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# Laurdan fluorescence spectroscopy in the thylakoid bilayer: The effect of violaxanthin to zeaxanthin conversion on the galactolipid dominated lipid environment

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**Abstract**

Laurdan (6-lauroyl-2-dimethylaminonaphthalene) fluorescence spectroscopy has been applied to probe the physical status of the thylakoid membrane upon conversion of violaxanthin to zeaxanthin. So far, only phospholipid-dominated membranes have been studied by this method and hereby we report the first use of laurdan in mono- and digalactosyldiacylglycerol-dominated membrane systems. The generalised polarisation (GP) of laurdan was used as a measure of the structural effect of xanthophyll cycle pigments in isolated spinach (*Spinacia oleracea*) thylakoids and in model membrane vesicles composed of chloroplast galactolipids. Higher GP values indicate a membrane in a more ordered structure, whereas lower GP values point to a membrane in a less ordered fluid phase. The method was used to probe the effect of violaxanthin and zeaxanthin in thylakoid membranes at different temperatures. At 4, 25 and 37 °C the GP values for dark-adapted thylakoids in the violaxanthin-form were 0.55, 0.28 and 0.26. After conversion of violaxanthin to zeaxanthin, at the same temperatures, the GP values were 0.62, 0.36 and 0.34, respectively. GP values increased gradually upon conversion of violaxanthin to zeaxanthin. Similar results were obtained in the liposomal systems in the presence of these xanthophyll cycle pigments. We conclude from these results that the conversion of violaxanthin to zeaxanthin makes the thylakoid membrane more ordered.


*Keywords:* Laurdan; Fluorescence; Thylakoid membrane; Galactolipids; Xanthophyll cycle; Zeaxanthin

## 1. Introduction

Carotenoids are lipophilic pigments occurring widely in nature. They are synthesized in plants, and are only introduced with diet to human and animals. Carotenoids have a broad range of functions; they are essential to plants for photosynthesis, acting in light-harvesting, act as powerful antioxidants and have a structural role in membranes. Moreover, carotenoids especially xanthophylls (oxygen containing carotenoids) also play a major role in photoprotection. The formation of zeaxanthin has been implicated in protecting the photosynthetic apparatus from overexcitation [1].

Zeaxanthin is formed by de-epoxidation of violaxanthin (Fig. 1) in the xanthophyll cycle (XC) that is located in the thylakoid membrane of higher plants. This conversion is catalysed by the enzyme, violaxanthin de-epoxidase (VDE) which requires low pH, ascorbic acid as a co-substrate and lipids that can form inverted hexagonal phase [2] such as mono-galactosyldiacylglycerol (MGDG). When the lumenal pH of thylakoids, upon overexcitation, drops to values below 6, a conformational change of VDE occurs presumably through protonation of conserved histidines [3]. VDE then docks to the membrane and starts converting violaxanthin to zeaxanthin via the intermediate antheraxanthin. The formed zeaxanthin plays a key role in non-photochemical quenching (NPQ) [1]. In the dark and at low light zeaxanthin is reconverted into violaxanthin. This

*Abbreviations:* DGDG, digalactosyldiacylglycerol; DMSO, dimethyl sulfoxide; DPPC, dipalmitoyl-phosphatidylcholine; EPR, electron paramagnetic resonance; GP, general polarisation; $H_{II}$, inverted hexagonal phase; HPLC, high performance liquid chromatography; laurdan, 6-lauroyl-2-dimethylaminonaphtalene; MES, 2-(N-morpholino)ethanesulfonic acid; MGDG, monogalactosyldiacylglycerol; MOPS, 3-(N-morpholino)propanesulfonic acid; NPQ, non-photochemical quenching; PG, phosphatidylglycerol; VDE, violaxanthin de-epoxidase; XC, xanthophyll cycle

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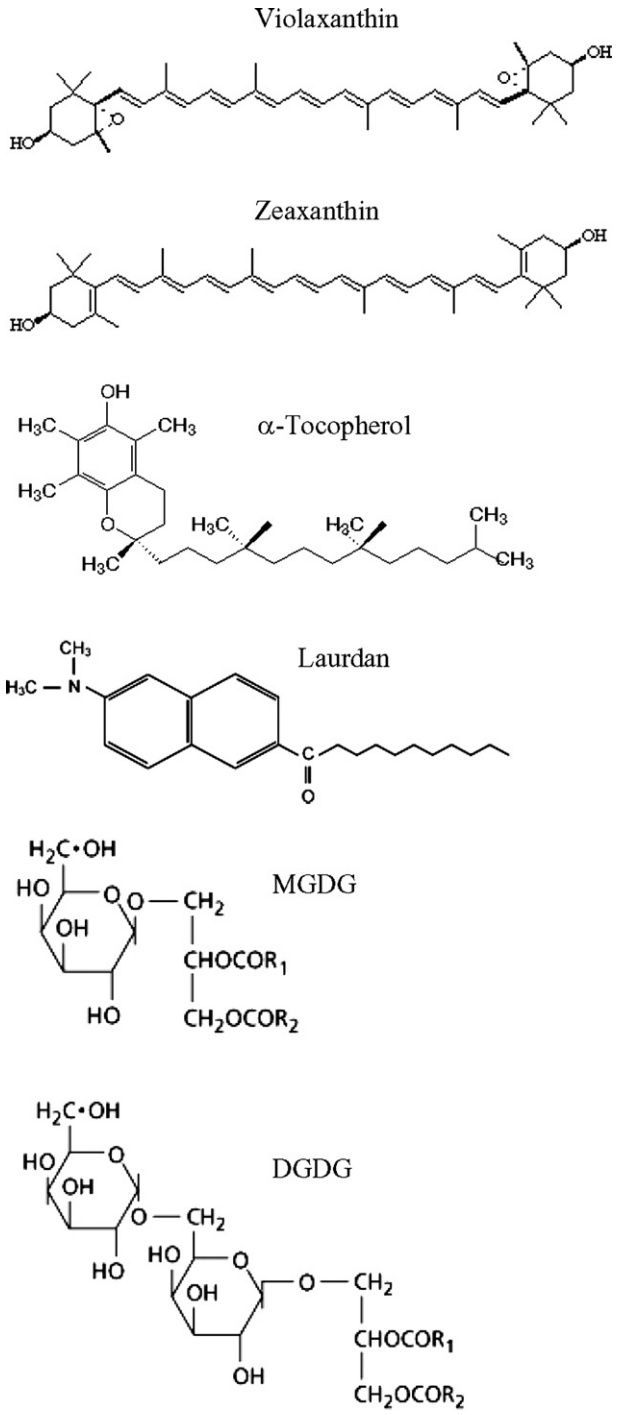


Fig. 1. Molecular structures of the xanthophyll cycle pigments violaxanthin and zeaxanthin; the antioxidant and membrane-rigidifier α-tocopherol; the fluorescent dye laurdan; and the main thylakoid lipids MGDG and DGDG.

back-reaction is catalysed by zeaxanthin epoxidase (ZE). For further details on the XC, there are several recent reviews available, e.g. by Demmig-Adams and Adams [4], Eskling et al. [5] and Grzyb et al. [6].

Thylakoids are unique membranes since they are dominated by galactolipids, digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG) (Fig. 1), while the diglycerides in other membranes in the plant cell such as plasma membrane and mitochondrial membrane are phospholipids. MGDG accounts for about 50 mol% of the total polar lipids, and the other major component, DGDG comprises about 30 mol% [7]. Notably, MGDG is a non-lamellar prone lipid forming inverted hexagonal phase ($H_{II}$) in aqueous media [8]. In the presence of DGDG and proteins the overall lamellar structure of thylakoids is formed even at such high non-lamellar lipid content.

The role of XC-pigments as antenna pigments and antioxidants is well known, but the location in the photosynthetic membrane is still under debate. One view suggests that XC-pigments are protein-bound as they can be found in pigment–protein complexes after fractionation or purification [9–11]. These pigments have been suggested to be present in the crystal structure of the major light harvesting complex [12], and required for non-photochemical quenching in the antenna systems [13]. However, the weaker binding of XC-pigments to proteins than that of other carotenoids [14] and the sub-stoichiometric quantities often found [15] indicate that XC-pigments are not necessarily protein-bound. The other view supporting the non-protein-bound location of violaxanthin and zeaxanthin is that they are free in the lipid matrix of the thylakoid membrane [16–18].

The latter assumption is based on the following findings, that (i) violaxanthin can be converted to the same extent from both sides of the thylakoid membrane [16], (ii) separation of different regions of the thylakoid membrane by mechanical fragmentation revealed that XC-pigments were almost evenly distributed between the different regions in contrast to other pigments in the membrane [16] (iii) and the amount of XC-pigments in the thylakoid membrane tend to vary in opposite direction to that of the basic set of antenna protein complexes [16]. A compromise would be if a small part of the XC-pigments were bound to proteins while the rest is free in the lipid matrix. An interesting question is then how the XC-pigments affect the lipid packing of the membrane.

Xanthophylls have been suggested to be membrane-spanning, rod-shape like molecules, acting like rivets that decrease membrane fluidity in the fluid phase [19–22]. Another molecule found in the thylakoid membrane, α-tocopherol has also been inferred to increase membrane rigidity [24]. The XC-pigments along with α-tocopherol were suggested to affect the fluidity of thylakoid membranes in a similar way to the action of cholesterol in animal cell membranes [23].

Recently, the involvement of violaxanthin and zeaxanthin in modulating membrane curvature stress has been proposed [18]. The presence of $H_{II}$ lipids in the thylakoid bilayer causes curvature stress as they are forced into unfavoured lamellar structures. It was found that the degree of conversion from violaxanthin to zeaxanthin could be altered with lipid packing modifiers such as linolenic acid, α-tocopherol and octaethylene glycol monohexadecyl ether, which all have a regulatory effect on the membrane curvature stress. Nearly 100% violaxanthin de-epoxidation was observed in the presence of linolenic acid via decreasing lipid packing, while α-tocopherol and cethylethers restricted the conversion of violaxanthin presumably by increasing lipid packing in the membrane [18]. The lipid

packing and curvature stress are partly overlapping phenomena. Factors releasing curvature stress are expected also to cause a more densely packed membrane and vice versa.

To test the hypothesis that xanthophylls, zeaxanthin in particular, make the thylakoid membrane more rigid and tightly packed, we utilised the reporter molecule, laurdan in combination with fluorescent spectroscopy. Laurdan (6-lauroyl-2-dimethylaminonaphthalene) (Fig. 1) is a polarity sensitive fluorescent probe widely used in phospholipid membrane studies to assess membrane physical properties. A great advantage of laurdan among other fluorescent probes is that it partitions into the lipid phase and its solubility in water is negligible [25]. Laurdan is incorporated into the membrane with the fluorescent naphthalene moiety situated at the level of the interface (glycerol backbone) region, and the lauric acid tail anchored in the hydrophobic core. Upon excitation in the UV-region, the dipole moment of Laurdan increases and water molecules in the vicinity of the probe will adopt to this new dipole [26]. In tightly packed, gel phase membranes with reduced molecular mobility in the hydrophobic region, the dipolar relaxation of these remaining water molecules is too slow to change the properties of fluorescent emission, thus the emission spectra have only one peak at 440 nm. However, in loosely packed fluid phase membranes, where the interior is fluid allowing the lipid molecules to move around, dipolar relaxation of water molecules occurs, and it is apparent as a red-shift about 50 nm, increased fluorescence at 490 nm, in the emission spectra of laurdan [27,28]. Hence, this phase dependent red-shift in the steady-state emission spectrum of laurdan reflects the excitation energy spent for relaxation of water molecules in the surroundings of the probe, and at the same time reports on the molecular dynamics in the membrane interface region [28].

In this study, we show for the first time that laurdan can be used also in galactolipid membranes, typical of thylakoids, to study membrane properties. We have used laurdan to elucidate the impact of violaxanthin to zeaxanthin conversion on the packing of thylakoid membrane components. Increasing amounts of zeaxanthin in the membrane correlated well with a less pronounced red shift in laurdan spectra revealing a more tightly packed membrane. Our hypothesis, regarding the lipid-phase location of XC-pigments, is supported by the data presented with laurdan fluorescence spectroscopy both in the thylakoid and in galactolipid model membranes.

## 2. Materials and methods

### 2.1. Isolation of thylakoid membranes

Thylakoids were isolated from spinach (*Spinacia oleracea*) essentially according to Yamamoto and Higashi [29]. Prior to isolation, leaves were dark-adapted for 12 h in order to convert all zeaxanthin to violaxanthin. Plant material was homogenized in 100-g portions in a blender with 200 mL medium A (400 mM sorbitol, 50 mM MOPS-NaOH, pH 7.0, and 10 mM NaCl). The homogenate was filtered through four layers of 20-μm nylon cloth and the filtrate was centrifuged at 2500×*g* for 7 min. The pellet was re-suspended in the same medium, centrifuged at 5000×*g* for 5 min, resuspended in 50 mL distilled water per 100 g plant material, followed by centrifugation at 37,000×*g* for 10 min. Finally, the thylakoid pellet was resuspended in medium B (400 mM sucrose, 50 mM MES-NaOH, pH 6.5, 15 mM NaCl). The chlorophyll content was determined according to Porra [30]. DMSO was added to a final concentration of 5% (v/v), and samples were stored at −80 °C until further use.

### 2.2. Violaxanthin to zeaxanthin conversion

The de-epoxidation reaction was performed at 37 °C, in 50 mM MES-NaOH, pH 5.1, in darkness. The reaction was initiated by the addition of ascorbate to a final concentration of 30 mM. Thylakoid samples, of 4 mL each, at 0.08 mg chlorophyll $mL^{-1}$ were incubated for 2, 15, 30 and 60 min to produce different amount of zeaxanthin in the thylakoid membrane. To terminate the reaction, the pH in the medium was adjusted with 1 M NaOH to give a pH above 7.0 where VDE is inactive. At this point, samples of 1 mL were withdrawn for pigment analysis. The rest of the thylakoid samples were sedimented by full speed centrifugation in an Eppendorf bench top centrifuge for 4 min, resuspended in 40 μL phosphate buffer (10 mM), pH 7.4, containing 100 mM sucrose, 5 mM NaCl and 1 mM $MgCl_2$ and kept on ice until use in the experiments with laurdan.

### 2.3. Pigment extraction and HPLC determination of the XC-pigments

The 1 mL samples of thylakoids were centrifuged for 4 min at 14000 rpm, resuspended in 130 μL 100 mM phosphate buffer, pH 7.0, and ice cold, 100% acetone (430 μL) was added to extract XC-pigments; the mixture was incubated on ice for 4 min in darkness and centrifuged as above. The obtained supernatant was withdrawn and further diluted with water to get 50% (v/v) acetone in the pigment extracts.

XC-pigments were analyzed by reversed-phase HPLC (Waters 600E) essentially according to Thayer and Björkman [31]. Pigment extracts (150 μL) were subjected to a Zorbax ODS 4.6×250 mm non-end capped cartridge column, where xanthophylls were separated in a methanol/acetonitrile mixture (15%/85%, v/v) run isocratically for 6 min, followed by a rapid elution of other pigments in methanol/ethyl acetate mixture (50%/50%, v/v) for 6 min. All solvents were HPLC grade. The flow rate was constant at 1 mL $min^{-1}$. Pigments were detected at 445 nm and quantified by peak area integration using data acquisition software (Waters Millennium).

### 2.4. Laurdan labelling of thylakoid membranes

Laurdan labelling was performed with spinach thylakoid membranes in violaxanthin-form and zeaxanthin-forms, at 0.02 mg chlorophyll $mL^{-1}$. These galactolipid-dominated membranes were incubated with 0.68 μM laurdan (Molecular Probes, Eugene, OR) for 30 min unless otherwise noted, in 10 mM HEPES–KOH, pH 7.0 buffer also containing 0.25 M sucrose and 10 mM KCl. Samples of 3 mL with labelled membranes were subjected to 4 °C, 25 °C and 37 °C under gentle mixing.

### 2.5. Liposome preparation

Multilamellar liposomes were prepared essentially as described in Parasassi et al. [28]. Lipids of DPPC (Sigma), DGDG and MGDG (Lipid Products, UK) in chloroform and laurdan in ethanol, with or without carotenoids (CaroteNature, Switzerland) and α-tocopherol (Sigma) in ethanol were mixed in a test tube. The solvent was evaporated under oxygen-free nitrogen flow. The total amount of lipids and laurdan was always kept the same, 250 nmol and 1.7 nmol respectively. XC-pigments, violaxanthin and zeaxanthin, of 1.8 nmol, α-tocopherol of 40 nmol were mixed with the lipids before addition of the fluorescent probe. To remove any possible trace of chloroform and alcohols, the lipid-laurdan(-pigment) films were incubated for 30 min in a speed vac (Savant) without heating. The dry films were hydrated with 500 μL of the HEPES–KOH buffer, pH 7.0, (see above in Laurdan labelling of thylakoid membranes). The samples were heated to 70 °C and liposomes formed by vortexing for 5×30 s, reheating the samples between every vortexing cycle. Using the same buffer, samples were diluted to 3 mL, equilibrated to a measuring temperature and used directly. Since acyl chains of plant galactolipids are highly unsaturated, and both the XC-pigments and laurdan are light sensitive, all preparations were carried out in dim light and nitrogen atmosphere.

### 2.6. Laurdan fluorescence spectroscopy

For interpreting the average fluorescence signals from fluid- and gel-phase embedded laurdan, Parasassi et al. [28] have developed the concept of general polarisation (GP) of laurdan fluorescence. It is important to note here that even if the name contains the word polarisation, polarisers are not required for the experiment; only steady-state fluorescence measurements are used. The definition of general polarisation is

$$GP = (I_g - I_f)/(I_g + I_f) \quad (1)$$

where $I_g$ and $I_f$ are the fluorescence intensity at the emission maxima of laurdan in gel and fluid phase, respectively. Higher GP values indicate a membrane in the gel phase or a more tightly packed structure, with a low rate of solvent relaxation. In the present work, the excitation GP values of the liposome system were calculated using fluorescent intensities at 440 and 490 nm, as gel and fluid phase, respectively. In the case of the thylakoid membrane modification we used fluorescent intensities at 460 nm and 516 nm, to represent the more and less tightly packed fluid phases, respectively. This change in measuring wavelength was suggested by Hellgen [32] to minimize interference from endogenous fluorescence of the thylakoids.

Thylakoid and liposome samples were placed in quartz cuvettes with optical pathways of 1 cm. Steady-state emission and excitation spectra measurements were performed with a FluoroMax-2 fluorometer (Spex spectrofluorometer system, Jobin Yvon, Longjumeau, France), equipped with a xenon arc lamp as a light source, using excitation at 340 nm for model membranes and 390 nm in the case of thylakoids. Some of the measurements (Tables 2 and 3, Fig. 5) were also performed using the Shimadzu spectrofluorometer (RF-1501, Shimadzu Corp, Kyoto, Japan). Both fluorometers were equipped with a thermostated sample chamber and a magnetic stirrer. The spectral bandwidths were 5 nm for the FluoroMax-2 and 10 nm for the RF-1501 instruments.

### 2.7. Lipid analysis

MGDG and DGDG (Lipid Products, UK) were analysed by two-dimensional thin-layer chromatography (TLC) and quantified from their acyl group composition as previously described [33], except that the trans-esterification of the fatty acids was performed at 80°C in 5% $H_2SO_4$ in dry methanol for 2 h.

## 3. Results

### 3.1. Laurdan fluorescence in liposomes of galactolipids

Most of the laurdan literature is based on phospholipid-dominated biological membranes, and model systems (e.g. [19,25,32,34,35]). However, the thylakoid membrane, we have used in this study, is dominated by galactolipids. The emission spectra of laurdan in the two lipid-systems (phospholipid and galactolipids) are shown in Fig. 2. Almost a three-fold higher fluorescence intensity was measured in the DPPC vesicles than in the DGDG liposomes (Fig. 2 insert) when excited at 340 nm. Both these lipids are lamellar-phase prone lipids, however the acyl chains of plant DGDG are mostly poly-unsaturated (Table 1) giving rise to a less ordered membrane, consistent with the lower and red-shifted fluorescence observed of laurdan. The fatty acid compositions of the galactolipids MGDG and DGDG are shown in Table 1. The major fatty acids in these lipids are palmitic (16:0), hexadecatrienoic (16:3) and linolenic (18:3) acids.

In phospholipid vesicles, the emission spectrum of laurdan is red-shifted around 50 nm going from gel phase to fluid phase as it was established by Parasassi et al. [27]. Similar results were

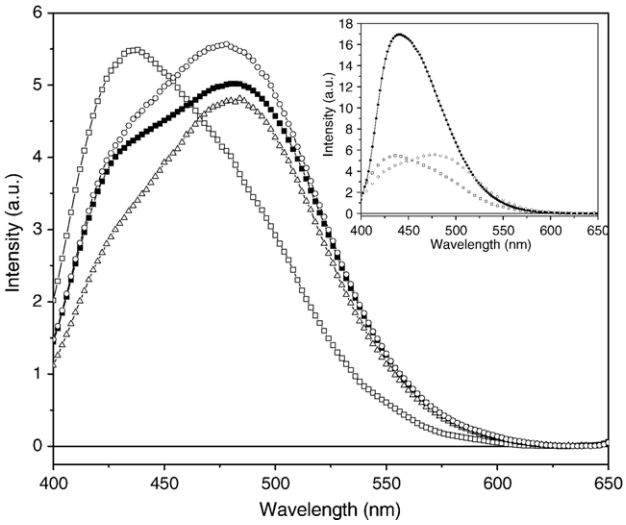


Fig. 2. Representative emission spectra of laurdan in pure DGDG liposomes at 10 °C (□), at 37 °C (○) and at 55 °C (△), and for DGDG : MGDG 70 : 30 mol% liposomes at 37 °C (■). The insert shows emission spectra of DPPC vesicles at 37 °C (●) together with DGDG liposomes at 10 °C (□) and at 37 °C (○). The excitation wavelength was 340 nm.

observed in pure DGDG-liposomes when the temperature was increased from 10 °C to 37 °C and further to 55 °C (Fig. 2). At 10 °C the emission spectrum of DGDG was dominated by a maximum at 435 nm, which looks like the DPPC emission spectrum in gel phase. However, at 10 °C DGDG could not be in the gel phase since 86% of the acyl groups are 16:3 + 18:3 [36]. Thus, the laurdan spectra for galactolipids are rather showing the packing or order of the lipids than the phase structure. This is also shown for DGDG mixed with 30 mol% MGDG at 37 °C (Fig. 2), which had a lower fluorescent intensity than pure DGDG at the same temperature. Since MGDG is an inverted-hexagonal phase prone galactolipid, the presence of large amounts of MGDG gives rise to curvature stress in the membrane. In a monolayer membrane, the presence of MGDG curves the membrane towards water giving rise to curvature stress. However, this bending is rather unlikely to occur in a bilayer, instead there is an increase in the lateral pressure in the centre of the bilayer and a decrease in pressure in the headgroup region [37]. The lower fluorescent intensity in the DGDG: MGDG (70:30 mol%) mixture is thus indicating a less ordered structure in the headgroup area. These results, in fact, show that laurdan fluorescence can be successfully used to report physical properties of model systems made of galactolipids.

### 3.2. Emission spectra of laurdan in thylakoid membranes

After establishing the method in the liposomal system of galactolipids, laurdan fluorescence was applied in a natural membrane composed mainly of MGDG and DGDG. Since laurdan needs time to partition to the lipid phase, it was important to follow the labelling kinetics of the fluorescence emission. For this purpose laurdan incorporation into thylakoid membranes in violaxanthin-form was followed at 25 °C (Fig. 3). The fluorescence emission intensity increased during the first

Table 1
Fatty acid composition of plant galactolipids

| | Purity | Fatty acid composition | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 16:1 | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
| MGDG | 99 | t | t | t | 25 | | | 1 | 73 |
| DGDG | 94 | 8 | t | t | 5 | t | 2 | 3 | 81 |

Both MGDG and DGDG are dominated by the polyunsaturated fatty acid, linolenic acid (18:3). The DGDG used in samples contained 4.5% phosphatidylglycerol (PG). Almost half of the fatty acids of PG were also 18:3 (47%). Values of purity, given in percentage, are calculated from the amount of total fatty acids in the samples. Less than 1%=*t*.

10 min and after 20 min stable GP values were obtained. This result corresponds to earlier findings, where laurdan fluorescent intensity in wheat plasma membrane became stable after 25 min incubation at 20 °C [32]. For further experiments a 30-min incubation time was therefore chosen.

### 3.3. Effect of temperature on laurdan fluorescence in thylakoids

The fluorescence spectra of isolated spinach thylakoid membranes labelled with laurdan were compared at different temperatures (Fig. 4). As depicted in panel A, part of the emission spectrum of laurdan in thylakoids in the violaxanthin-form was about 50 nm red-shifted from 450 to 500 nm, when the temperature was increased from 0 °C to 37 °C. The same temperature increase in the membrane containing 63 mol% of XC-pigments as zeaxanthin (panel B) retained its fluorescence dominance at around 460 nm although with decreased maximum intensity. Thus, laurdan fluorescence clearly responds to the temperature-caused structural changes in the galactolipid-dominated thylakoid membrane.

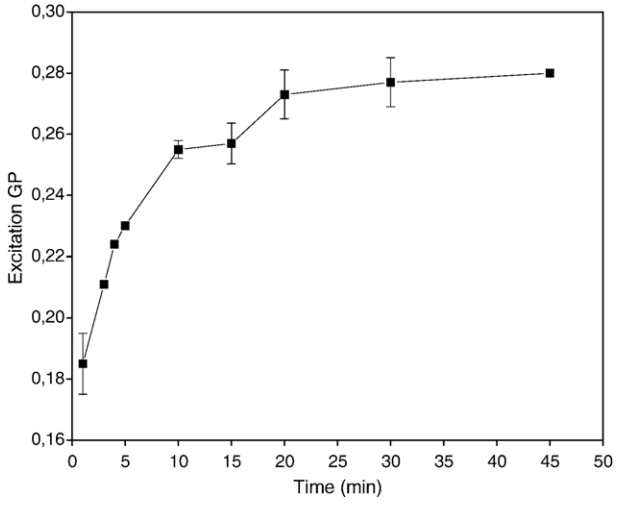


Fig. 3. Laurdan (0.8 μM) GP values measured in the thylakoid membrane as a function of time during labelling. Membranes at 0.02 mg chlorophyll $mL^{-1}$ concentration were incubated in a buffer containing 0.25 M sucrose, 10 mM KCl and 10 mM HEPES–KOH, pH 7.0, at 25 °C. Fluorescence intensity was continuously measured during 45 min. GP values were calculated from emissions at 460 and 516 nm using excitation at 390 nm.

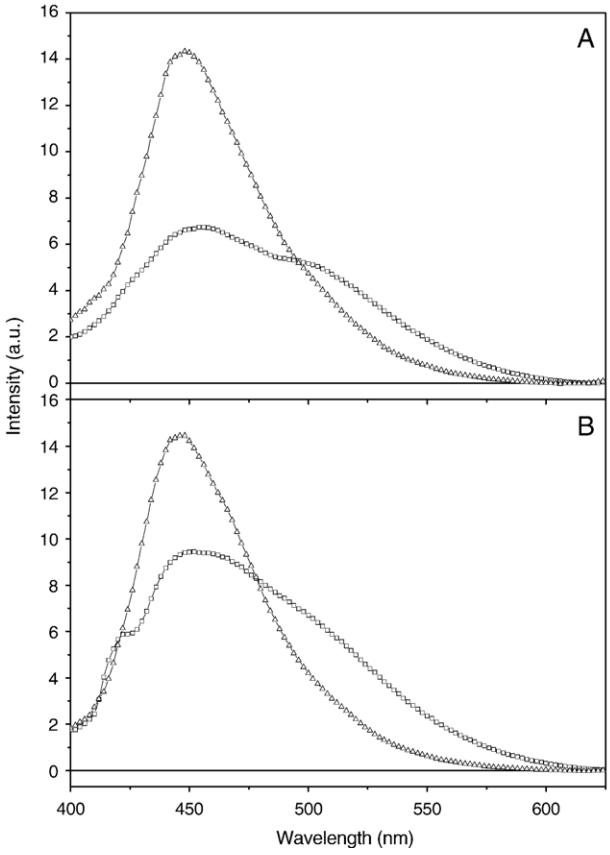


Fig. 4. Emission spectra of laurdan in thylakoids in the violaxanthin-form (A) and zeaxanthin-form (B) incubated for 30 min at 0 °C (Δ) and at 37 °C (□). Excitation wavelength was 390 nm, and the emission was monitored between 400 and 625 nm. The spectra were acquired using the FluoroMax-2 fluorometer, with 5-nm excitation and emission band passes, and continuous mild stirring of the samples. The cell holder was thermostated at 0 and 37 °C±0.1 °C using a circulating water bath.

### 3.4. Effect of different amounts of zeaxanthin in the thylakoid membrane

Since there was a difference, in terms of emission spectra, between the violaxanthin- and zeaxanthin-form of thylakoids at elevated temperature (Fig. 4) this raised the question whether the method could be sensitive enough to detect gradual changes in the amount of zeaxanthin in the membranes. Thylakoid membranes with 4, 15, 32, 52 and 63 mol% zeaxanthin were therefore produced (for experimental details see Violaxanthin to zeaxanthin conversion) and labelled with the fluorescent probe for 30 min at 25 °C. Fluorescence spectra of laurdan was recorded and the corresponding GP values were calculated (Eq. (1)), using emission at 460 and 516 nm. The GP values gradually increased with the amount of zeaxanthin in the membranes (Table 2). This strongly suggests that zeaxanthin makes the membrane structure more and more ordered or densely packed.

### 3.5. The combined effect of temperature and zeaxanthin

General polarisation (GP) was used to compare membranes in the violaxanthin- and zeaxanthin-form at different temperatures.

Table 2
The amount of zeaxanthin (Z) formed at 37 °C and GP values measured in the same thylakoid membrane at 25 °C

| V to Z conversion time at 37 °C (min) | mol% Zeaxanthin (Z/VAZ) | GP values (30 min at 25 °C) |
|---|---|---|
| 0 | 4.0±0.4 | 0.25±0.01 |
| 2 | 15.3±0.7 | 0.25±0.03 |
| 10 | 31.7±1.5 | 0.29±0.01 |
| 30 | 52.1±1.2 | 0.32±0.01 |
| 60 | 63.2±1.1 | 0.36±0.01 |

The enzyme reaction, converting violaxanthin to zeaxanthin, was started by the addition of 30 mM ascorbate to the thylakoid samples in 50 mM MES-NaOH buffer, pH 5.1. The de-epoxidation reaction was stopped after 2, 10, 30 and 60 min. Pigments were analysed by RP-HPLC and the amount of zeaxanthin was expressed as mol% of the total XC-pool pigments (Violaxanthin–Antheraxanthin–Zeaxanthin). The laurdan labelling of the membranes was carried out at 25 °C for 30 min. The GP values were calculated from emission at 460 and 516 nm using excitation at 390 nm. Data are average from three sets of experiments (±SE).

In Fig. 5, GP values for thylakoid membranes in violaxanthin- and zeaxanthin-form at 4, 25 and 37 °C is shown. Increasing the temperature from 4 °C to either 25 or 37 °C resulted in more fluid membranes, seen as lower GP values. However, regardless of the temperature applied, higher GP values were obtained for thylakoids in the zeaxanthin-form, reflecting a comparatively more ordered or densely packed membrane.

### 3.6. Effect of XC-pigments on the excitation GP in liposomes of galactolipids

The effect of XC-pigments was tested in the liposomal system resembling the galactolipid composition of the thylakoids. In Fig. 6, the more ordered DGDG emission spectrum (recorded at 10 °C) is compared with the spectrum of DGDG-MGDG

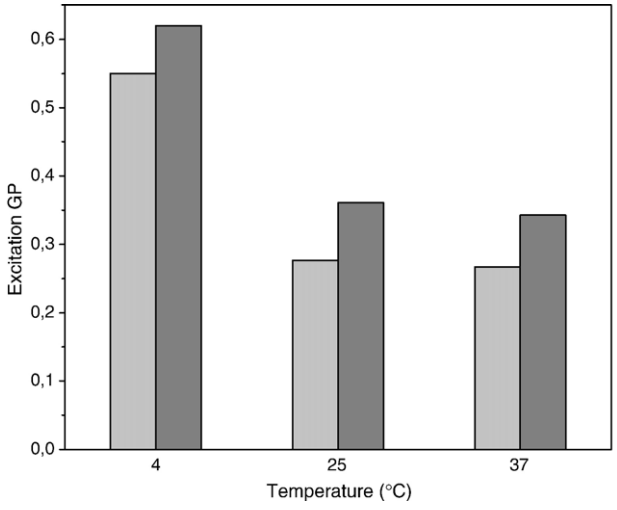


Fig. 5. GP values of laurdan-labelled thylakoid membranes at different temperatures containing either none (light grey bars) or 63 mol% zeaxanthin (dark grey bars). Membranes at 0.02 mg chlorophyll $mL^{-1}$ concentration were incubated with 0.68 μM laurdan for 30 min prior to fluorescent measurements. GP values were calculated from emission at 460 and 516 nm using excitation at 390 nm.

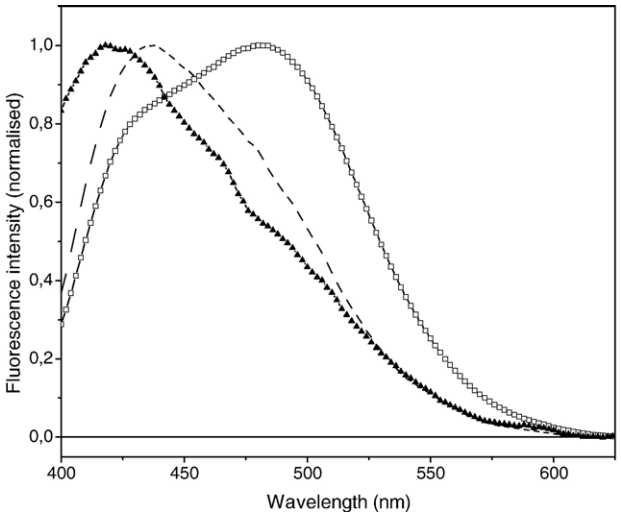


Fig. 6. Typical normalised emission spectra of laurdan-labelled DGDG : MGDG liposomes (70 : 30 mol%) at 37 °C (□), the same proportion of DGDG and MGDG mixed with 1.8 nmol zeaxanthin at 37 °C (▲) and pure DGDG-vesicles at 10 °C (dashed line). Excitation λ=340 nm. General polarisation (GP) has been calculated for the different lipid–pigment samples (see Table 3.).

liposomes at 37 °C in the absence and presence of zeaxanthin. DGDG-MGDG liposomal preparation without zeaxanthin showed a red-shifted fluorescent maximum revealing a fluid phase membrane. The same membrane system mixed with zeaxanthin revealed a one-peak-dominated emission spectrum at shorter wavelength, indicating a more ordered fluid phase membrane structure.

The ordering effect of zeaxanthin at 25 °C is also depicted in Table 3 in terms of the increased GP values. In Table 3, GP values in different proportions of DGDG-MGDG liposomes and the effect of XC-pigments and α-tocopherol are summarised. Vesicles of the two lamellar phase forming lipids, DPPC and DGDG, showed a large difference in their GP values. This is in agreement with the emission spectra shown in the insert of Fig. 2, DPPC with saturated acyl chains and in gel phase, formed a more tightly packed membrane structure than DGDG with unsaturated acyl chains, in liquid crystalline phase (Table 1). When DGDG, mixed with increasing proportions of

Table 3
Excitation GP values of laurdan in multilamellar liposomes made from DPPC, DGDG, and DGDG mixed with MGDG in the absence and presence of violaxanthin (1.8 nmol), zeaxanthin (1.8 nmol) and α-tocopherol (40 nmol)

| Model membranes (25 °C) | Addition | GP values | |
|---|---|---|---|
| DPPC | | 0.43 | 0.46 |
| DGDG | | 0.16 | 0.14 |
| DGDG : MGDG 95:5 (mol%) | | 0.16 | 0.15 |
| 85:15 (mol%) | | 0.06 | 0.06 |
| 70:30 (mol%) | None | 0.04 | 0.05 |
| | + Violaxanthin | 0.23 | 0.25 |
| | + Zeaxanthin | 0.27 | 0.26 |
| | + α-Tocopherol | 0.08 | 0.07 |

The GP values have been calculated from emission at 440 and 490 nm (Eq. (1)) using excitation at 340 nm, measured at 25 °C. For further details on the liposome preparation see Liposome preparation. Data are shown of two separate sets of experiments.

MGDG, the GP value decreased on average from 0.15 for pure DGDG to 0.15, 0.06 and 0.04 for the mixtures. This decrease reflects a less ordered membrane, with higher curvature stress, in the presence of MGDG. Nevertheless, increased GP values were acquired, 0.24, 0.27 and 0.08, when either violaxanthin, or zeaxanthin or α-tocopherol, respectively, was incorporated in the DGDG-MGDG liposomes (30 mol% MGDG). This indicates that both violaxanthin and zeaxanthin made the membrane more ordered, however violaxanthin was somewhat less efficient in this respect. The impact of α-tocopherol (40 nmol), dissolved in the hydrophobic part of the membrane, was smaller than that of the membrane-spanning xanthophylls (1.8 nmol).

## 4. Discussion

Fluorescence spectroscopy was used to characterise the membrane probe, laurdan, in galactolipid systems. In the literature, generalised polarisation (GP) has been reported to be an appropriate parameter reflecting membrane dynamics, i.e. membrane rigidity and fluidity [32,34,35,38]. After incorporating into natural or model membranes of phospholipids, laurdan molecules show a fluorescent red shift from 440 to 490 nm when solvent dipole relaxation takes place [27,28], e.g. when a phase transition from the tightly packed gel phase to the fluid phase of membrane phospholipids occur. The fluorescent red shift can be described by the calculation of GP values [28].

The results presented in this paper show that laurdan fluorescence spectroscopy is applicable in natural and model membranes made of galactolipids, i.e. thylakoid membrane and liposomes, respectively. The GP value for laurdan in thylakoid membranes decreased with temperature, but increased in response to zeaxanthin formation. The presence of xanthophyll cycle pigments or α-tocopherol had an ordering effect of lipids in liposomes of DGDG-MGDG, zeaxanthin being slightly more effective in that respect.

The effect of carotenoids in biological membranes has been extensively discussed in the literature [19,39,40]. Although opinions are divided regarding those carotenoids of the XC, as described in Introduction. Gruszecki et al. [39] showed by spin label EPR (electron paramagnetic resonance) studies that zeaxanthin and violaxanthin decrease membrane fluidity in the fluid phase of the phosphatidylcholines membranes. It was also concluded in the same study that carotenoids might modulate the physical properties of the natural membranes not containing cholesterol. The same authors have reported that the changes in the carotenoid pigments composition in the thylakoid membranes as an effect of the activity of the xanthophyll cycle result in distinct modification of the fluidity of these membranes [40]. Furthermore, Socaciu and co-workers [19] have found, using laurdan fluorescence spectroscopy, that zeaxanthin incorporation resulted in a more ordered structure of the phospholipid bilayer. Our results, with the laurdan fluorescence method, are in agreement with the above study by Socaciu and co-workers [19]. However we found, in addition, that zeaxanthin was more efficient than violaxanthin to increase the GP values (Fig. 5 and Table 3). Zeaxanthin incorporation, especially at 25 and 37°C, decreased the rate of dipolar solvent relaxation, revealing that the molecular mobility of water molecules decreased at the hydrophobic/hydrophilic interface of the laurdan-embedded membrane. The more pronounced effect of zeaxanthin could be explained by the increased hydrophobic length in the molecule as compared to violaxanthin. In zeaxanthin (Fig. 1), the distance between the oxygen atoms in the hydroxyl groups is 30.2 Å as measured by $^{1}$H-NMR [41]. In violaxanthin (Fig. 1), on the other hand, the distance between the epoxi-groups is expected to be 5–7 Å shorter. The increased hydrophobic length in zeaxanthin could allow the fatty acid chains of lipid molecules to stretch somewhat more leading to tighter packing [18] and release of curvature stress. The effect of α-tocopherol as a membrane-rigidifying agent is known [24]. α-Tocopherol, not spanning the membrane, exerts its rigidifying effect at higher concentrations than needed for zeaxanthin.

Temperature and lipid chain saturation is known to have a strong influence on the type of bilayer structure formed by membrane lipids [7,8]. DPPC with saturated dipalmitoyl chains assembles into a highly ordered lamellar gel phase structure up till 41.5 °C. When increasing the temperature above the main transition point, gel phase lipids melt to form a liquid crystalline phase. This change is seen as a red shift in laurdan fluorescence for phospholipids [27,28]. A fluid lamellar liquid crystalline phase structure is formed by the plant DGDG containing highly unsaturated fatty acids from temperatures well below 0 °C [7,36]. Although the emission spectrum of laurdan in pure DGDG liposomes at 10 °C resembles the spectrum of DPPC in gel phase (Fig. 2), DGDG forms a liquid crystalline phase at this temperature. The presence of MGDG, a lipid that can form inverted hexagonal phase ($H_{II}$) when mixed with water per se, induce a curvature stress in the vesicle, and a decrease of the GP-value. Increased temperature resulted in decreased GP values for the violaxanthin- and zeaxanthin-form of thylakoids (Fig. 5). The difference between the effect of violaxanthin and zeaxanthin was the same when the membrane was more ordered at 4 °C and less ordered at 25 and 37 °C, respectively. Although the GP values have only been used in a qualitative way here, we can compare the relative effect of changing temperature with that of changing the zeaxanthin content. This can give an indication of how efficiently zeaxanthin could compensate for an increase in temperature. As seen in Fig. 5, the sample containing zeaxanthin (63%) at 37 °C had a higher GP value (0.34) than membranes containing violaxanthin at 25 °C (0.28). This suggests that the zeaxanthin-containing membrane was more tightly packed than the violaxanthin-containing membrane, despite the 12 °C temperature-difference. Thus, zeaxanthin appears to have good capacity to reduce the curvature stress that develops at higher temperatures. At high light, plants may suffer from both over-excitation and temperature stress.

In conclusion, this is the first time when a galactolipid-dominated plant membrane is investigated by means of laurdan fluorescence spectroscopy. The results presented here further support the hypothesis that xanthophyll cycle pigments are located in the thylakoid membrane, influence membrane packing and that zeaxanthin provides protection not only through quenching of excess light energy but also by decreasing the curvature stress in the membrane.

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