electrostatic repulsion from the phosphate and sulfonate groups. Conical and cylindrical shapes for MGDG and DGDG have been obtained by minimum energy modelling of these lipids [126]. This analysis showed the areas occupied per hydrocarbon chain/head group to be 0.96 nm^2/0.54 nm^2 for MGDG and 0.95 nm^2/0.85 nm^2 for DGDG. This means that unsaturated MGDG and DGDG occupy very similar areas in monolayers [127], but their aqueous dispersions should have inverted and lamellar packing respectively based on shape considerations. Very similar phase behaviors are observed for the monoglucosyl- and diglucosyldiacylglycerols isolated from *Acholeplasma laidlawii* [128,129].

III-G. Lipid shape and thylakoid architecture

In thin-section transmission electron microscopy granal thyalkoids show a characteristic appearance as closely appressed flattened sacs (Fig. 1). Although it is not apparent from most two-dimensional drawings of thylakoid structure, the connecting stroma lamellae are thinner in diameter, more rounded and form a system of anastamosing tubules (references in Ref. 140). In the granal thylakoids the appressed regions have zero membrane curvature (very large radius of curvature); and the unappressed end margins have a very small radius of curvature. It has been proposed that the wedge-shaped MGDG should be preferentially located on the inner monolayer of the end margin to facilitate the high curvature in this region [24,105], and that the margins are mostly lipidic. This implies that the more cylindrically-shaped lipids DGDG, SQDG and PG would be enriched on the outer monolayer of the end margins as well as in the zero curvature areas of the appressed membranes. However, the data in Table I indicate the opposite: the appressed regions are more likely to be enriched in MGDG and depleted in DGDG. The lipid composition of margins has not been determined, since they make up only a small percentage of the thylakoid membrane system and cannot be purified intact. However, Webber et al. [130] succeeded in selectively solubilizing the margin fraction and were able to show that it did contain proteins, being enriched for coupling factor and a small amount of PS I, in agreement with immunocytochemical studies [2].

Other factors besides lipid shape must contibute to the different degrees of curvature found within the thylakoid system. Gruner and co-workers [123,131] have shown that the tendency for a pure lipid monolayer to have a very small radius of curvature is affected by factors which alter the balance of lateral packing energies in the head-group and hydrophobic domains. The sum of packing energies at different depths within the bilayer due to different acyl chain shapes gives rise to a bending energy and, thereby to an intrinsic radius of curvature for the monolayer. The packing energies also complicate the prediction of lipid phase behavior. In addition to this, when there are adhesive forces between bilayers the lateral tension will cause the bilayers to flatten and conserve vesicle surface area [132]. These factors are discussed in more detail in Section VII.

In the intact thylakoid the proteins must also contribute to thylakoid morphology. Several *Arabidopsis thaliana* mutants deficient in galactolipid unsaturation, i.e., with a lesser tendency to form non-bilayer structures, have been isolated [133–137]. Their defects had no consistent effect on the ratio of appressed/non-appressed membranes. A mutant with hardly any di- or tri-unsaturated fatty acids and therefore only half the normal unsaturation (*fad*C), had a lower ratio [134], the reverse of what would be predicted from their higher content of cylindrical lipids. However, it also had a decreased protein/lipid ratio, which partially compensated for a decrease in fluidity (see Section IV). The *fad*B mutant, whose defect mainly decreased the unsaturation of MGDG, had increased stability at high temperature but no change in thylakoid ultrastructure or fluidity [135].

In other literature, there is one report that catalytic hydrogenation of lipids does not cause a change in thylakoid morphology [138]. Removal of galactolipids by galactolipase digestion has been reported to cause granal swelling, but not de-stacking of the grana [139]. Therefore, although lipid shape may have something to do with the morphology of the thylakoid membrane, the effects of changing part of the lipid population are too subtle to be detected with the means currently available.

There is an interesting suggestion that reversed cubic phase lipid may be responsible for the unusual morphology of the prolamellar body [96]. This is the three-dimensional lattice of lipoprotein tubules formed in the protoplastid when seedlings are allowed to grow in the dark for a few days after germination. On exposure to light, and in parallel with chlorophyll syn-

thesis, this membrane system rearranges within a few hours to give a regular thylakoid network. Micrographs of the prolamellar body [140] show a striking resemblance to the geometry expected of reversed cubic phase lipid [96]. The lipid composition of the prolamellar body is similar to that of thylakoids, although the MGDG/DGDG is about 20% higher and the lipid/protein ratio 25% lower [141]. Hydration of a mixture of purified prolamellar body lipids can give rise to the reversed cubic phase, although it appears to occupy a rather limited region of the phase diagram [96]. However, there is as yet no direct evidence that the lipid in the prolamellar body itself is in the reversed cubic phase.

IV. Fluidity

It is commonly believed that a high degree of thylakoid lipid unsaturation is critical for membrane fluidity and for the low-temperature thermal tolerance of photosynthesis. Fluidity is loosely defined as the degree of unhindered mobility experienced by the acyl chains of lipid molecules [24]. It is commonly measured by electron paramagnetic resonance (EPR) spectroscopy using a spin-label probe, or by flourescence polarization using a fluorescent probe such as diphenylhexatriene (DPH) that partitions into the bilayer. The experimental results are used to calculate an order parameter or a measure of the rate of molecular movement such as a rotational correlation time or a rotational diffusion constant. The equations for doing these calculations depend on the model of molecular motion used. It should be noted that rotational motion of a probe is not equivalent to long-distance translational diffusion [142].

It is generally assumed that a decreased order parameter means decreased microviscosity in the neighborhood of the probe and increased rotational and diffusional motion. Unfortunately, this is not always the case. Rigorous angle- and time-resolved analysis of DPH probe motion in PC bilayers has shown that increased acyl unsaturation may decrease the order parameter associated with the hydrocarbon region, yet also decrease the rate of probe rotational diffusion [113,114]. The addition of cholesterol was found to increase the order parameter of DGDG and SQDG liposomes (as previously reported for many other lipids) but at the same time increase the rotational diffusion coefficient. In addition, the order parameters and diffusional motion rates were very similar in the DGDG and SQDG vesicles in spite of the fact that DGDG acyl chains are much more unsaturated [114]. Furthermore DGDG had a higher order parameter than di-18:1-PC. Both DGDG and SQDG had probe diffusion rates strikingly similar to those of di-18:1-PC and markedly lower than those of di-18:2-PC. In other words, there was no consistent correlation between unsaturation and either order parameter or diffusion rate. These workers have concluded that there is "no connection between molecular order and dynamics" [113]. This should be kept in mind in interpreting the data discussed below.

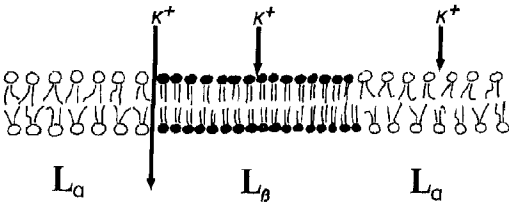

Fig. 5. Schematic representation of the dependence of bilayer permeability on lipid phase behavior. Solutes are envisaged as being unable to pass through lipid bilayers in either the L_α (lipids with open circles) or the L_β phase (lipids with closed circles). However, solutes may permeate through the packing irregularities proposed to exist in the interfacial region between them.

IV-A. Thylakoid lipid fluidity and temperature

Many tropical and subtropical species of higher plants show a wide variety of stress symptoms, and frequently plant death, when exposed to low, nonfreezing, temperatures in the 10°C to 12°C range. Physiological changes associated with chilling injury include immediate cessation of protoplasmic streaming, changes in respiratory rates, and extensive changes in general metabolite levels (reviewed in Refs. 143,144). Early attempts to link this range of physiological responses with a single trigger or site of damage were those of Lyons [143] and Raison [145]. Both workers suggested that the primary plant response to cold was a lipid phase transition of cellular membranes from the L_α to L_β phase. This theory, particularily that of Lyons, predicted that significantly increased cellular permeability to ions and solutes would result from the formation of "cracks and channels" in the solid state lipid (Fig. 5).

A recent version of Lyon's original hypothesis specific to chloroplast membranes has been proposed by Murata and co-workers [92,93,146,147]. The hypothesis suggests that elevated proportions of high-melting-point molecular species of PG in thylakoids promotes L_β phase formation at chilling temperatures in sensitive plants. The hypothesis has been supported by the detection of phase transitions in the PG purified from chilling-sensitive plants, but not in the PG from chilling-resistant plants, nor in other thylakoid lipids [147]. In addition, phase transitions have been detected by DSC in total polar lipids from chilling-sensitive plants [107,108] as well as by fluorescence methods in thylakoid, mitochondrial, and plasma membrane preparations and their lipid extracts [91,106,148,149]. Some

workers have found positive correlations between chilling sensitivity and the proportions of the high-melting-point fatty acids (16:0, 16:1*tr*, and 18:0) and/or with the high-melting-point molecular species of PG [92,93,146]. Other workers have found no correlation between the content of high-melting-point fatty acids and chilling sensitivity [150,151] and have challenged the generality of the hypothesis. In addition, some investigators have been unable to find L_β phase formation in liposomes with compositions mimicking those of chilling-sensitive plants [23] or have observed no correlation between chilling stress and phase behavior of thylakoids or their polar lipid extracts [109] (subsection III-E). In a recent review, Somerville and Browse concluded that the degree of lipid unsaturation cannot account for naturally occurring cold-sensitivity in plants [133].

A more direct connection between acyl unsaturation, L_β phase formation and cellular death has been established in the cyanobacteria (reviewed in Ref. 152). In the cyanobacterium *Anacystis nidulans* the thylakoid lipids are comprised exclusively of saturated and monounsaturated fatty acids. The degree of unsaturation of the lipids is increased by adaptation to lower growth temperature. In *A. nidulans* there is a strong correlative relationship between the occurrence of the L_β phase in the plasma membrane, as detected by freeze-fracture electron microscopy as well as ESR spectroscopy and other methods, and leakage of cytoplasmic solutes to the external medium. Phase transitions were reported to occur in the thylakoid membranes as well but were not directly related to cellular death. In contrast, the cyanobacterium *Anabena variabilis* possesses lipids whose acyl chains are di- and trienoic. Its plasma membranes have no phase transitions above 0°C and show neither electrolyte leakage nor physiological damage in the same temperature range [152]. Direct evidence in favor of unsaturation being responsible for chilling resistance comes from experiments where *A. nidulans* was transformed with the gene for a Δ^{12}-desaturase from *Synechocystis* and showed a substantial increase in chilling resistance [153].

Low-temperature-induced reduction of lipid acyl chain motion in thylakoids may be expected to lower the rate of PQ diffusion and, hence, of electron transport [154]. In plants, therefore, an adaptive response to a reduction in growth temperature would be a compensatory increase in acyl unsaturation and decrease in membrane microviscosity [145,154,155]. This has been observed in wheat root phospholipids [156], pine chloroplasts [157], microsomes of *Dunaliella salina* [158]; whole cotyledon lipids of linseed and soya-bean [159]; ivy leaves and spruce needles [160] and in leaves of *Vicia faba* [161]. Other reports show no correlation between fatty acid unsaturation and temperature in chloroplasts and plasma membranes of *Pinus sylvestris* [154,162], nor in cold-hardened *Brassica napus* [163]. Other temperature responses of membranes may include changes in phospholipid composition [164,165], glycolipid composition [166], fatty acid positional distribution [167], and non-acyl-lipid membrane components [168]. Several workers have suggested that low-temperature stress may be avoided by the seasonal accumulation of the 'free-water binding' lipid, DGDG [154,157], an assertion not supported by direct determinations of DGDG hydration (see Section VII).

There is better evidence that MGDG unsaturation may be involved in high-temperature instability of thylakoids. At temperatures of 40–50°C irreversible denaturation of chlorophyll-protein complexes is observed [169] and correlates with loss of oxygen evolution, PS II activity, and photophosphorylation [170]. These temperatures also cause granal destacking, lipid phase separation and extensive H_{II} phase formation [171]. Protection of PS II activity and prevention of H_{II} phase formation were both conferred by in vivo hydrogenation of the membrane lipids [172,173]. Some mutants of *Arabidopsis thaliana* with reduced levels of acyl unsaturation exhibit enhanced stability at elevated temperatures [133–135], similar to that conferred by in vivo hydrogenation.

IV-B. Alteration of thylakoid fluidity

Alteration of thylakoid fluidity in vivo has been attempted by several groups. Growth of barley seedlings in the presence of the pyridazinone compound SAN 9785 led to greatly decreased total lipid unsaturation and strong effects on several photosynthetic parameters [174]. However, when the lipids were extracted and reconstituted into liposomes, the effect on bilayer fluidity as measured by DPH fluorescence was very small. Restall et al. [138] reported that hydrogenation of 40% of the double bonds in spinach thylakoids, affecting primarily the 18:3 fatty acids, had no effect on the rate of photosynthetic electron transport, nor on thylakoid ultrastructure.

Recent work using a removable water-soluble catalyst has suggested inhibition of electron flow from PS II to PS I after 10% hydrogenation, inhibition through PS II at higher levels of hydrogenation, and insensitivity of PS I electron flow to as much as 50% loss of acyl double bonds [175,176]. These effects were apparently not due to catalyst interactions with antennal or reaction-center complexes [176], or with chlorophylls, carotenoids or PQ [177], and were attributed to an effect of bilayer fluidity on PQ diffusion from PS II to PS I [176]. However, at 10% hydrogenation levels, at which full-chain electron transport was inhibited by 30%, EPR measurement of bilayer order with a C_{12} spin-labelled stearic acid probe, 12-SASL, showed de-

creased not increased, acyl order [175]. In addition, even with 40% hydrogenation of double bonds, EPR measurements of rotational correlation times (τ_0) with a C_{16} spin-labelled stearic acid probe, 16-SASL, showed only a minor increase of τ_0 from 1.75 ns to 2.0 ns [176]. This appraisal is consistent with conclusions reached by Van de Ven et al. [178] and Koole et al. [179], as well as those discussed above [113,114], that the reduction of double bonds in DGDG does not decrease hydrocarbon region fluidity, at least as measured by these techniques. That hydrogenation affects some other, as yet unexamined, electron transport component remains a significant possibility.

The addition of cholesterol [180] or cholesterol hemisuccinate [181,182] to thylakoids reduced whole-chain electron transport rates, proton uptake, Q_B oxidation, and cytochrome *f* reduction. It was concluded that this was due to a reduction in the rate of PQ diffusion through a reduction in bilayer fluidity as measured by DPH fluorescence. However, as was stated above, long-distance translational diffusion of PQ in the hydrocarbon region of the bilayer is not equivalent to the rotational motion measured by DPH fluorescence.

IV-C. Proteins and thylakoid fluidity

The results discussed above suggest that acyl unsaturation is not the primary factor influencing overall thylakoid fluidity. This is supported by several comparisons showing that there is higher microviscosity, a higher degree of acyl orientational order, and lower fluidity in whole thylakoids than in their purified and reconstituted lipids [183–185]. These results suggest that proteins play the central role in decreasing overall membrane fluidity. This is supported by the higher fluidity of stromal membranes compared to granal membranes. Stroma lamellae have a higher lipid/protein ratio and higher fluidity than grana lamellae but are nearly identical in acyl unsaturation [45,54]. A recent EPR study [186] of the mobility of spin-labelled PC, PG, and MGDG in PS II preparations showed the rotational correlation times to be about four orders of magnitude slower than in L_α phase lipid bilayers. There is some evidence that plants may adjust their thylakoid fluidity in response to lowered growth temperature by increasing the lipid/protein ratio of the membrane rather than adjusting acyl unsaturation [187]. This result is consistent with a recent EPR investigation showing that increased fatty acid unsaturation did occur in low temperature-grown *Synechococcus* sp. but was not related to changes in bilayer fluidity nor thermal adaptation [188]. The *fad*C mutant of *Arabidopsis thaliana*, with a 50% reduction in di-and tri-unsaturated fatty acids, also has a 30% increase in lipid/protein ratio with the result that whole-chain electron transport rates and PS I rates are unaffected, although there is a 20% reduction in PS II activity [134]. In other words, the plant is able to compensate for decreased fluidity by adjusting it in another way during the development of the thylakoid membrane. Another *A. thaliana* mutant deficient in palmitic acid desaturation showed no fluidity changes (as measured by DPH), nor differences in whole-chain, PS II, or PS I electron transport rates [135].

In summary, the above discussion shows that only in a few organisms, and perhaps only in specific membrane systems, can fatty acid unsaturation be considered a primary response to lowered growth temperature. Even when decreased temperature does lead to increased unsaturation, an associated increase in bilayer fluidity does not necessarily occur. The thylakoid lipids may be highly unsaturated in part to facilitate rapid PQ diffusion but primarily to counter the ordering effect obtained from the very high protein content of this membrane. This would serve to ensure that the membrane is in the liquid-crystalline lamellar phase, but might also confer high temperature instability to the thylakoid due to the H_{II} transition of MGDG at higher temperature. This suggestion is similar to the 'homeophasic adaptation' proposal of Silvius and McElhaney [189]. It is unfortunate that few of the studies on lipid unsaturation and thermal adaptation have compared lipid/protein ratios. Alternative roles for unsaturated thylakoid lipids are outlined in the next section.

V. Lipid–protein interactions in thylakoids

The heterogeneity of lipid species in the thylakoid and other membranes has prompted many workers to look for specific interactions between lipids and membrane proteins. These interactions are usually envisaged as involving a so-called 'boundary lipid' that may be preferentially associated with a given protein. Such a lipid might be expected to act as a specific activator of protein or enzyme function or act to facilitate protein packing in the membrane [24,25,105]. Electron spin resonance can detect lipids which exchange less rapidly with bulk lipid, provided the lipid with a spin-label can be introduced into the membrane. Such a study using labelled MGDG, PC and PG (lacking 16:1*tr*) showed that a larger fraction of PG than PC or MGDG was motionally restricted in thylakoids, detergent-derived PS II particles and Yeda-press produced PS I stroma membranes [190]. The association constants calculated for PG were several-fold higher than those for PC and MGDG, suggesting that there was specific association of PG with proteins of both photosystems. Saturation transfer ESR measurements on PS II membranes using the same labels showed that all the lipids had very restricted rotational motion [186]; as

would be predicted from the high protein/lipid ratio. In this system, PC was more motionally restricted than PG or MGDG but it is not clear how this relates to normal thylakoids given the uncertainty regarding the presence and location of PC (subsection II-A).

Many investigators have looked for the co-purification of a specific lipid during the purification of a protein or chlorophyll-protein complex from the thylakoid. Digestion with specific lipolytic enzymes has also been used. Others have attempted to reconstitute a purified protein or complex with different lipids and lipid mixtures. We will first review the literature according to the experimental approach, then examine in detail the question of the association of PG with LHCII.

V-A. From thylakoids to subthylakoid particles

As discussed in subsection II-C, there is little consistent difference between PS-II-enriched grana lamellae and PS-I-enriched stroma lamellae prepared by mechanical fractionation. When PS II membranes were prepared by treatment with the non-ionic detergent Triton X-100, five different groups obtained five different results (Table I). These differences were probably due to the influence of residual detergent on the separation of lipid classes. Murata et al. [50] discovered that Triton X-100 interferes with the separation of lipid classes by chromatography on silica gel, and used reversed-phase HPLC to remove it prior to quantitative analysis. We add only that similar criteria should be applied prior to analysis by gas chromatography, since detergents such as Triton X-100 are partially comprised of long-chain polyethoxy groups. The effect of detergents on analysis of fatty acids by gas chromatography does not appear to have been investigated.

PS II membranes can be further fractionated to remove LHCII, some non-essential polypeptides and 90% of the remaining lipids to obtain oxygen-evolving PS II cores (Table I). Murata et al. [50] found PS II cores highly enriched in PG and somewhat depleted in MGDG. The fatty acids of the remaining lipids were more highly saturated than total thylakoid lipids. The PS II reaction centre particles produced by extensive Triton treatment and ammonium sulfate precipitation retained approximately one MGDG molecule per reaction center. Its fatty acids were unusually highly-saturated. There was no SQDG or PC in the PS II cores or reaction centers. Although the lipid compositions of Triton [52] and octyl glucoside [50] PS II cores are different, both papers report that the MGDG is more highly saturated than that of whole thylakoids. This suggests that there may be a special role for saturated MGDG molecule in PS II.

The anionic glycolipid SQDG has been reported to co-purify with CF_0-CF_1 from both spinach and *Dunaliela salina* [49], and increase the activity of the isolated enzyme in vitro (see below). Sigrist et al. [191] have reported the co-purification of SQDG with *Chlamydomonas reinhardii* LHCII. In addition, SQDG has been reported to co-purify with spinach PS I [192] and with a PS II core from spinach [52]. Therefore SQDG has been reported to co-purify with every major protein or pigment-protein complex of the thylakoid membrane! This surprising result may suggest that methodological rather than biological factors are at work. For example, the disruption of normal protein–protein interactions by detergents may expose positively charged groups which could then associate with a negatively charged lipid.

LHCII can be purified by a method involving Triton X-100 solubilization followed by cation precipitation: in view of the analytical problems caused by Triton, it is not surprising that two reports using this method [193,194] are in disagreement (Table I). Alternatively, LHCII in monomer and oligomer forms can be resolved by SDS-PAGE from thylakoids solubilized with SDS or octyl glucoside [26,27]. Electrophoresis in the presence of SDS would be expected to replace anionic lipids with SDS, but no depletion of either PG or SQDG has been reported [192,195,196] (Table I). In fact, it appears that the oligomeric form of LHCII (CPII*) is enriched in PG, as was one of the Triton preparations which had been redissolved and reprecipitated twice [194]. CPI, the reaction-center complex of PS I is relatively stable on SDS-PAGE, and all workers seem to agree that it is enriched in SQDG [192,195,196]. We have not found any published data showing the specific co-isolation of DGDG with any thylakoid associated protein complex.

V-B. Effect of lipases on photochemical activity

Galactolipase digestion of thylakoids was found to inhibit whole chain electron transport and electron flow through PS I, but these effects were largely due to enzymatic release of free fatty acids and not to galactolipid removal per se [71,72,139]. Galactolipid digestion has also been correlated with the release of plastocyanin from thylakoids [197] and the dissociation of chlorophyll-protein complexes as detected by gel electrophoresis [198], but the experiments were not controlled for the possible detergent activity of released free fatty acids. Free linolenic acid at levels as low as 100 μM is a potent inhibitor of PS II [199]. Galactolipid digestion did have a significant impact on granal stack morphology: digested thylakoids became significantly swollen but remained stacked and the behavior was not due to free fatty acid release [139,200]. It has even been claimed that thylakoids remained intact and normally appressed after hydrolysis of 100% of the galactolipids, with the resulting free fatty acids still part of the membrane bilayer [200]. Perhaps this is not

so surprising in view of the high protein content of the thylakoid membrane, particularly in the appressed regions, which means that there may not be a lot of lipid in between protein macrocomplexes [25,32,186]. Interestingly, functional CF_0-CF_1 ATPase activity appears to modify the susceptibility of MGDG to lipase hydrolysis in thylakoids [63]. In view of the reported effects of SQDG on CF_0-CF_1 (see below), it is unfortunate that there is no enzyme which specifically hydrolyzes SQDG.

There have been reports that PG co-purifies with PS I [192] and that it is required for PS I activity [201]. However, the removal of 75% of thylakoid PG by phospholipase A_2 digestion had no effect on PS I, although it inhibited electron flow through PS II and whole-chain electron transport by 50% [202]. When hydrolysis products were removed during digestion, there was little effect of an identical extent of phospholipase A_2 digestion on whole-chain electron transport [12]. Extended digestion to remove the portion of PG that is presumably localized on the inner monolayer (16% of total PG) did lead to an 80% inhibition of non-cyclic electron flow, but since this lipid had to move from inner to outer leaflet to be accessible to the enzyme, it is not clear what other structural changes might have been provoked by its relocation.

V-C. Reconstitution of photochemical activity

Most macrocomplexes of the photosynthetic apparatus have been reconstituted into phospholipid vesicles: LHCII [193,203–207], cytochrome b_6/f [205], CF_0-CF_1 [49,205,208], PS II cores [206,207], sonicated fragments of PS II membranes [206], and PS I particles [209–212]. Functional PS I activity has been coupled to CF_0-CF_1 ATP synthetase activity with reconstituted soybean phospholipids [213]. Similarly, reconstitution into bilayer DGDG vesicles has been accomplished for the cytochrome b_6/f complex [205], CF_0-CF_1 [205], PS II cores [207] and LHCII [207,214]. The only indication of a specific requirement for DGDG was that PS II core particles were more successfully reconstituted into DGDG liposomes than into PC liposomes [207]. Larkum and Anderson [215] used purified thylakoid lipids to reconstitute PS II cores, PS I and LHCII into liposomes, and were able to demonstrate energy transfer from LHCII to both PS I and PS II by fluorescence measurements. The relevant literature up to 1985 has been carefully reviewed by Ryrie [216].

A general comment is required at this point regarding the nature of reconstituted lipid-protein systems. While the ideal reconstituted system [217] may not be necessary for all types of study, some degree of caution is obligatory. It is not uncommon, for instance, for workers to claim the reconstitution of a given protein with unsaturated MGDG without showing the existence of a lamellar phase instead of the H_{II} phase, nor showing that the protein actually was integrated into a hydrophobic, non-detergent milieu [218–222]. Furthermore, the problem of the quantity and location of residual detergent is rarely addressed, and some reconstitutions would appear to contain 50% or more detergent [219,220,223]. Low levels of the non-ionic detergent octyl glucoside have been shown to significantly enhance bilayer Cl^- permeability [224] and undoubtably affect lipid phase behavior at higher levels. Many detergents, such as cholate and Triton X-100, are not easily removed. Significant amounts of cholate were found to be associated with reconstituted cytochrome oxidase vesicles even after extensive dialysis [225]. Therefore, the biological relevance of some reconstituted systems is unclear. At the very least, they should be treated as ternary systems, i.e., the contribution of the detergent to the bilayer should be explicitly recognized and measured.

There are a number of reports suggesting that SQDG and MGDG may influence the activity of ATPases. The earliest was that of the ability of SQDG to protect CF_1 against thermal inactivation [226]. The ATPase activity of isolated spinach CF_0-CF_1 can be stimulated by chloroplast lipids but not by phospholipids [221,222]. It can also be activated by the detergent octyl glucoside [227]. These workers reported [222] that MGDG by itself (in the presence of cholate) had the most stimulatory effect, but since MGDG is an H_{II}-phase lipid, the enzyme's physical environment is unknown. They also found a stimulatory effect of the further addition of DGDG and SQDG, although SQDG alone inhibited ATPase activity. They suggested that SQDG may affect apparent ATPase activity and ATP–P_i exchange rates by directly affecting membrane proton permeability [222]. However, they did not provide convincing evidence that the enzyme was reconstituted into an intact liposome in the experiments where proton permeability was measured, nor investigate the effect of cholate.

The ATPase of *Synechoccus* 6716 has also been reported to require a mixture of bilayer-forming lipids and MGDG for optimal reconstitution [228]. It should be noted that the MGDG from this organism is only monounsaturated (16:0/18:1) and its tendency to go to the H_{II} phase at the assay temperature of 50°C is not clear. However, a requirement for H_{II}-phase-preferring lipids, such as MGDG and PE, over lamellar phase lipids DGDG and PC has also been reported for the Ca^{2+}-ATPase of sarcoplasmic reticulum [229].

It should be noted that reconstitution of electron transfer can be achieved in systems where no attempt is made to form liposomes. For example, Lam and Malkin [230] simply added purified cytochrome b_6f complex to PS II membranes, diluted and measured light-induced reduction of cytochrome f. In another study, they combined PS II membranes, cytochrome

b_6/f, purified PS I and the three soluble proteins required to complete the electron transfer chain (plastocyanin, ferredoxin and ferredoxin-NADP reductase) and were able to detect reduction of $NADP^+$ without any added electron donor [231]. All three membrane complexes would have contained residual detergent. They reported that the addition of sonicated phospholipids had no effect on activity. In order to study the interaction between plastocyanin, PS I and the components of the cytochrome b_6/f complex, Rich et al. [232] mixed the three purified components (without added lipids) and used spectroscopic methods to assay the redox states. The ability of isolated cytochrome b_6/f complexes (in the presence of cholate) to reduce plastocyanin was stimulated by added plastoquinone and this effect was increased by including PC, DGDG or PG but not MGDG [233]. No information about lipid phases was given. Addition of total thylakoid polar lipids, PC, DGDG or MGDG increased the activity of PS II membranes, although they were certainly not forming sealed vesicles [48]. Matsuda and Butler [234,235] showed that the high-potential form of cytochrome *b*-559, either purified or in PS II cores, could be partially restored by either DGDG or PC under conditions which probably led to the formation of liposomes. Addition of DGDG liposomes to PS II membranes improved the rate of O_2 evolution as well as the fraction of high potential cytochrome *b*-559.

Since the physical environments of the reconstitutions detailed in the last paragraph are unknown (e.g., pure bilayer, bilayer with intramembranous particles, H_{II} cylinders, micelles, macromolecular aggregates or various mixtures thereof), it is difficult to know how to evaluate the data in terms of what they might tell us about the normal function of these complexes in a normal membrane bilayer environment. It must be pointed out once again that all these experiments contained an unmeasured level of residual detergent(s). It should also be pointed out that all the usual detergents are very effective in extracting plastoquinone. A reported requirement for lipids in restoring stable charge separation in PS II particles extracted with sodium cholate and high salt [223] was probably due to reconstitution of plastoquinone [236].

V-D. PG and LHCII

The remainder of this section will concentrate on PG(16:1*tr*) and its interactions with LHCII, a subject that has been reviewed by Dubacq and Tremolieres [237]. The earliest suggestions of a role for PG (16:1*tr*) in granal stacking were based on the correlation between the level of this fatty acid in PG and the extent of granal stacking during the greening of etiolated leaves [43,238]. This correlation extends to the accumulation of chlorophyll *b* and LHCII in developing leaves and can be obtained under different developmental conditions of temperature, light intensity, and illuminating wavelength and periodicity [237]. At the biochemical level, PG and 16:1*tr* are enriched in some preparations of LHCII made with Triton X-100 followed by divalent cation precipitation [194] or by SDS solubilization followed by SDS-PAGE [195,196] (Table I), but not in others [192,193]. This may have something to do with the degree of oligomerization of LHCII, since PG is enriched in the oligomer form (CPII*) but not the monomer form (CPII) after electrophoresis in the presence of SDS (Table I). However, that cannot explain the lack of agreement on whether PG is enriched in PS II membranes and grana fragments (Table I), both of which have high LHCII contents. PG(16:1*tr*) is-reported to be associated with purified fucoxanthin-chlorophyll *a/c* light-harvesting assemblies of the brown alga *Fucus* [239].

A correlation between 16:1*tr* levels, LHCII oligomer and freezing tolerance of some species of cereals has been reported [240]. Low-temperature development of rye leads to the formation of LHCII which has a significantly lowered 16:1*tr* content when isolated. The thylakoids have a much lower LHCII oligomer to monomer ratio after separation of chlorophyll-proteins by SDS-PAGE [194,240–243]. Enzymatic removal of PG from purified LHCII resulted in the complete conversion of the oligomer to the monomer on subsequent SDS-PAGE separation [194], although dissociation due to the release of hydrolysis products was not controlled. Similar results have been obtained by phospholipase A_2 digestion of thylakoids [196]. Finally, the monomeric LHCII has been reported to reassociate into the oligomeric form in PG(16:1*tr*) liposomes to a much greater extent than in other types of liposome [196,214].

However, there are alternative explanations for many of the observations above. Co-isolation of PG with LHCII may result from reassociation of the charged lipid with protein after detergent solubilization of thylakoids [245]. Oligomerization of LHCII monomer by PG might also result simply from lipid-induced charge neutralization and/or hydrophobic association. The correlation between the amount of 16:1*tr* PG in cold-hardened plants and the amount of LHCII oligomer on SDS-PAGE could reflect the effect of PG on the sensitivity of the LHCII oligomer to detergent solubilization. In a recent EPR study using spin-labeled PG reincorporated into thylakoid membranes, it was found that membrane destacking, protein phosphorylation and changes in illumination, all of which provoke changes in the lateral distribution of thylakoid membrane proteins and especially of LHCII, had no detectable effect on PG-protein interactions [244].

In addition, the involvement of PG and 16:1*tr* in LHCII oligomer formation and granal stacking has

been seriously challenged by genetic studies. Reduced membrane appression was found in a barley mutant lacking chlorophyll *b* and LHCII but with normal levels of PG and 16 : 1*tr* [246]. A mutant of *Arabidopsis thaliana* specifically lacking in PG(16 : 1*tr*) showed no changes in membrane appression or energy transfer from LHCII to PS II [137,247]. PG and 16 : 1*tr* levels were observed to decline in mutants of *Chlamydomonas reinhardii* deficient in LHCII [248] but also in those deficient in PS II and PS I [249]. In addition, some plant species with no 16 : 1*tr* present in their PG still have normal ratios of LHCII oligomer to monomer [240]. Rye seedlings developed under intermittent light, which prevents chlorophyll *b* and LHCII apoprotein accumulation, had normal levels of PG(16 : 1*tr*) in spite of the absence of LHCII [243]. The authors suggested that the high levels of PG(16 : 1*tr*) facilitated the development and assembly of LHCII when plants were subsequently exposed to continuous illumination.

V-E. Summary of lipid–protein interactions in thylakoids

The evidence reviewed in this section leads us to conclude that there are no unambiguously known instances of specific lipid–protein interactions in thylakoids. Similar conclusions have been reached for other membranes [250]. There is some evidence for the association of MGDG and SQDG with CF_0-CF_1 and for the association of PG with PS I and LHCII. In all cases, however, there is either significant contradictory evidence and/or important methodological inadequacies that limit the reliability of the results. It is unlikely that the results obtained from different techniques will yield consistent conclusions regarding lipid–protein interactions while significant methodological considerations are being overlooked, particularly as regards reconstituted systems. The mutants of higher plants and algae offer the least invasive method for probing lipid–protein interactions and appear to support the idea that there are few specific interactions. However, they may also indicate the flexibility and developmental adaptiveness of plants which can apparently compensate in a variety of ways for environmentally or genetically-induced metabolic deficiencies [133].

VI. Permeability of thylakoid acyl lipids

Very little work has been done on the permeability properties of the thylakoid lipids. Early work by Graziani and Livne [112] showed black lipid films of MGDG to be only slightly more permeable to water than those of PC. These workers obtained a water permeability coefficient of $7.31 \cdot 10^{-3}$ cm s^{-1} at 37°C for MGDG compared to $6.41 \cdot 10^{-3}$ cm s^{-1} for plant PC. A significantly higher activation energy for water permeation through MGDG (17 kcal mol^{-1}) was taken to indicate the existence of extensive intermolecular H-bonds in the MGDG bilayers.

Multilamellar preparations of DGDG and binary mixtures of DGDG and MGDG have been examined for permeability to KCl by a turbidimetric method [251]. These authors concluded that the galactolipids have about the same permeability to KCl as does PC. In contrast, we have reported that large unilamellar DGDG vesicles have permeability characteristics very different from PC as determined by radiotracer efflux experiments [100]. DGDG was found to be 100-fold more permeable to $^{86}Rb^+$, 50–80-fold less permeable to $^{36}Cl^-$, and similar in terms of glucose permeability when compared to PC liposomes. Furthermore, our results clearly showed that liposomes composed of lipid mixtures similar to those found in vivo were approximately three orders of magnitude more permeable to $^{86}Rb^+$ than were PC liposomes. While a mechanistic explanation for these observations is not at hand, the results raise the intriguing possibility that high intrinsic lipid permeability to alkali metal cations is responsible for the low $\Delta\Psi$ in energized thylakoids [100]. This suggestion is in contrast to the conclusions of Barber [252], Ball et al. [253] and Ort [254] that the apparent leakiness of the thylakoid arises from the exceptionally high surface area/volume ratio of thylakoid membranes (approx. 10^6). In our experiments with both galactolipid and PC vesicles, the surface area/volume ratios were identical to those of thylakoids at 10^6, yet they had very different solute permeabilities [100]. However, voltage-dependent, specific K^+ [262] and Cl^- [263] channels have recently been detected in thylakoid membranes. Such ion-specific channels could permit enough ion movement to explain why the electrical component of the protonmotive force is small compared to the chemical component (ΔpH), irrespective of the rate of leakage through the lipid bilayer [100].

Proton permeability measurements in thylakoid lipid multilamellar vesicles suggested that galactolipids have similar permeability to protons as PC [251]. On the other hand, Pick et al. [221,222] claimed that the proton permeability of chloroplast lipids is far higher than that of the phospholipids. These workers, however, only reported measurements of proton flux in cholate/CF_0-CF_1/lipid proteoliposomes and not in the pure lipid liposomes. Proton flux measurements are notoriously sensitive to contaminants, including detergents [255]. If proton flux is strongly linked to H_2O permeation of bilayers [255] then the similar H_2O permeability of MGDG and PC [112] suggests that the galactolipids should not be significantly more permeable to protons than are the phospholipids. In vivo measurements of proton flux in non-phosphorylating thylakoids were interpreted to show that nonspecific leakage contributes only 1% of the total thylakoid proton flux with

a permeability coefficient of $2.0 \cdot 10^{-5}$ to $3.0 \cdot 10^{-5}$ cm s^{-1} [256,257]. This calculation involved a number of assumptions, but the values obtained were in the range of 10^{-3} to 10^{-5} cm s^{-1} reported for a variety of lipid vesicles and membrane systems [255]. With some reservations, the data available to date may suggest that the thylakoid membrane is about as leaky to H^+ as pure lipid bilayers, but considerably more leaky to other ions.

Bilayer permeability may also be affected by lipid phase behavior. Irregularities of lipid packing similar to those expected at the lipid/protein interface may also occur at the interfacial region between L_α and L_β phases. Therefore, greatly increased solute permeability would be expected when L_α and L_β phases coexist (Fig. 5). In fact, L_β phase formation results in greatly increased ion and sugar permeability in saturated PC's [258,259] and PG's [260] but not in saturated PE's [258,259]. This was interpreted as indicating that hydrogen-bonding between adjacent PE head-groups significantly reduced lateral lipid density fluctuations presumed to be the cause of increased solute permeability in the other lipids [259]. In lipid mixtures the temperature of the permeability maximum closely followed that of phase separation [260]. There was no maximum in mixtures of phospholipids and cholesterol which do not demonstrate phase separation [261]. As discussed in subsection III-E, the occurrence of L_β phase separation in higher plant thylakoid membranes is still in dispute. We did not find peaks or maxima of $^{86}Rb^+$ permeability in liposomes composed of normal proportions of purified lipids (50 mol% MGDG, 25 mol% DGDG, 12 mol% SQDG, and 12 mol% PG), in experiments where disaturated PG varied from 0 to 12 mol% and over a temperature range of 7°C to 30°C [23]. We have concluded that L_β phase separations, and hence increased solute permeability, probably do not occur in these lipids [23].

In an intact membrane, it is often envisaged that lipid packing defects could arise at the interface between lipids and proteins and that these defects would facilitate solute passage. This idea is supported by some data from reconstitution of glycophorin [264–266] and cytochrome oxidase [225] into liposomes of different composition. The results were interpreted to suggest a lipid-shape-dependent sealing of the protein/lipid interface, particularly by wedge-shaped unsaturated PE's. However, the usual caveats about the effects of residual detergent apply to these experiments, as well as the possibility that the incorporated proteins are induced to aggregate, forming pores through which solutes might pass [264–266]. It has similarly been suggested that specific thylakoid lipids, especially MGDG, may be found at lipid/protein interfaces, fitting around the large pigment-protein complexes and reducing non-specific membrane permeability as suggested in Fig. 6 [24,25,72,267]. It is very difficult to get at the whole question of lipid–protein interactions experimentally without introducing additional factors, as discussed at length in Section V. Once more is known about specific ion channels, it might be possible to block all known channels, including CF_0-CF_1, and determine the passive permeability through the bilayer. The possibility of doing patch clamping on thylakoids [263], and planar bilayer reconstitutions in the absence of detergent [262] represents significant technical ad-

Fig. 6. Effect of shape on employment of MGDG in lipid bilayers. With permission of TAB and Elsevier [267].

vances. In the meantime, it is surprising how little work has been done on the permeability of lipid bilayers made from thylakoid lipids, despite the obvious biological relevance. Early results suggest that the permeability properties of the thylakoid lipids may be very different from those of the phospholipids [100]. As it is now possible to produce uniformly sized unilamellar vesicles containing in vivo levels of MGDG without formation of H_{II} phase [100], it is to be hoped that some detailed studies on the H^+ permeability of the thylakoid lipids and solute permeability in well-controlled reconstituted lipid-protein systems will be undertaken.

VII. Surface properties of galactolipids

The physical chemistry of interactions between bilayer membranes is the subject of extensive current research. The principles have been comprehensively reviewed by Rand [268], Rand and Parsegian [269], and Israelachvili [132] in relation to phospholipid bilayers and by Barber [270] as well as Thorne and Duniec [271] in relation to thylakoids.

VII-A. Principles of surface interactions

There are several forces which determine how close bilayer membranes can approach. The long-range attractive Van der Waals forces arise from attraction between intrinsic fluctuating dipoles, and depend on the polarizability of the lipid molecules. Since they decay somewhat more slowly than the inverse sixth power of the distance, they have an effective range of approx. 15 nm. The relative magnitude of the attractive Van der Waals force is often expressed in terms of the Hamaker coefficient and varies with the chemical nature of the lipid. Bilayers containing charged lipids are also subject to a medium-range electrostatic repulsive force, or electrostatic potential, arising from the presence of fixed charges on the membrane surface. Mobile counter-ions will be attracted to the fixed surface charges, giving rise to the so-called double-layer. The magnitude and decay length of the electrostatic potential depends in a complex fashion on the concentration and valency of the counterions, and on the pH of the medium. Electrolytes, particularly divalent counterions, screen the surface charges and reduce the electrostatic potential that prevents close approach of the bilayers. Since Van der Waals forces are longer range than repulsive electrostatic forces, vesicles should be expected to aggregate at the distance at which attractive forces equal repulsive forces. These forces have been measured directly for PG monolayers adsorbed onto mica surfaces immersed in NaCl and $CaCl_2$ solutions [272]. The bilayers jumped to a minimum separation once the electrostatic repulsion was overcome.

Fig. 7. Freeze-fracture electron micrograph of DGDG vesicles dispersed in water, extruded to a mean diameter of 100 nm then aggregated by the addition of $MgCl_2$ to 10 mM final concentration. Note that the aggregated liposomes show both flattened areas with zero bilayer curvature and 'end margin' areas with a very small radius of curvature. See also Fig. 1 in Ref. 277. Bar represents 100 nm.

The above highly simplified version of DLVO (Deryaguin, Lifshitz, Verweey, Overbeek), or electrostatic double-layer theory has been supplemented with the recent recognition of a rapidly decaying repulsive hydration force acting at very short distances (< 2 nm) from the bilayer surface [268]. The force arises from the energy required to dehydrate polar groups on the surface and dominates short-range interactions between approaching membranes. The strength of the hydration force is expected to be a function of the solvent, rather than of the solute. Hydration repulsion is probably the force preventing the aggregation of neutral lipid bilayers at direct molecular contact, as shown for PC and PE bilayers by surface force measurements [272]. Several additional forces probably act between approaching bilayers, including steric repulsive forces due to interactions of the head groups of opposing membranes [273], repulsion due to thermal undulation of bilayers [274,275] and the recently proposed hydration-attraction force [276].

VII-B. Studies with purified lipids

We have found that large unilamellar vesicles of DGDG aggregate in the presence of both mono- and divalent cations (Fig. 7) [248]. The effective concentration for monovalent cations depended strongly on its hydrated ionic radius. This result would not be expected if aggregation were simply due to traces of charged contaminants being electrostatically screened according to electrical double-layer theory. Furthermore, different monovalent anions had markedly different effects. These data were interpreted to suggest that liposome aggregation resulted from an effect of salts on head-group–water interactions. We have found recently that mixed MGDG/DGDG liposomes aggregate to a similar extent in the presence of salts with the difference that the aggregation was not reversible if MGDG made up more than 25 mol% of the total lipid [278]. The addition of PG or SQDG to MGDG/DGDG vesicles increased the cation concentrations needed for aggregation, as expected for electrostatic repulsion.

While it is possible that galactolipid liposomes may aggregate due to salt–dipole interactions this seems unlikely, as MGDG and DGDG monolayers have surface potentials and dipole moments close to those of PC [279]. This is consistent with a small, positive outside, dipole on the galactose of galactocerebrosides [280] that is similar in direction and magnitude to that expected for the phosphate dipole of phospholipids. Galactose–water-ion interactions are also consistent with reported interactions of ions with the hydration of cerebroside [281] and diglucosyldiacylglycerol [282] monolayers through changes in water structure. In addition, an effect on the ion-binding of a disaturated PG by dimannosyldiacylglycerol from *Micrococcus luteus*, possibly mediated by hydration effects, has been reported [283]. It is usually taken for granted that galactolipid bilayers cannot adsorb cations, but this has not been tested directly.

At the light microscope level, Mutz and Helfrich [274] observed salt- and temperature-dependent DGDG bilayer adhesion in hydrated multilayered systems and have estimated the bending energy of DGDG bilayers at $0.12 \cdot 10^{-12}$ to $0.21 \cdot 10^{-12}$ erg. This value is similar to that expected for phospholipid bilayers [276] and the recently measured value of $1.8 \cdot 10^{-12}$ erg for egg PC [284]. According to their calculations, DGDG bilayers were very close to balancing attractive and repulsive forces, depending on the choice of Hamaker coefficient and bending modulus [274].

The free energy of adhesion between giant unilamellar vesicles (10–20 μm) of DGDG has been found to be twice that of PE vesicles and 20-fold higher than PC vesicles [275,285]. The Hamaker coefficient of $3 \cdot 10^{-21}$ J for DGDG vesicles calculated from micropipette aspiration experiments [285] was very close to the $7.5 \cdot 10^{-21}$ J measured using surface force determinations [286,287]. These values are 2.5- to 6-fold higher than those found for PC and PE monolayers. Force of adhesion measurements between MGDG and DGDG monolayers yielded values of 3.0 and 1.8 mN m^{-1}, respectively [286,287] and showed that MGDG has a deeper energy minimum than DGDG at short separations [286]. These values were expected to be closely related to the degree of hydration of the galactosyl head-group, the less hydrated MGDG possessing the higher force of adhesion. These force measurements are consistent with our conclusion that MGDG promotes vesicle aggregation and adhesion to an extent similar to or greater than that observed for DGDG [278]. It is likely that the high forces of adhesion for these lipids originate from extensive inter-lipid H-bonding and/or low hydration of the galactosyl head-groups, and could be considered evidence in favor of the hydration-attraction concept [276].

The degree of hydration of the galactolipids is therefore central to their surface interactions. Most workers agree that the extent of hydration of DGDG is low [269,288,289] and possibly similar to that of PE at 7–12 H_2O/lipid [290], and diglucosyldiacylglycerol at 8 H_2O/lipid [282]. A value of 14–15 H_2O/lipid for DGDG and 12–13 H_2O/lipid for MGDG has been measured using ^2H_2O-NMR [96]. These values are remarkably similar to values of 14.5 and 12.1 H_2O/lipid for DGDG and MGDG, respectively, that can be calculated from the limiting hydration at 22% (w/w) water as determined by X-ray diffraction [81]. A very high value of 50 H_2O/DGDG was measured in micelles of DGDG in hexane [290]. Distearoyl-MGDG has been reported to hydrate to a lower extent, 5 H_2O/lipid [86].

Attractive interactions between galactolipid bilayers will also depend on the specific details of head-group orientation. McDaniel [288] concluded that the digalactosyl group of DGDG lies parallel to the plane of the bilayer based on neutron diffraction data showing a 0.8 nm thick polar group region. Marra has reached the same conclusion from surface force measurements [287]. A flat orientation for the glucosyldiglycerides of *A. laidlawii* has been suggested based on the low water binding capacity of these lipids [282]. In a series of ^{2}H-NMR experiments of 1,2-di-*O*-tetradecyl-3-*O*-glycolipids, Jarrell et al. [291,292] found different head-group orientations for α- and β-D-glucosyl groups as well as for α-D-mannosyl and lactosyl head-groups [292]. Differences in L_β-L_α transition temperatures have been reported for several galactose vs. glucose ether-linked glycolipids [293,294]. These results strongly suggest extensive inter-head-group H-bonding that is higher in galactolipids than in the glucolipids. Iwamoto et al. [295] pointed out the importance of head-group conformation in affecting the phase transition temperature and fluidity of several glycerolipids containing different head-groups and proposed that head-group conformation would also affect the relative hydration of a head-group. In light of the significant effects of small changes in sugar structure on the orientation of the head-group, it is interesting to note that *Anabena variabilis* synthesizes its MGDG by epimerization of the intermediate monoglucosyldiglycerides [296,297]. The possibility that there are specific physical reasons for the presence of galactose, as opposed to other sugars, in plant thylakoids has not been considered in the literature.

VII-C. Thylakoid stacking

The aggregated DGDG vesicles in Fig. 7 have a remarkable resemblance to thylakoids in that they have flattened regions with zero curvature and 'end margin' regions with very small radii of curvature. This does not mean that thylakoid membranes form grana stacks because of the interactions between the galactolipids, but it does mean that the same principles of surface interaction apply to both. The extent to which the liposomes will deform on aggregation will depend in a complex fashion on adhesion energy, the area elastic modulus of the bilayer, the balance of interbilayer attractive and repulsive forces, and liposome radius [132]. In the case of thylakoids, there will be osmotic forces as well as intrabilayer packing stresses due to the complex interactions among the many components of the membrane. There is a substantial body of evidence (reviewed in Ref. 2) that much of the adhesive force between granal thyakoids is due to interaction between LHCII units on apposing bilayers, and that divalent cations are required to screen negative surface charges due to other proteins (and possibly lipids). The attractive interactions between LHCII units will pull thylakoids together and promote the lateral segregation of LHCII into the regions of appression. This will introduce a lateral tension which will cause thylakoids to flatten as they adhere, conserving surface area [132,275,285]. In agreement with this argument, thylakoids swell when grana are 'unstacked' by removal of cations [244,298]. The degree to which attractive forces between the galactolipids contributes to stacking is unknown. However, it should be pointed out that when the ability of LHCII to promote stacking is removed by trypsin treatment, thylakoids can still be induced to stack by addition of higher levels of cations [299]. Furthermore, in chlorophyll-*b*-less mutants which have no LHCII these membranes demonstrate almost normal appression in vivo and require only slightly higher than normal divalent cations to stack in vitro [300]. This could be due to the influence of other proteins as well as to the membrane lipids.

VIII. Perspectives and summary

We now know a great deal about the phase behavior of the major thylakoid acyl lipids and the effect of both head-group size and acyl unsaturation on this behavior. A variety of in vivo and in vitro studies indicate that the H_{II}-phase preference of purified, unsaturated MGDG contributes to thylakoid instability at elevated temperatures. At reduced temperatures, however there is no reproducible relationship between temperature, acyl unsaturation, and either bilayer fluidity or acyl order. The glycolipids of the cyanobacteria appear to be good exceptions to this generalization. It seems likely that decreased acyl order and increased unsaturation in response to lowered growth temperature is both species- and membrane-specific, but is not a general phenomenon. Acyl unsaturation may be high in thylakoids simply to counter the ordering effect due to the high protein content of this membrane. In view of the fact that some plants can alter their protein/lipid ratio in response to lower unsaturation it is unfortunate that more studies have not determined this ratio.

There is still very little detailed information regarding certain important physical parameters such as head-group orientation and hydration, the extent of lipid-lipid H-bonding, head-group interaction with aqueous phase components, and bilayer permeability characteristics. In 1983 Quinn and Williams [78] observed that there was "an urgent requirement for more detailed thermodynamic and structural data". This is still the case and is particularly true for mixtures approaching those found in vivo. Until more is known about the properties of the isolated lipids, and their interactions with each other and with the other membrane components, it will be difficult to assess the contribution of lipids to the intact, functioning thyl-

akoid membrane. In particular, the H^+ permeability of thylakoid lipid mixtures should be rigorously investigated, as should the possibility of significant ion adsorption to uncharged galactolipid bilayers.

In terms of lipid distribution with the membrane, the bulk of the evidence favors transverse asymmetry of PG and possibly DGDG, but the evidence for lateral asymmetry is weak. A consensus on the enigmatic issue of specific lipid–protein interactions has been elusive. It could be argued that transverse asymmetry must result from interactions with the protein components of the bilayer. Several groups have now reported an association of PG with LHCII and its oligomer as well as with PS I. It is possible that the negatively-charged PG and SQDG associate with positively-charged protein groups in the membrane, but it will be necessary to show that such an association is not due to electrostatic reassociation during detergent solubilization. We are confident that the results obtained from the range of different methodologies currently in use will converge towards a consensus. However, all of the techniques used in this field are very invasive and in order to obtain reliable, interpretable data it will be necessary for workers to pay close attention to important methodological details such as lipid phase structure as well as detergent levels and activities. Fast spectroscopic techniques such as EPR and fluorescence energy transfer (reviewed in Ref. 250) hold promise provided that suitable probes can be incorporated in membranes without perturbing the native structure.

A major task for future work will be to find plausible physical explanations for why galactolipids are the dominant components of the bilayer, and particularly why a non-bilayer lipid (MGDG) should make up such a large fraction of the total lipid. Perhaps the specificity of headgroup H-bonding and orientation, and the low level of hydration of these lipids is required for certain specific interactions in the membrane. It has been suggested by several authors that wedge-shaped MGDG might fit around the intrinsic proteins to assure a non-leaky membrane [25,72] (Fig. 6). Its tendency toward the H_{II} phase may suggest a role in promoting vesicle fusion during development of the mature chloroplast from the proplastid or etioplast. There is evidence that signal peptides disrupt bilayer structure during penetration [301] and this could mean that a high proportion of non-bilayer lipid might facilitate the insertion of nascent membrane proteins. The idea that highly unsaturated lipids are specifically required during development is supported by recent work on cold-sensitive chloroplast development in *Arabidopsis* mutants deficient in lipid unsaturation [133]. PG may also have a role in protein insertion as it is required for signal peptide translocation in *E. coli* [302]. In the case of the chloroplast there is a dramatic increase in PG (16:1*tr*) correlated with chloroplast development but not specifically with LHCII.

We also have to consider the possibility that the answer to some of these questions lies in long-abandoned evolutionary pressures on the ancestors of the blue-green algae, one or more of whose descendants became the successful symbionts we now call chloroplasts. Since MGDG, DGDG and PG (16:1*tr*) are found only in the photosynthetic membranes of oxygen-evolving species, perhaps their common ancestors lived in a 'galactolipid world'. Upon the establishment of the green photosynthetic endosymbiont, the genes for galactolipid synthesis were transferred to the nucleus. This could explain the apparent duplication of many steps of lipid synthesis in green plants [133].

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