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Transversal localization of monogalactosyldiacylglycerol and digalactosyldiacylglycerol in spinach thylakoid membranes

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The lipase from *Rhizopus arrhizus* has been used to determine the transmembrane distribution of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) in spinach thylakoid membranes. The hydrolysis kinetics, expressed as semilog plots, were carried out at constant bulk pH under a wide range of salt concentrations and temperatures. DGDG always showed two pools of constant amplitude, regardless of the conditions used. The behavior of MGDG was more complex. Depending on environmental conditions, two or three pools could be evidenced. We have developed a simple rationale which allows to distinguish between kinetic pools (identified as different populations of lipid molecules being degraded at different hydrolysis rates within the outer monolayer) and topological pools (corresponding to the classical outside/inside distribution). It is suggested that the surface potential and temperature are involved in the control of the packing pressure of thylakoid membrane lipids, as probed by the lipolytic enzyme. We defined a limited set of conditions under which it is possible to obtain reliable estimations of the transversal distribution of galactolipids in thylakoid membranes. For spinach, the outside/inside ratios are 15/85 for DGDG and 65/35 for MGDG. Taking into account the contribution (in mol%) of these lipids to the total acyl lipid composition, it is deduced that total galactolipids are equally distributed within the two monolayers of the thylakoid membrane. The implications of these results for the overall molecular organization of the thylakoid membrane will be discussed.

Introduction

The acyl lipids of thylakoid membranes provide the functional membrane components (chlorophyll-protein complexes, electron carriers, coupling factor) with an adequate environment for optimal activity. At least two mechanisms – fine tuning of excitation rate of both photosystems via protein (de)phosphorylation and plastoquinone diffusion – require a membrane fluidity compatible with the need for lateral movement. There is now good evidence that the membrane lipid to protein ratio is involved in the control of membrane fluidity [1,2].

A better understanding of the role of acyl lipids in thylakoid functions will obviously depend on the knowledge of their molecular organization in the membrane. In particular, more detailed information on acyl lipid topography (both lateral and transversal) would be desirable in view of the

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Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; Mops, 4-morpholinepropanesulfonic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

rather controversial data obtained so far [3–8]. Although there is now increasing evidence for the preferential localization of phosphatidylglycerol in the outer monolayer [9,10], studies about galactolipid transversal distribution are both scarce and equivocal [6–8]. Although this may be attributed to the use of different techniques, we feel that both the substrate itself (the thylakoid membrane) and the nature of incubation media should be considered more closely. Indeed, the available data on galactolipid localization have been obtained with starting materials as different as intact thylakoids [6], sonicated thylakoids [7] or randomized vesicles of opposite sidedness [8]. Moreover, the incubation conditions were highly variable, especially with respect to salt conditions.

In this paper, we have undertaken a detailed study of the hydrolysis of galactolipids in intact spinach thylakoid membranes treated with a lipase from *Rhizopus arrhizus*. Special attention has been paid to the incubation conditions. Lipolytic treatments have been carried out at constant bulk pH under a wide range of salt concentrations and temperatures. This allowed to assess the role of surface potential in the control of packing properties of membrane lipids and enabled us to define a set of conditions under which it was possible to obtain reliable estimations of galactolipid transmembrane distribution. We shall now describe the basic rationale of our approach.

Rationale

When the transmembrane distribution of acyl lipids in thylakoid membranes is studied with lipolytic enzymes, the resulting hydrolytic pattern is the consequence of a rather complex interplay of various parameters, as described in Fig. 1. The hydrolysis kinetics is governed by two environmental factors, ionic conditions and temperature.

Ionic conditions are known to affect the overall morphology of thylakoids by controlling their degree of stacking [11]. Moreover, changes in salt concentration modify the lateral distribution of intrinsic chlorophyll-protein complexes [11]. The type and concentration of cations also dictate, at a given bulk pH and temperature, the values of the surface potential and pH [12]. In addition, salts may interact with polar headgroups of lipid molecules and can therefore alter their physical properties [13]. Finally, salts may also control the activity of lipolytic enzymes themselves [14,15]. One may thus suspect salts to modulate at least one of the following parameters in thylakoid membranes: (a) The initial packing pressure of lipid molecules. This may occur either by a direct effect (e.g.,

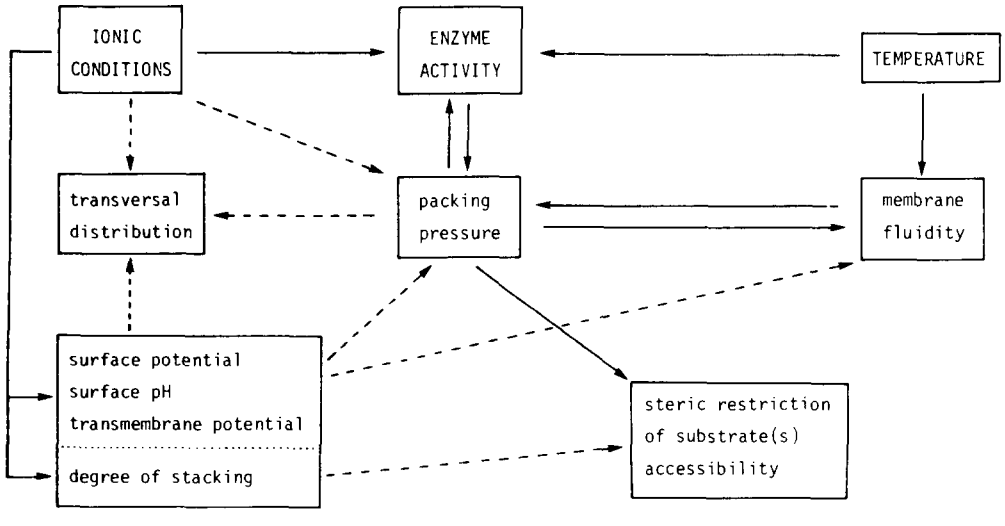


Fig. 1. Interplay of the various factors involved in the hydrolysis of thylakoid membrane lipids by a lipolytic enzyme. Key: →, one-way action; ⇄, reciprocal action; ---→, putative action. See text for further details.

change in hydration level of polar headgroups [16]) or via changes in surface potential and/or pH [17]. As a consequence, the fluidity may also be affected. (b) The transversal distribution of a given lipid class, especially that of negatively charged lipids [18]. (c) The availability of lipid substrate(s) for lipolytic enzymes, particularly with highly appressed membranes.

Temperature may affect simultaneously the membrane fluidity and the lipolytic enzyme activity in a parallel fashion, whilst having much less influence on the electrical properties of the membrane surface. An often neglected, but typical effect of a temperature increase is to (partly) release the build-up of the packing pressure of membrane lipids due to the lipase-induced formation of such hydrolysis products as lysolipids and free fatty acids [14].

These two extrinsic, independent factors obviously have a direct influence on a third one, the activity of the lipolytic enzyme.

Keeping these considerations in mind, we must now introduce another concept. Expressed as semilog plots [10,19], the hydrolysis pattern of a given lipid class is often polyphasic, thus revealing the presence of several lipid 'pools'. We suggest that these pools may fall into two groups: (a) Kinetic pools reflect populations of lipid molecules being degraded, within the outer monolayer only, at different hydrolysis rates which can be due firstly to environmental control (by ions and temperature) of the initial packing pressure of the membrane lipids and, later, to an increase in packing pressure following the formation of lysolipids and free fatty acids generated within the membrane by the enzyme action. For such kinetic pools, a change in incubation temperature should affect not only their hydrolysis rate, but also their amplitude.

(b) Topological pools correspond to those populations of lipid molecules localized entirely in the outer and in the inner monolayer of the thylakoid membrane, respectively. Any temperature change will thus affect the hydrolysis rate without changing the extent of such pools. This should be especially valid for the inner pool, the hydrolysis of which being possible only via outwards transbilayer movement, provided that the enzyme action is restricted to the outer membrane surface.

Materials and Methods

Lipase (EC 3.1.1.3) from *Rhizopus arrhizus* was purchased from Boehringer (Mannheim, F.R.G.) and used without further purification. This lipase splits the fatty acyl chain in sn_1 position, leaving a 1-lysolipid. Its substrate specificity on detergent-solubilized polar lipids has been shown to be MGDG > DGDG = SQDG > PG, PC [20]. The stock enzyme was diluted with the desired incubation medium (see Table I) to give a working solution containing 15 units/10 μl.

Spinach leaves were harvested from 14-week-old, hydroponically grown plants in a controlled greenhouse. Chopped leaves (50 g) were homogenized for 5 s (Waring blendor) in 150–200 ml of ice-cold 300 mM sorbitol buffered with 10 mM Mops/KOH (pH 7.6). After filtration on 4 muslin layers and 2 Miracloth layers, the juice was centrifuged 30 s at $2000 \times g$. Supernatants were discarded. The smooth pellets were resuspended in a 10-fold dilution of the above medium and recentrifuged for 4–5 min at $4000 \times g$. The greenish supernatants were discarded and the pellets were resuspended in the desired incubation medium (see Table I for the composition of incubation media). Chlorophylls were determined [22] and adjusted to 0.5 mg/ml. Thylakoid membranes were allowed to equilibrate with respect to the imposed salt concentration for 15 min at 20°C in darkness. They were then brought to the desired incubation temperature. After sampling a zero time control, the lipase was added (30 units/mg chlorophyll). Incubations were carried out in the dark under constant stirring.

To check for the complete hydrolysis of glycolipids when both sides of the membrane are exposed to the enzyme, thylakoid membranes were unstacked (medium 4, Table I) and sonicated in the presence of the lipase (Labsonic 1510, Braun, three pulses of 5 s at 100 W). The sonicated vesicles were then incubated and hydrolysis was determined as described below.

At timed intervals, aliquots (280 μl) of the suspension were withdrawn and the lipids extracted as in Ref. 23. We have checked that solvent extraction immediately stopped the enzyme activity. The chlorophyll concentration of each total lipid extract was determined [22]. Known aliquots

of these extracts (about 40 μg chlorophyll) were spotted on Type 60 silica gel-coated glass plates using an automatic sample applicator fitted with a Hamilton syringe (Linomat III from Camag, Muttenz, Switzerland). Plates were developed in chloroform/methanol/acetic acid/water (85 : 15 : 10 : 3, v/v) and briefly dried. After slight staining with I_2, lipid zones corresponding to MGDG, DGDG and SQDG were circled with a carbon pencil, I_2 was blowed off, spots were scraped into test-tubes and their sugar content was determined with a microassay derived from the method of Dubois et al. [24], using galactose as standard. The chlorophyll content of each spotted aliquot served as an internal standard. The hydrolysis kinetics were expressed as the amount of remaining undegraded acyl lipid (in % of its initial amount), plotted on a log scale versus incubation time.

The colorimetric determination of each glycolipid was in good agreement with the gas-chromatography estimation of their fatty acyl chain content. Typically, the respective amounts of MGDG, DGDG and SQDG in thylakoid membranes (in μmol/μmol chlorophyll) were 1.54 ± 0.15, 0.73 ± 0.06 and 0.26 ± 0.05 ($n = 13$). This corresponds to 55, 27 and 8.8 mol% of the total acyl lipids.

The surface charge density of spinach thylakoid membranes was determined by the 9-aminoacridine method as in Ref. 25 and found to be -0.025 C/m^2. Surface potentials, calculated as described in Ref. 12, should be considered within a 3 mV range to account for the various incubation temperatures.

Results and Discussion

We have checked for the complete degradation of lipids when both sides of the thylakoid membrane were accessible to the enzyme. This was achieved by a brief sonication of unstacked thylakoids in the presence of the lipase. Vesicles were thus formed, both sides of which were exposed to the lipase. Under these conditions, an extensive hydrolysis of glycolipids occurred during incubation. More than 95% of MGDG were destroyed in about 30 min, whereas 60 min were necessary to obtain a comparable degradation of DGDG and SQDG, showing that protective interactions of glycolipids with membrane proteins were negligible. The requirement that lipids should be fully degradable when both sides of the membrane are exposed to the enzyme attack was therefore satisfied.

The enzymatic degradation of thylakoid membrane lipids was always carried out in the presence of an excess of lipase. In addition, under all salt conditions used (see Table I), the surface con-

TABLE I

CHEMICAL COMPOSITION OF INCUBATION MEDIA (BUFFERED TO pH 7.6) AND THE RESULTING VALUE OF THE THYLAKOID MEMBRANE SURFACE POTENTIAL

Medium	Component	(mM)	Surface potential (mV)
1	sorbitol	300	−130
	Mops/NaOH	0.8	
2	sorbitol	300	−87
	Mops/NaOH	5	
3	sorbitol	300	−72
	NaCl	5	
	Mops/NaOH	5	
4	sorbitol	300	−63
	NaCl	10	
	Mops/KOH	5	
5	sorbitol	300	−52
	NaCl	10	
	$MgCl_2$	1	
	Mops/KOH	5	
6	sorbitol	300	−40
	NaCl	10	
	$MgCl_2$	5	
	Mops/KOH	5	
7	sorbitol	300	−33
	NaCl	10	
	$MgCl_2$	10	
	Mops/KOH	5	
8 [a]	sorbitol	100	−14
	NaCl	5	
	KCl	100	
	$CaCl_2$	30	
	$MgCl_2$	70	
	Mops/NaOH	5	

[a] This medium reflects the ionic environment of thylakoid membranes bathed in the stroma of illuminated chloroplasts (taken from Ref. 21).

centration of Na^+ (calculated according to Ref. 12) was high enough to provide the 3–10 mM Na^+ required for full lipase activity [15]. Therefore, the lipase action was essentially independent of both temperature and Na^+ concentration. One may thus conclude that the observed differences in hydrolysis rates will reflect differences in the molecular organization of thylakoid membrane lipids.

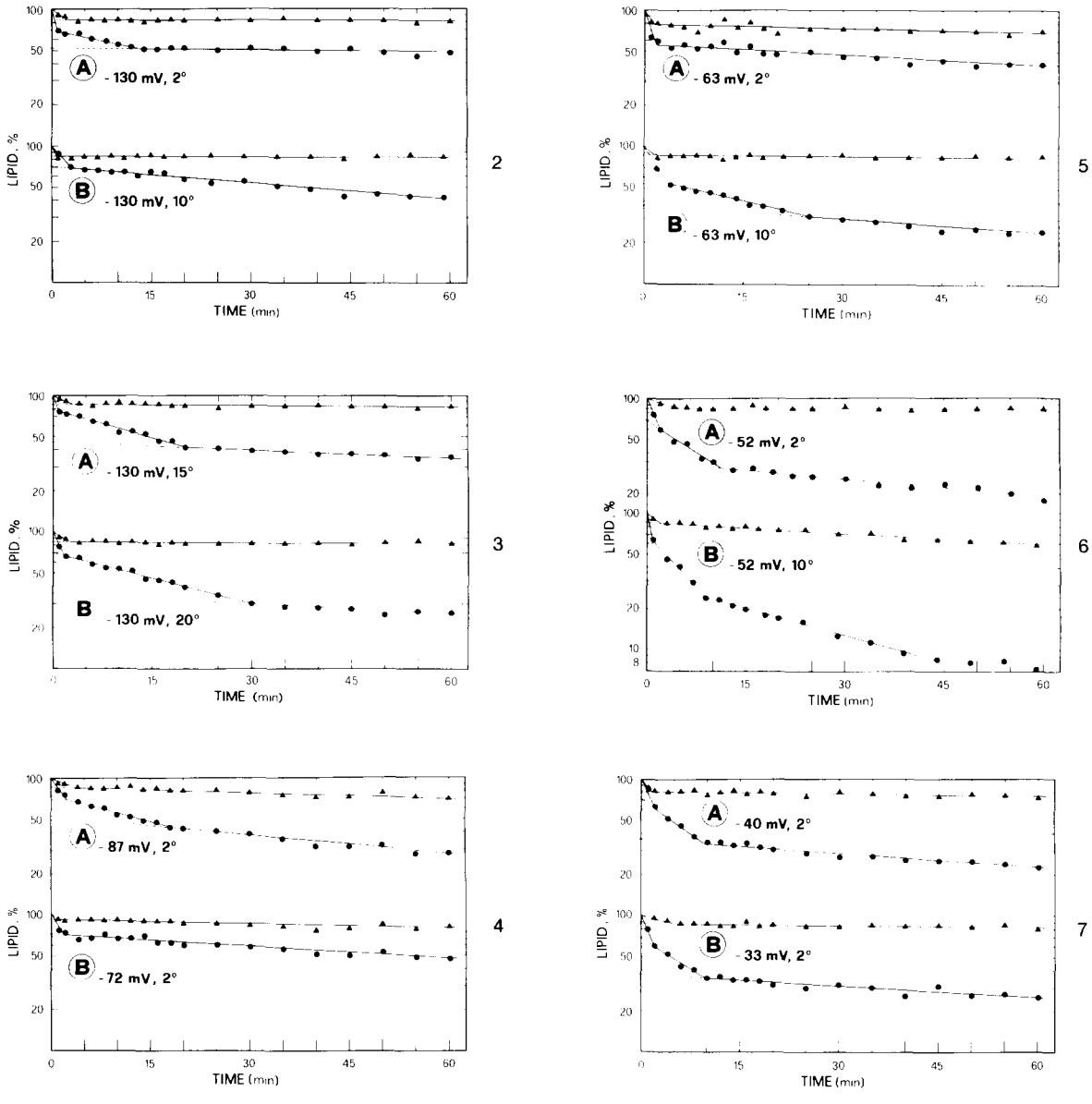
The degradation of DGDG resulted in a simple, biphasic pattern which was observed for all salt combinations over a wide range of temperatures (Figs. 2–7). Moreover, the amplitude of the two DGDG pools was constant, regardless of the temperature and ionic conditions. This behavior is typical of topological pools (see Rationale). Consequently, the first DGDG pool ($15 \pm 3\%$) may be attributed to the outer monolayer and the second ($85 \pm 3\%$) to the inner one. The second phase of the DGDG hydrolysis pattern was always quasi-horizontal, suggesting that essentially no transbilayer movement of inner DGDG molecules occurred during the time-scale of the experiment, whatever the conditions used.

The susceptibility of detergent-solubilized SQDG to the *Rhizopus* lipase was shown to be comparable to that of DGDG [20]. Nevertheless, no significant degradation of SQDG (less than 5% after 60 min of incubation) could be observed when thylakoid membranes were treated with the lipase (not shown). We tentatively conclude that SQDG is almost completely localized in the inner monolayer of the membrane. An alternate, but less probable view, would be that the lateral surface pressure of any outer SQDG domain(s) may exceed the maximum pressure at which the lipase is still able to penetrate the lipid monolayer.

The hydrolytic behavior of MGDG was most remarkable. Indeed, depending on the incubation conditions, two (Figs. 2B, 4B and 5A) or three pools (Figs. 2A, 3, 4A, 5B, 6 and 7) could be evidenced. These pools showed two main features. Firstly, for all conditions tested, the hydrolysis rate of each successive pool was progressively decreased. This indicates that no lysis of thylakoid membranes occurred during the time-scale of the experiment. Should lysis have taken place, an abrupt increase in hydrolysis rate would have been expected [26] as the result of exposure of both membrane faces to the lipase. In our conditions, the enzyme action was therefore restricted to the outer monolayer only. Secondly, there was a large difference in the amplitude of the MGDG pools from one condition to another, except for the third pool depicted in Figs. 3B, 5B, 6 and 7 (see also Table II, last column).

For the sake of clarity, and in line with the Rationale described above, we shall term these different pools in the following way. For each environmental condition, the first pool (with a 22–44% range) is K_a and the second pool (with a 18–72% range) is K_b (see Figs. 2–7 and Table II). The wide range of amplitude with respect to the various incubation conditions (salt and temperature) illustrates the kinetic nature of these first two pools (as defined in Rationale). The third pool (when present) corresponded to the difference $100\% - (K_a + K_b)$ and displayed a dual nature according to the incubation conditions. For instance, the third pool will be a kinetic pool K_c in Figs. 2A, 3A and 4A, since its amplitude can be clearly shifted towards lower values by increasing the temperature only (compare Figs. 2A and 3A with Fig. 3B) or by altering both salt and temperature conditions (compare Fig. 4A with Fig. 5B). On the other hand, the third pool corresponds to the (inner) topological pool T in Figs. 3B, 5B, 6 and 7 (see also Table II, last column) since the amplitude of this pool ($35\% \pm 2$) is remarkably constant and independent of environmental conditions of salt and temperature. One must be aware of the fact that whenever it may not be reached under certain conditions (Figs. 2, 3A, 4 and 5A), the inner topological pool T is nevertheless always implicitly included in the kinetic pool K_b (Figs. 2B, 4B and 5A) or K_c (Figs. 2A, 3A and 4A).

We have emphasized in Fig. 1 the role of the lateral surface pressure of membrane lipids. The importance of this parameter in the study of membrane lipid topography via enzymatic techniques has been carefully studied [27] and recently reviewed [14]. It is sensible to assume that the packing pressure of bulk membrane lipids (with the possible exception of a few molecules tightly bound to membrane proteins) should be homogeneous, especially when dealing with membrane lipids in a liquid-crystalline state. This assumption is certainly valid in our experimental conditions. The fact that pancreatic phospholipase A_2 is able to



Figs. 2–7. Hydrolysis patterns of MGDG (●) and DGDG (▲) in thylakoid membranes treated with the lipase from *R. arrhizus*. Pool amplitudes are given in Table II.

Fig. 2. Incubations were carried out in medium 1 (see Table I) at 2°C (A) and 10°C (B).

Fig. 3. Incubations were carried out in medium 1 (see Table I) at 15°C (A) and 20°C (B).

Fig. 4. Incubations were carried out at 2°C in medium 2 (A) and medium 3 (B) (see Table I).

Fig. 5. Incubations were carried out in medium 4 (see Table I) at 2°C (A) and 10°C (B).

Fig. 6. Incubations were carried out in medium 5 (see Table I) at 2°C (A) and 10°C (B).

Fig. 7. Incubations were carried out at 2°C in medium 6 (A) and medium 7 (B) (see Table I).

TABLE II

SUMMARIZED DATA OF MGDG HYDROLYSIS UNDER VARIOUS ENVIRONMENTAL CONDITIONS, GIVEN BY THE AMPLITUDE (IN % OF THE TOTAL MGDG) OF KINETIC POOLS K_a, K_b AND K_c AND OF THE INNER TOPOLOGICAL POOL T

	MGDG hydrolysis												
Pool													
Environmental conditions													
Total C^+ (mM)	0.8				5	10	15		15		15	15	110
Total C^{2+} (mM)	–				–	–	–		1		5	10	100
State [a]	s				us	us	us		s		s	s	s
Surface potential (mV)	−130				−87	−72	−63		−52		−40	−33	−14
Temperature (°C)	2	10	15	20	2	2	2	10	2	10	2	2	10
Conditions of Fig.	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B	7A	7B	–
K_a	30	29	22	30	25	28	44	40	31	30	28	31	26
K_b	18	71	32	34	22	72	56	23	35	38	36	32	38
K_c	52	–	46	–	53	–	–	–	–	–	–	–	–
T	nr [b]	nr	nr	36	nr	nr	nr	37	34	32	36	37	36

[a] As shown by cation-induced chlorophyll fluorescence; s, stacked and us, unstacked membranes.

[b] nr, means that the topological pool T, although implicitly included in K_b or K_c, was not reached under the specified condition.

hydrolyze phospholipids in spinach thylakoids [9,10] suggests that the lateral surface pressure of their diacyl lipids must be somewhat below 16 dynes/cm [27]. The high relative amount of MGDG (a non-bilayer forming, cone-shaped lipid) may account for this rather low value. Monolayer studies have shown that at 15 dynes/cm, both MGDG and DGDG (with a double-bond index of 2.9 and 2.6, respectively) are already packed to about 70% of their maximum compressibility [28]. It is thus conceivable that the formation of free fatty acids and lysogalactolipids during the enzymatic treatment will soon increase the lateral surface pressure to an extent that will decrease the rate and extent of hydrolysis, as suggested earlier [29,30].

The hydrolytic patterns of MGDG presented in Figs. 2–7 are an illustration of this effect. In a salt-depleted medium at 2°C (Fig. 2A), the pool K_a (30% of the total MGDG) was rapidly hydrolyzed. The subsequent accumulation of degradation products caused an increase in the packing pressure which resulted in a slower hydrolysis rate of pool K_b (18% of the total MGDG). A further increase in the packing eventually led to a 'high pressure' equilibrium state at which the lipase action was completely stopped. Although expressed by a horizontal plateau, the third hydrolytic phase (Fig. 2A) does not represent a topological equilibrium at which 48% of the total MGDG would be localized outside and 52% inside. This is clearly demonstrated by the hydrolysis patterns of Figs. 2B and 3. When the lipolytic treatment was carried out in the same salt-depleted medium at 10°C (Fig. 2B), the pool K_a (29% of the total MGDG) was again rapidly degraded but, due to the temperature increase, the build-up of the packing pressure was somewhat released so that the second phase (giving rise to pool K_b) was no longer interrupted. Moreover, the extent of MGDG hydrolysis after 60 min was 60% at 10°C as compared to 50% at 2°C (Fig. 2). At 15°C, the effect of temperature was more pronounced so that about 60% of the total MGDG were already degraded after 20 min only (Fig. 3A, intersect of 2nd and 3rd phase). The extent of hydrolysis was 64% after 60 min of incubation (Fig. 3A). At 20°C (Fig. 3B), the second hydrolytic phase ended up between 25 and 30 min together with a complete degradation of the MGDG in the outer monolayer (corresponding to $K_a + K_b = 64\%$). The third phase represents in this case (Fig. 3B) the hydrolysis of inner MGDG molecules which become available at the outer surface of the membrane via a (slow)

outwards transbilayer movement. Such a compensatory mechanism has already been described [31]. Similar interpretations can be given for the other individual results (Figs. 4–7).

Fig. 8 presents an experiment that has been designed to provide a direct evidence of the involvement of the lateral surface pressure in the control of lipid hydrolysis in thylakoid membranes. When control membranes were treated with the lipase at 20°C, the hydrolysis of MGDG and of DGDG were biphasic and the inner (topological) pools of each lipid reached their expected values (38 and 85%, respectively). The biphasic hydrolysis of MGDG was due here to a set of favorable conditions (high temperature and high salt concentration, see also below). When thylakoid membranes were preloaded with an amount of linolenic acid (500 nmol/mg chlorophyll) comparable to that accumulated in pools K_a (see Table II), MGDG hydrolysis was much slower. Even under these favorable environmental conditions, an intermediate kinetic pool K_b was present, and 35 min were required to reach the inner (topological) pool (35% of MGDG). Moreover, DGDG was not degraded at all. On the other hand, when bovine serum albumin was included in the incubation medium, the hydrolysis of MGDG

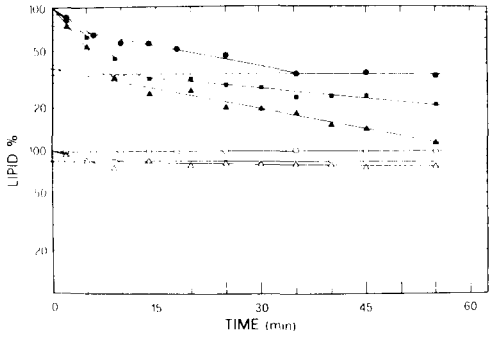


Fig. 8. Influence of bovine serum albumin and of linolenic acid on the hydrolysis patterns of MGDG (closed symbols) and DGDG (open symbols) in thylakoid membranes treated with the lipase from *R. arrhizus*. Membranes were preincubated for 10 min at 20°C in a medium comprising 300 mM sorbitol, 5 mM NaCl, 50 mM KCl and 5 mM Mops/NaOH (pH 7.6) (■, □) or supplemented with either 1 mg bovine serum albumin/mg chlorophyll (▲, △) or 500 nmol linolenic acid/mg chlorophyll (●, ○). Then, the lipase was added and hydrolysis was followed at 20°C in the dark. The surface potential provided by the incubation medium was −40 mV.

occurred faster and its extent was greater than in control membranes. The outer (topological) pool (62% of MGDG) was degraded in one phase only and the inner pool (38% of MGDG) was rapidly reached. However, the presence of bovine serum albumin had no noticeable effect on the hydrolysis pattern of DGDG (Fig. 8). These results show clearly that when the packing pressure of membrane lipids was increased (e.g., by preloading with linolenic acid), the lipid hydrolysis was significantly delayed and reduced. Conversely, when the build-up of the lateral surface pressure due to split products (e.g., free fatty acids and lysolipids) was prevented by the presence of bovine serum albumin (which acts as a removal agent for split products, see Ref. 32), hydrolysis was significantly enhanced.

We have shown that under salt-depleted conditions, the inner topological pool T could be reached simply by a proper choice of the temperature (Fig. 3B). However, the inner pool T can also be obtained at a constant (and even low) temperature by manipulating the ionic composition of the incubation medium. Spinach thylakoid membranes were equilibrated under different salt conditions (Table I) which allowed to alter both their gross morphological state (stacked versus unstacked) and their electrical surface properties (Table II). The hydrolysis patterns of MGDG were then determined. The results show two salient features. Firstly, the hydrolysis rate and/or extent of the second and third phases tended to increase with the ionic strength of the medium (thus with decreasing surface potential). This can be seen by comparing the hydrolysis rates and extents at 2°C when the imposed surface potentials are −130 mV (Fig. 2A), −87 mV (Fig. 4A), −52 mV (Fig. 6A), −40 and −33 mV (Fig. 7) or at 10°C when the potentials are −130 mV (Fig. 2B), −63 mV (Fig. 5B) and −52 mV (Fig. 6B). Secondly, the inner topological pool T can be reached not only at high temperature (20°C, Fig. 3B), but also at intermediate (10°C, Figs. 5B and 6B; see also Table II, last column) and even at low temperature (2°C, Figs. 6A and 7), provided that an adequate ionic environment is supplied to thylakoid membranes.

It is very unlikely that the modification of the environmental conditions stimulated the activity of the lipase, which was always present in excess

and was supplied with optimal Na^+ (surface) concentration. Therefore, a simple explanation would be to assume a salt-induced decrease in the lateral surface pressure of the membrane lipids. Since the enzymatic degradation of membrane lipids is an interface phenomenon, this salt-induced effect would be mediated by a surface parameter (such as surface potential) rather than by a bulk property of the medium (such as ionic strength). The involvement of the surface potential in the control of the packing pressure of membrane lipids may be indirectly supported by the fact that the presence of a small amount of divalent cations (1 mM $MgCl_2$) in the incubation medium enabled the lipase to reach the inner topological MGDG pool at 2°C (−52 mV, Fig. 6A), whereas in absence of Mg^{2+}, an incubation temperature of 10°C was required (Fig. 5B), although the ionic strengths of these both media did not significantly differ (0.018 and 0.015, respectively). Presently, we cannot decide whether this is a direct effect of salts (via surface potential) on galactolipid headgroups or a more general membrane property. Since the negative surface charge of thylakoids implies that anions must be repelled from and cations attracted to the membrane [12], we would expect cations to play the major role in the putative salt-induced decrease of the lateral surface pressure of membrane lipids. Headgroup interactions are recognized as a predominant factor in determining the packing properties of MGDG and DGDG [28]. Possible interactions of divalent cations (Mg^{2+}, Ca^{2+}) with sugars [33] and glycolipids [34] and also of Na^+ with MGDG [35] have been reported. It was also suggested that above the transition temperature, metal cations might induce some conformational change in galactolipid headgroups, thereby increasing their hydration level [16]. This would in turn weaken the mutual interaction of polar headgroups and consequently the lateral packing pressure, thus favoring the enzyme activity. A more direct support of our contention comes from the recent demonstration that the fluidity of rabbit intestinal brush-border membranes increases with salt concentration, presumably via their surface electrical properties [36].

The apparent insensitivity of DGDG hydrolysis patterns to the various salt/temperature conditions is merely due to the small relative amount of this lipid in the outer monolayer (15%) as compared to that of MGDG (65%). Indeed, the first decrease in the MGDG hydrolysis rate occurred when the outer (topological) DGDG pool was (almost) completely degraded (Figs. 2–7).

We have already suggested that the inner topological MGDG pool became available at the outer surface of the membrane presumably via an outwards transbilayer movement. A comparison of the relative hydrolysis rate of this inner pool under various environmental conditions reveals an interesting dependence of the transbilayer movement of MGDG molecules on the salt conditions. For instance, under salt-depleted conditions, the relative rate was 0.16 at 20°C (−130 mV, Fig. 3B), whereas in the presence of salt (−52 mV, Fig. 6), the relative rates were 0.27 at 2°C and 0.58 at 10°C. A decrease in the outer surface potential appears thus to enhance the capacity of inner MGDG molecules for transbilayer movement even at low temperatures. This phenomenon was not observed for DGDG and may be thus envisaged as a specific property of MGDG, possibly related to its non-bilayer character. Hunt and Tipping [37] have described a similar transmembrane effect of salts by showing that the interaction of metal cations with the outer polar headgroups of dipalmitoylphosphatidylcholine liposomes could be transmitted to the molecules of the inner monolayer so that the temperature and the extent of the phase transitions were affected in both outer and inner monolayers. The bilayer stability (as reflected by the packing tightness of lipid molecules) was thus apparently increased under low-salt as compared to high-salt conditions. This interpretation may be endorsed, although indirectly, by the works of Träuble and Eibl [13] and of Gounaris et al. [38].

We conclude from the above results that specific requirements with respect to salt concentration and temperature must be fulfilled in order to obtain a reliable estimation of the transmembrane distribution of galactolipids in thylakoid membranes (Table II): namely, high-salt conditions (surface potential $\leqslant -63$ mV) plus low to high temperature (2–20°C) or low-salt conditions (surface potential = −130 mV) plus high temperatures (20°C). Note that most of these conditions will favor the formation of stacked membranes,

which do not appear to be a limiting factor for the penetration of the lipase into the partitions. These environmental requirements illustrate the validity of our working model (Fig. 1) and may also provide a clue to explain the divergent results obtained by various authors [6–8].

What are the consequences of the asymmetric localization of galactolipids for the molecular organization of thylakoid membranes? The relative proportions of MGDG and DGDG in the outer and inner monolayer (65/35 and 15/85, respectively) correspond to relative amounts (in mol% of total acyl lipids) of 36/19 for MGDG and 4/23 for DGDG (Table III). The two galactolipids would thus account for 40 and 42 mol% of total acyl lipids in the outer and inner monolayer of the thylakoid membrane, respectively. In other words, the total galactolipids (MGDG + DGDG) are almost equally balanced between both membrane sides (outside/inside ratio = 0.95). The transmembrane distribution of total acyl lipids will certainly reflect closely that of total galactolipids since among anionic lipids (which account for about 15% of the total acyl lipids), phosphatidylglycerol is found preferentially outside [6,9,10] whereas SQDG adopts most probably the opposite distribution (this report; see also Ref. 6).

The relative amount of MGDG localized in the inner monolayer (19 mol% of the total acyl lipids) represents one-third of the total MGDG (19/55 = 0.345, see Table III). Morphological and compositional considerations have led Murphy [39] to suggest that the presence of (more or less) pure MGDG in the tightly concave inner thylakoid margin would considerably stabilize this structure, and that this inner MGDG pool would account for about one-third of the total MGDG. One cannot prejudge, from the present results, the exact lateral localization of the inner (topological) MGDG pool. However, matching our results with Murphy's prediction implies that most (if not all) inner MGDG molecules would be localized in the concave portions of thylakoid margins. Whether this is true or not must clearly await further investigation.

Table III also shows that the molar ratios MGDG/DGDG in the outer and inner monolayer are 9 and 0.8, respectively. Due to their low relative amount in the thylakoid membrane, neither phosphatidylglycerol (via its outer pool) nor SQDG (via its inner pool) can significantly decrease the proportion bteween non-bilayer-forming lipids and bilayer-forming lipids, particularly in the outer monolayer, although they are known to stabilize the bilayer configuration of vesicles made of total polar lipid extracts [38]. From our results, we can estimate the probable lipid composition of the inner monolayer of thylakoid membranes (in mol% of total acyl lipids): MGDG (19), DGDG (23), SQDG (8) and PG (2) (Table III; see also Refs. 10 and 11). Therefore, the inner monolayer appears to contain enough bilayer-forming lipids to accommodate the non-bilayer-forming MGDG [40]. The 'flat' inner regions of the membrane would even include bilayer-forming lipids only, if the inner MGDG molecules were strictly localized in the margins.

On the other hand, a mixture of MGDG and DGDG in a molar ratio of about 9 (Table III) is not expected to form by itself any lamellar phase in the outer monolayer [41]. However, both the low lipid/protein ratio of stacked thylakoid membranes [2] and the calculations of Murphy [39] and Murphy and Woodrow [4] indicate that the occur-

TABLE III

DISTRIBUTION AND RELATIVE AMOUNTS OF GALACTOLIPIDS IN THE THYLAKOID MEMBRANE

Lipid (% total lipids)	Relative amount in thylakoid membrane	Outer monolayer		Inner monolayer	
		distribution (% lipid class)	relative amount (% total lipids)	distribution (% lipid class)	relative amount (% total lipids)
MGDG	55	65	36	35	19
DGDG	27	15	4	85	23
MGDG+DGDG	82	–	40	–	42
Molar MGDG/DGDG ratio	2	–	9	–	0.8

rence of free lipid domains in the thylakoid membrane is rather unlikely. We must therefore envisage that most of the outer MGDG molecules would interact with the large (pigment)-protein complexes so as to provide them with the required seal and orientation in the membrane, as already suggested by various authors [42–46]. The boundary layer model proposed by Williams et al. [42] would be particularly well-suited for this purpose. Finally, we must point out that the overall asymmetry described here does not exclude the possibility that galactolipids may adopt a different transmembrane distribution in grana and in stroma membranes.

Experiments are now in progress which address the issue of the galactolipid localization in thylakoid membranes of various plant species.

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References

1 Hiller, R.G. and Raison, J.K. (1980) Biochim. Biophys. Acta 599, 63–72
2 Ford, R.C., Chapman, D.J., Barber, J., Pedersen, J.Z. and Cox, R.P. (1982) Biochim. Biophys. Acta 681, 145–151
3 Gounaris, K., Sundby, C., Andersson, B. and Barber, J. (1983) FEBS Lett. 156, 170–174
4 Murphy, D.J. and Woodrow, I.E. (1983) Biochim. Biophys. Acta 725, 104–112
5 Tuquet, C., Guillot-Salomon, T., De Lubac, M. and Signol, M. (1977) Plant Sci. Lett. 8, 59–64
6 Unitt, M.D. and Harwood, J.L. (1982) in Biochemistry and Metabolism of Plant Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C., eds.), pp. 359–362, Elsevier Biomedical Press, Amsterdam
7 Radunz, A. (1981) Ber. Deutsch. Bot. Ges. 94, 477–489
8 Sundby, C. and Larsson, C. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. 3, pp. 115–118, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
9 Rawyler, A. and Siegenthaler, P.A. (1981) Biochim. Biophys. Acta 635, 348–358
10 Giroud, C. and Siegenthaler, P.A. (1984) in Structure, Function and Metabolism of Plant Lipids (Siegenthaler, P.A. and Eichenberger, W., eds.), pp. 413–416, Elsevier Biomedical Press, Amsterdam
11 Staehelin, L.A. and Arntzen, C.J. (1983) J. Cell Biol. 97, 1327–1337
12 Barber, J. (1980) Biochim. Biophys. Acta 594, 253–308
13 Trauble, H. and Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71, 214–219
14 Roelofsen, B. (1982) J. Toxicol. Toxin Rev. 1, 87–197
15 Sémériva, M. and Dufour, C. (1972) Biochim. Biophys. Acta 260, 393–400
16 Wieslander, R., Ulmius, J., Lindblom, G. and Fontell, K. (1978) Biochim. Biophys. Acta 512, 241–253
17 Israelachvili, J.N. (1973) Biochim. Biophys. Acta 323, 659–663
18 McLaughlin, S. and Harary, H. (1974) Biophys. J. 14, 200–208
19 Rawyler, A. and Siegenthaler, P.A. (1984) in Structure, Function and Metabolism of Plant Lipids (Siegenthaler, P.A. and Eichenberger, W., eds.), pp. 409–412, Elsevier Biomedical Press, Amsterdam
20 Fischer, W., Heinz, E. and Zeus, M. (1973) Z. Physiol. Chem. 354, 1115–1123
21 Barber, J. (1980) in Plant Membrane Transport, Current Conceptual Issues (Spanswick, R.M., Lucas, W.J. and Dainty, J., eds.), pp. 83–96, Elsevier, Amsterdam
22 Bruinsma, J. (1961) Biochim. Biophys. Acta 53, 576–578
23 Rawyler, A. and Siegenthaler, P.A. (1980) J. Biochem. Biophys. Methods 2, 271–281
24 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356
25 Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 589, 346–352
26 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
27 Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97–107
28 Bishop, D.G., Kenrick, J.R., Bayston, J.H., MacPherson, A.S. and Johns, S.R. (1980) Biochim. Biophys. Acta 602, 248–259
29 Fujii, T. and Tamura, A. (1979) J. Biochem. 86, 1345–1352
30 Roelofsen, B., Sibenius-Trip, M., Verheij, H.M. and Zevenbergen, J.L. (1980) Biochim. Biophys. Acta 600, 1012–1017
31 Demant, E.J.F., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1979) Eur. J. Biochem. 95, 613–619
32 Haest, C.M.W., Plasa, G. and Deuticke, B. (1981) Biochim. Biophys. Acta 649, 701–708
33 Weigel, H. (1963) in Advances in Carbohydrate Chemistry (Wolfrom, M.L. and Tipson, R.S., eds.), Vol. 18, pp. 61–97, Academic Press, New York
34 Hauser, H., Levine, B.A. and Williams, R.J.P. (1978) Trends Biochem. 1, 278–281
35 Kuiper, P.J.C. (1969) Plant Physiol. 44, 968–972
36 Ohyashiki, T. and Mohri, T. (1983) Biochim. Biophys. Acta 731, 312–317
37 Hunt, G.R. and Tipping, L.R.H. (1978) Biochim. Biophys. Acta 507, 242–261
38 Gounaris, K., Sen, A., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1983) Biochim. Biophys. Acta 728, 129–139
39 Murphy, D.J. (1982) FEBS Lett. 150, 19–26
40 Sprague, S.G. and Staehelin, A. (1983) in Biosynthesis and Function of Plant Lipids (Thompson, W.W., Mudd, J.B.

and Gibbs, M., eds.), pp. 144–159, American Society of Plant Physiologists, Rockville
41 Quinn, P.J. and Williams, W.P. (1983) Biochim. Biophys. Acta 737, 223–266
42 Williams, W.P., Gounaris, K. and Quinn, P.J. (1984) in Advances in Photosynthetic Research (Sybesma, C., ed.), Vol. 3, pp. 123–130, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
43 Siefermann-Harms, D., Ross, J.W., Kaneshiro, K.H. and Yamamoto, H.Y. (1982) FEBS Lett. 149, 191–196
44 Pick, U., Gounaris, K., Admon, A. and Barber, J. (1984) Biochim. Biophys. Acta 765, 12–20
45 Krupa, Z. (1984) Photosynth. Res. 5, 177–184
46 Gounaris, K., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1984) Biochim. Biophys. Acta 766, 198–208