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Galactolipid multibilayers modified with xanthophylls: orientational and diffractometric studies

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Oriented multibilayers of chloroplast galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) modified with violaxanthin and zeaxanthin were examined by X-ray diffractometry and linear dichroism. The results obtained suggest that zeaxanthin, in contrast to violaxanthin, has a significant ordering effect on galactolipid bilayers. The best ordered system consists of DGDG and zeaxanthin. In this case, the angle between the long axis of zeaxanthin molecule and the normal to the plane of bilayers amounts to 9° and system has a periodicity of 61.7 Å. The analogous angles in systems MGDG + violaxanthin, MGDG + zeaxanthin and DGDG + violaxanthin are clearly wider (35°, 17° and 28°, respectively) but diffractograms show no distinct maxima.

Introduction

Carotenoids are an important group of photosynthetic pigments [1]. It is generally accepted that their physiological function in chloroplasts is to serve as antennae absorbing light and transfering excitation energy to chlorophyll [2], and to protect the photosynthetic apparatus against a lethal photo-degradation [3]. Recently, a new biologically important action of carotenoid pigments with respect to lipid membranes has been postulated [4–6]. This mechanism consists in a reinforcement of a membrane and an ordering effect similar to that of cholesterol. Such an idea finds support from a spin-label study [6]: it appears that a small addition of carotenoids (3 mol%) drastically decreases the fluidity of a liquid crystalline state and blurs out phase transition on the model lipid membranes.

It is generally accepted that all photosynthetic pigments are bound *in situ* to proteins [7]; however, the pigments of the so-called xanthophyll cycle are supposed to be present as such within a lipid matrix of thylakoid membranes [8–10]. In particular zeaxanthin, the pigment synthesized by the xanthophyll cycle enzymes upon a strong illumination, is assumed to act directly within lipids since it is produced by de-epoxidation of violaxanthin at the inner surface of the thylakoid membrane and is continously turned into violaxanthin at its stroma surface.

In the present study, the modifying effect of the two main pigments of the xanthophyll cycle is examined as they are incorporated into multibilayers formed of the natural, plant galactolipids. A similar study performed previously using synthetic lecithin as the lipid component showed no essential difference between the effect of violaxanthin and zeaxanthin on the structural properties of a membrane [5]. The fact that the lipids used now are natural components of thylakoid membranes makes the present model study more valuable from the physiological point of view.

Materials and Methods

Zeaxanthin (β,β-carotene-3,3′-diol) was purchased from Hoffman La Roche (Basel). Violaxanthin (5,6,5′6′-diepoxyzeaxanthin) was extracted from fresh nettle leaves and purified on silica gel-covered plates Kieselgel 60 (Merck, Darmstadt). The solvent mixture: benzene/ethyl acetate/methanol (75:20:5, v/v/v) was applied as a developing phase [11]. Zeaxanthin was recrystalized before use. The purity of both pigments was tested according to the absorption maxima reported [1,11]. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were extracted from fresh nettle leaves with chloroform/methanol mixture (2:1, v/v), and separated by preparative TLC on silica gel plates as in the case of the pigments. The

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solvent system acetone/benzene/water (91 : 30 : 8, v/v/v) was used as a developing phase according to the procedure of Pohl et al. [12]. The concentration of MGDG and DGDG was evaluated by the galactose test as reported by Dubois et al. [13].

The concentration of the xanthophylls applied in the present work (10 mol%) was shown not to exceed the threshold values reported for the pigment miscibility within the lipid phase of dimyristoylphosphatidylcholine (about 17 mol% in the case of zeaxanthin and 11 mol% in the case of violaxanthin) [14].

Oriented multibilayers were prepared according to the method developed and tested previously [5]. The calculated concentration of a lipid solution and its volume deposited on a support enabled the estimation of the number of bilayers forming a single multilayer. The limiting area of a single MGDG and DGDG molecule used for calculations was taken from a monolayer study [15].

Linear dichroism measurements were performed as was described previously in detail [5]. The dichroic ratio r was calculated as the ratio of the relative absorbance (sample minus control) measured with a polarized light ($A_{\perp}/A_{\parallel}$) at 455 nm (22000 cm^{-1}).

Diffractometric measurements were carried out with a DRON 2D apparatus with the Fe X-ray radiation $\lambda = 1.9373$ Å.

Absorption spectra were registered with a Specord UV VIS spectrophotometer (Carl-Zeiss, Jena). All measurements were performed at 25°C ± 1.

Results and Discussion

Linear dichroism measurements

The amount of lipids corresponding to 100 single bilayers was deposited to a support for the linear dichroism measurements. This relatively small number as compared with the similar study performed previously with the xanthophyll-modified synthetic lecithin multibilayers [5] was chosen to minimize the risk of possible perturbations of the parallel orientation of all bilayers with respect to the support. Such perturbations should be taken into consideration with large numbers of layers because of the variety of lengths of the acyl chains as well as the variety of unsaturation indexes in the case of the natural galactolipids [16]. Additionally, the samples do not contain any excess of water which, forming a separate phase, could 'smooth out' the surface of a layer by minimalizing the free energy of interaction. The method of mulibilayer preparation employed in this work implies that only the water molecules tightly linked to polar groups of a hydrophilic head of each lipid molecule are present in the sample. On this basis, one can estimate the content of water in the layer system studied as not higher than 20% by weight.

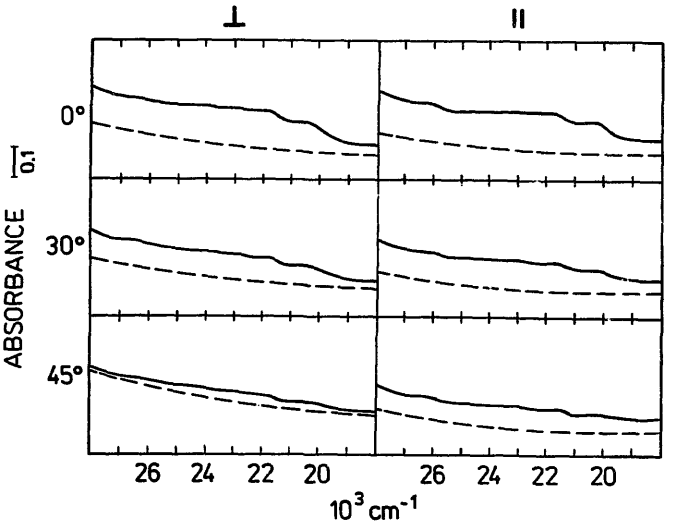

Fig. 1. Absorption spectra of multibilayers formed with DGDG (– – –) and DGDG multibilayers modified with violaxanthin (———). Symbols || and ⊥ refer to the light electric vector parallel and perpendicular, respectively, to the plane of incidence. The angle of incidence: 0°, 30° and 45° are indicated. The series of spectra presented in this figure refers to Table I, position DGDG+VIOL, No. 1.

The multibilayers of MGDG and DGDG modified with violaxanthin and zeaxanthin were examined by measuring the absorbance of polarized light. The absorbance of the samples registered in the region of the electronic transition of the pigments depends on the angle between a light beam and the axis normal to the plane of a layer as well as on the orientation of the electric vector of an exciting light with respect to the plane of incidence. Such a dependence is exemplified by the absorption spectra of violaxanthin modified DGDG multibilayers presented in Fig. 1. A different absorption of light polarized in two perpendicular directions by the samples containing carotenoid pigments was employed several times to determine their orientation [17–19]. The average angle ν of the transition dipole of carotenoid molecule and the axis normal to the surface of multibilayer can be calculated according to the theory developed and tested previously [5]. The value of this angle can be derived from the following formula (Eqn. 4 in Ref. 5):

$$\tan^2\nu = 0.0822(1 + r_0) \Big/ (r_0/r_2 + 0.3966 - 1.3145 r_0/r_1) \quad (1)$$

where r_0, r_1 and r_2 denote the dichroic ratios ($A_{\perp}/A_{\parallel}$) at the angles of incidence 0°, 30° and 45° respectively. The measured values of dichroic ratios and calculated angles ν are presented in Table I. Each preparation was duplicated and the last column of Table I gives the mean value of the angle ν for violaxanthin and zeaxanthin in MGDG or DGDG multibilayers. As may be seen from the data presented, both

TABLE I

Results of the measurements of linear dichroism of MGDG and DGDG multibilayers containing 10 mol% of violaxanthin (VIOL) or zeaxanthin (ZEA)

Sample	No.	$A_\perp / A_\parallel$			ν [a]	$\bar{\nu}$ [b]
		0°	30°	45°		
MGDG+VIOL	1	0.83	0.95	0.88	41.2°	35.4°
	2	1.53	2.60	1.50	29.6°	
MGDG+ZEA	1	0.86	1.17	0.25	13.0°	16.8°
	2	0.88	1.88	0.67	20.6°	
DGDG+VIOL	1	0.94	0.88	0.50	23.2°	28.0°
	2	1.10	1.00	0.75	32.8°	
DGDG+ZEA	1	0.57	0.86	0.11	9.5°	8.8°
	2	1.00	5.00	0.13	8.1°	

[a] The maximum experimental error in determining the angle of ν was estimated to be not higher than 4°.

[b] $\bar{\nu}$ stands for mean value.

carotenoids tend to be oriented rather narrowly with respect to the normal, similarly as in lecithin multibilayers [5]. In the case of MGDG the angle ν was, however, found to be wider than in the case of DGDG, for violaxanthin and zeaxanthin. The different structures of the lipid molecules in MGDG and DGDG imply a different proportion between the volume of the hydrophobic and hydrophilic parts, which could lead to different packing in the membrane; this is probably responsible for the different effects observed. Another finding concerns the evident difference of the orientation of zeaxanthin as compared to violaxanthin in all the samples examined (see Table I). Zeaxanthin possesses only two polar groups located at the opposite ends of the molecule (hydroxyls at the positions 3 and 3'). The distance between the two hydroxyls, 31.7 Å [20], seems to be large enough to span the hydrophobic core of the bilayer. It should be noted that the orientation of the xanthophyll pigment molecule in which the distance of its two opposite hydroxyls suits the thickness of a hydrophobic core could be defined in two different ways. The axis connecting carbon atoms at the position 3 and 3' to which the polar groups are bonded was estimated to form an angle with the chromophore (approximately axis 6–6', see Fig. 2) equal to about 11° [5]. It means that a parallel orientation of the axis 3–3', with respect to the normal to the plane of bilayer corresponds to the angle of a chromophore equal to approximately 11°. The orientation of a chromophore can be obtained from linear dichroism measurements and the value of the angle ν found for zeaxanthin in the present study (Table I) is close to the predicted one.

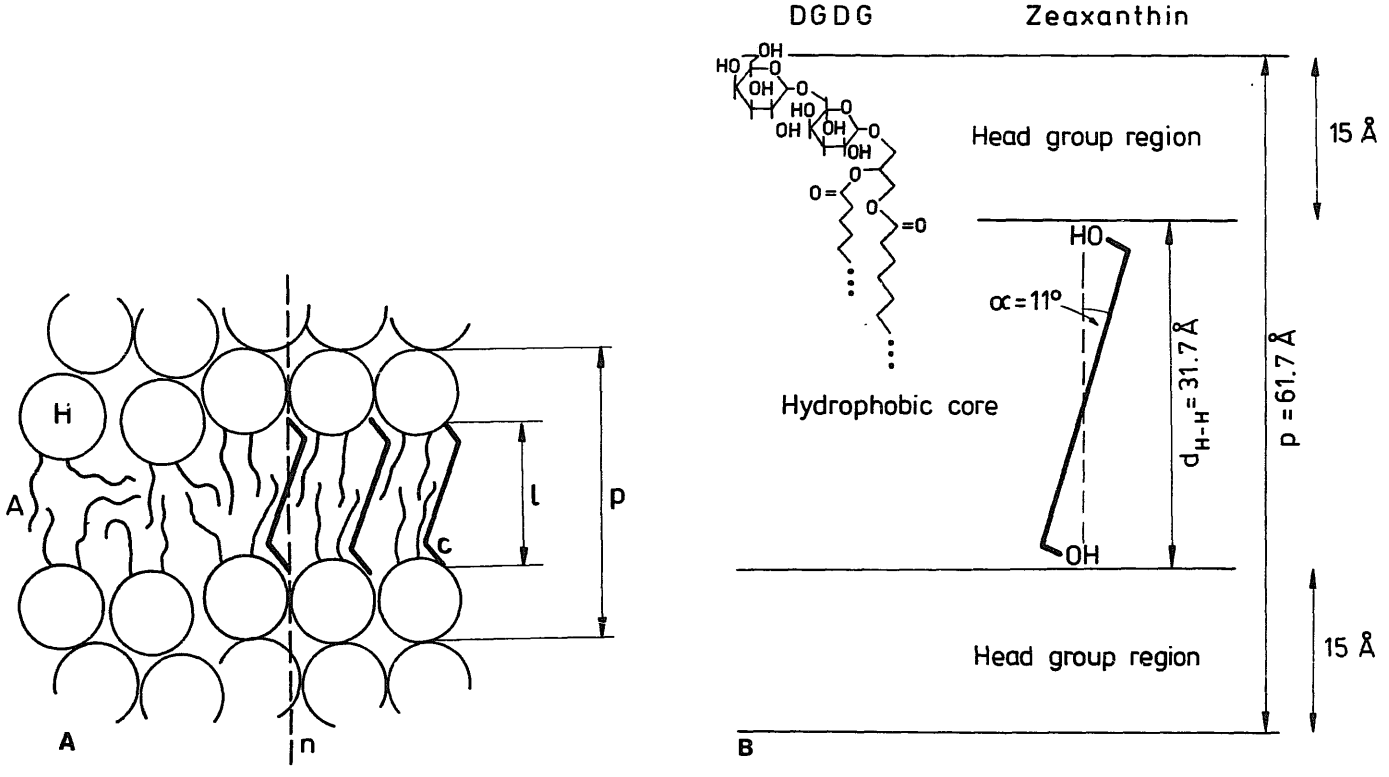

Fig. 2. (A) Schematic representation of a single bilayer from multibilayer modified with carotenoids. c, Carotenoid molecule as a broken line connecting carbon atoms at positions 3-6-6'-3' in the projection of β-carotene molecule on the crystallographic plane (1, 0, 0) [28]; H, polar head of a lipid molecule; A, acyl chain; *l*, thickness of a hydrophobic core [5]; *p*, periodicity of a multibilayer. Dashed line, axis normal to the plane of a layer. (B) Schematic reprezentation of DGDG bilayer containing zeaxanthin. The values of the multilayer periodicity (*p*) and the distance between the peripheral hydrogen atoms in OH groups of zeaxanthin (d_{H-H}, [20]) are indicated. For more explanations see text.

Violaxanthin has two additional polar groups, 5–6 and 5′–6′-epoxides. If the distance between two epoxides of violaxanthin is not large enough to span the hydrophobic core, at least two possible pictures of action of the pigment with respect to a membrane could be postulated. The first one is a decrease in the thickness of a bilayer in order to push out both polar groups from a hydrophobic region. The second one is the effect of the disintegration of the surface region of hydrophobic core by introducing the polar oxygen atoms of epoxides. Perturbations of the interface between the polar and unpolar part of the membrane should affect obviously the membrane structure and increase the permeability towards small polar molecules, like water. One can predict, additionaly, a third possibility consisting in the combination of both mechanisms discussed above. In any case, we can expect that the galactolipid multibilayer containing violaxanthin will be a poorly ordered system. This lack of the order is most probably responsible for the large differences between the values of the angle ν obtained in different experiments with violaxanthin.

Diffractometric measurements

The samples composed of a number of 100 bilayers, when subjected to a spectrophotometrical measurements appeared not to give clear diffractometric data. This is related to the lack in the samples examined of any heavy atoms, which could be 'visible' by the method. We attempted to obtain a diffractogram from samples prepared by the deposition of an amount of lipid corresponding to 400 bilayers, neglecting the above-discussed risk of imperfections in the ideal order along the axis normal to the support since it is not as important for the diffractometric method. The original traces from these diffractometric measurements are presented in Fig. 3. The range of scanning starting at a position $2\theta = 3°$ does not make it possible to follow a periodicity in the system when it is larger than 37 Å, considering the first-order diffractometric maxima and with the wave length of the radiation used in the present work. A simple estimation of the periodicity of multibilayers formed with MGDG and DGDG indicates that this value should be expected to be much higher than that the limit calculated above. In the small-angle region of the traces presented in Fig. 3, one may find some small peaks being possibly related to higher order maxima representing the multibilayer periodicity. However, only in one case (plot No. 6) a maximum appearing at 5.4° is sufficiently evident to be assigned as a diffractometric peak since it is reproducible in all measured samples of the same composition. Considering that this is probably a third-order diffractometric maximum, it corresponds to a periodicity value equal to 61.69 Å. This parameter should be assigned to the periodicity of a multibilayer along the

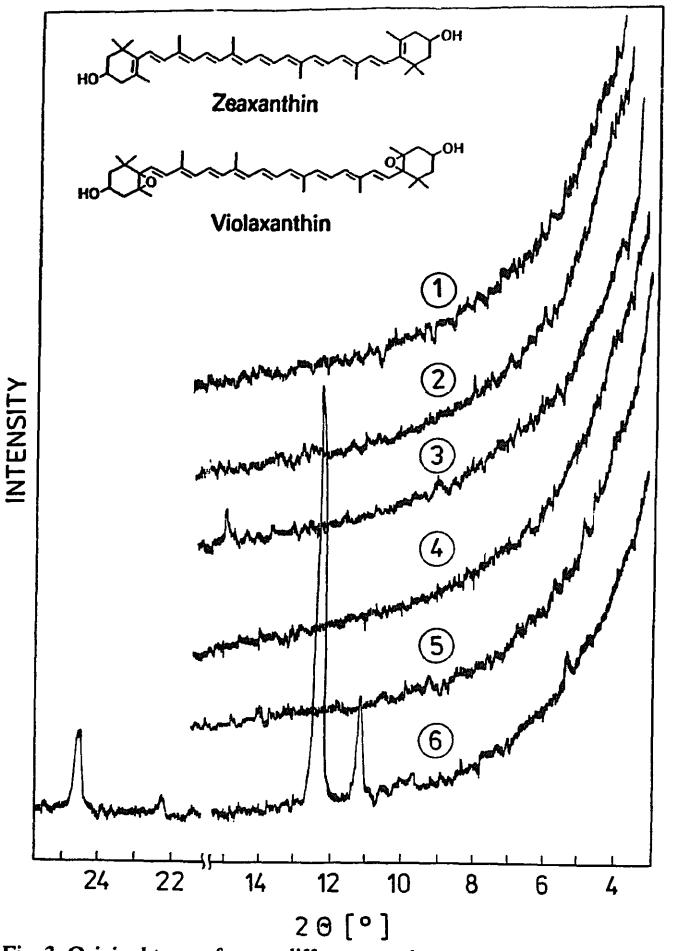

Fig. 3. Original traces from a diffractometric measurements of multibilayers of the following composition: 1, MGDG; 2, MGDG + VIOL; 3, MGDG + ZEA; 4, DGDG; 5, DGDG + VIOL; 6, DGDG + ZEA. + VIOL denotes modification with violaxanthin and + ZEA modification with zeaxanthin. The time constant of the scan 5 s. Inset, the formulas of examined xanthophylls.

normal to support (see parameter p, Fig. 2) as it corresponds to the thickness of a single bilayer. The almost normal orientation of zeaxanthin in this case, determined by linear dichroism measurements (Table I), indicates that the thickness of a hydrophobic core in such a bilayer equals the distance between two opposite-side polar groups, $l = 31.7$ Å (see parameter l, Fig. 2). In contrast to the small maxima considered above appearing in a small-angle region of the diffractogram very clear peaks are found in a wide-angle region (Fig. 3). These maxima correspond to an acyl chain subcell packing but appeared only in the diffractograms of multibilayers containing zeaxanthin (plots No. 3 and 6, Fig. 3). All the measured samples containing no carotenoids, or containing violaxanthin, do not demonstrate in the packing of acyl chains any order which could be registered by a diffractometric method. The position of the wide-angle maxima in a diffractogram from a zeaxanthin-modified multibilayer depends, however, on the lipid component. The first-order maxima are found at 14.875° and 14.650° in the case of

MGDG, and at 11.139° and 12.175° in the case of DGDG. In the latter case the diffractometric peaks are relatively high, so the second-order maxima are also detectable at 22.386° and at 24.500°. To determine accurate values of the parameters of hydrocarbon chain subcell packing structure, the wide-angle region of the diffractometric maxima was scanned with a twice larger time constant and registered on a 4-fold larger scale. Some results are presented in Fig. 4. Fig. 5 presents a schematic representation of the structure of an acyl chains subcell packing. The value of parameters *a* and *b* (see Fig. 5) determined on the basis of a position of diffractometric maxima according to Bragg's law are $a = 7.48$ Å and $b = 7.60$ Å for MGDG and $a = 9.13$ Å and $b = 9.98$ Å for DGDG. The area of the membrane occupied by one molecule, calculated according to the model presented in Fig. 5, is 65.6 Å^2 in the case of MGDG and 105.2 Å^2 in the case of DGDG. These values correspond well to the areas occupied by MGDG and DGDG at an air/water interface during compression [15]. The analysis of the width of the diffractometric peaks (Fig. 4A), based on the Scherrer formula [21], made it possible to estimate the mean diameter of the crystallites giving rise to such maxima not to be larger than 600 Å. This gives a number of about 60 DGDG molecules along the diameter of one identically oriented and well-ordered 'patch' in a single zeaxanthin-modified bilayer. Analyzing the intensity of the X-ray

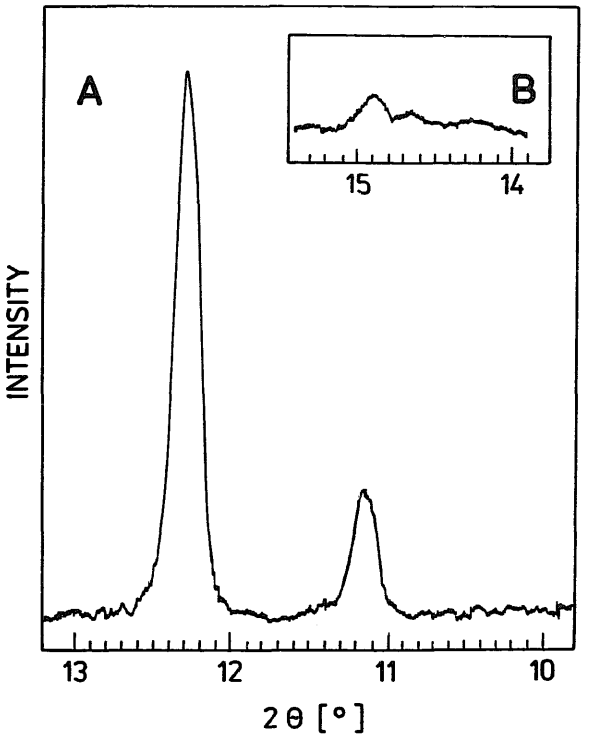

Fig. 4. Original traces from a diffractometric measurements of DGDG (A) and MGDG (B) multibilayers modified with zeaxanthin. The time constant of the scan 10 s.

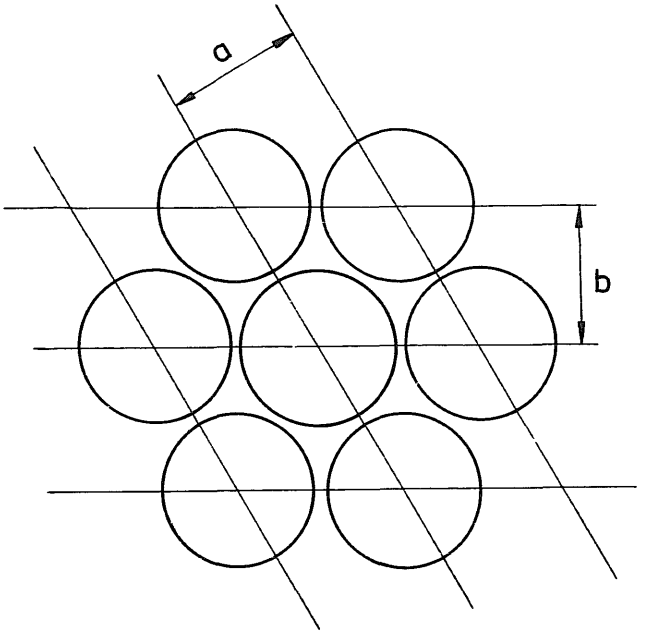

Fig. 5. Schematic representation of the hydrocarbon chain subcell packing of galactolipids in zeaxanthin-modified multibilayer. Parameters *a* and *b* refer to the Bragg's spacings related to the wide-angle maxima (see Fig. 4) and equal to 7.48 Å and 7.60 Å in the case of MGDG and 9.13 Å and 9.98 Å in the case of DGDG.

diffraction maxima obtained for zeaxanthin-containing MGDG and DGDG multilayers one may conclude that the ordering effect is much more evident in the latter case. This situation is probably related with the ability of DGDG to spontaneously form well-ordered lamellar structures in a water medium in contrast to MGDG, postulated to be organized as tube-like hexagonal structures in similar conditions [22]. In the present study, the tendency of spontaneous organization is highly limited by the geometry of support (glass slide), the procedure of lipid hydration and the absence of water as a free fraction. Considering, however, the effect of zeaxanthin, it seems that it is easier to establish order within the fluid region of DGDG layer than between cone-like MGDG molecules.

In previous studies [5] concerning the effect of violaxanthin and zeaxanthin on structural properties of synthetic lecithin membranes, we have found small differences between the pigments examined. The present investigations show that such differences are much more pronounced in membranes formed with the natural, chloroplast galactolipids. The two xanthophyll pigments, as long-chain, rigid molecules, are oriented differently in a membrane. Violaxanthin forms an angle wider by about 20° with respect to the normal of a layer than zeaxanthin. Taking into account the angle between the chromophore and the axis connecting the opposite hydroxyl groups, the orientation of zeaxanthin determined by linear dichroism leads to the conclusion that the pigment spans the hydrophobic core with its

polar groups lying on the axis normal to the plane of a membrane. Such an orientation was demonstrated by the diffractometric findings to affect strongly the structure of the hydrophobic core of the bilayer. The effect of ordering of the acyl chains by zeaxanthin found in the present study was also reported as a conclusion from a model study using the EPR spin-label technique [6] and observing the mechanical properties of liposomes [23,24]. The difference of the effect of violaxanthin and zeaxanthin as membrane-modifying agents sheds a new light on the results of the study concerning the physiological meaning of the xanthophyll cycle. Blocking the accumulation of zeaxanthin in chloroplasts by the inhibition of violaxanthin de-epoxidation results in an increase of fluidity of thylakoid membranes monitored by an increase of the motion of a stearic acid spin-label (in preparation). The accumulation of zeaxanthin within thylakoid membranes was also correlated with the photoacoustically monitored level of photosynthetic oxygen evolution [25]. This finding was explained in terms of the dependence of thylakoid membrane fluidity on the presence of zeaxanthin. A decreased fluidity was postulated to affect the migration of the moving elements of the photosynthetic electron transport chain.

All the results presented above, including the finding of the present study, suggest that the modifying effect of zeaxanthin, to decrease the membrane fluidity by ordering of the hydrocarbon acyl chains, may be considered as one of the crucial mechanisms of the xanthophyll cycle. The membrane fluidity can obviously regulate all physiological processes like diffusion or enzymatic activity [26]. Limiting the penetration of oxygen and free radicals within a highly unsaturated lipid core may be understood as a protective mechanism against lethal degradation of the structural elements of a membrane. Such an effect, in addition to the well known efficiency of carotenoids as photoprotectors [3], seems to be physiologically important since the accumulation of zeaxanthin is related to light intensities exceeding those optimal for photosynthesis [8–10]. Illumination of chloroplasts with such a strong light is known to result in chlorophyll triplet formation, photosensitization and photodestruction [27].

Acknowledgements

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