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Orientation of xanthophylls in phosphatidylcholine multibilayers

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Oriented multibilayers of dimyristoyl phosphatidylcholine (DMPC) modified with violaxanthin or zeaxanthin were examined by X-ray diffractometry and linear dichroism. It appears that pigment molecules and the normal to the bilayer plane form an angle of 24–25°. It was also observed that rather small concentrations of added xanthophylls (molar fraction up to 3%) increase the pigmented bilayer thickness by a value of about 2 Å as compared with that of the pure DMPC bilayer. The observed nonzero linear dichroism at normal incidence of light suggests the possibility of nonhomogeneous orientation of transition dipoles in the plane of the bilayer.

Introduction

The influence of carotenoids on the structure and properties of artificial lipid membranes has already been examined many times [1–4]. The fact that thylakoid carotenoids are usually incorporated into pigment-protein complexes [5] obviously restricts the significance of such a kind of investigations as a modelling of in vivo photosynthetic apparatus. However, there are a few carotenoids whose presénce in the membrane lipid matrix seems to be implicated by their participation in the transmembrane xanthophyll cycle [6,7].

The cycle consists of two two-step reactions: de-epoxidation of violaxanthin to zeaxanthin and epoxidation of zeaxanthin to violaxanthin. The reactions are catalyzed by two different enzymes and take place at opposite surfaces of thylakoid membrane [6,7]. So, the both mentioned xanthophylls must migrate across the membrane. On the other hand, violaxanthin, as the most polar carotenoid in photosynthetic membranes, is weakly attached to protein-pigment complexes [5]. Moreover, the de-epoxidase of violaxanthin is active in vitro only in the presence of thylakoid lipids, from which monogalactosyldiacylglycerol is the most effective [8]. Thus, it is highly probable that, in contrast to β-carotene and lutein, violaxanthin and zeaxanthin are present in the membrane lipid matrix. For the physiological role of the cycle, it is relevant that different from zero steady-state concentrations of zeaxanthin occur only under illumination saturating photosynthesis, when the interior of thylakoids is acidified. Experiments with photobleaching of chlorophyll a in lecithin liposomes in the presence of violaxanthin or zeaxanthin [9] showed that at pH 7.5 violaxanthin is more effective as a photoprotector than zeaxanthin. At pH 5.0, zeaxanthin is a more effective photoprotector than violaxanthin. These facts suggested that photoprotection of the photosynthetic apparatus could be a good proposal for the unknown physiological role of the xanthophyll cycle. Both xanthophylls have sufficiently long systems of conjugated bonds to be effective as photoprotectors. The differences in their photoprotective action in liposomes and, likely, in thylakoids must originate from differences in their interactions with membranes. This is why it is important to understand how zeaxanthin and violaxanthin interact with lipid membranes.

This paper belongs to the series of works devoted to investigations of two main pigments of the xanthophyll cycle in lipid membranes. It considers the orientation of molecules of the two xanthophylls in lipid bilayers. Most of the investigations of carotenoid orientation in lipid membranes dealt with β-carotene [1–4]. As a rule, these investigations led to the conclusion that β-carotene molecules are oriented rather parallely than perpendicularly to the plane of the membrane. However, Van de Ven et al. [1] concluded that the orientation of β-carotene molecules in a lipid bilayer depends on the kind of lipid. According to them, the angle between the long axis of β-carotene molecule and the normal to the plane of bilayer was clearly narrower in DMPC and soybean phosphatidylcholine, slightly narrower in di-

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galactosyldiacylglycerol, slightly wider in egg phosphatidylcholine and distinctly wider in dioleoylphosphatidylcholine than the magic angle 54.74°, which is for dichroic measurements equivalent to homogeneous distribution. We will mention the papers concerning xanthophylls in lipid multilayers in the section Results and Discussion.

Materials and Methods

L-β-γ-dimyristoyl-α-phosphatidylcholine (DMPC) was obtained from Fluka. The xanthophyll pigments: zeaxanthin (3,3′-diol-β-β-carotene) and violaxanthin (5,6,5′,6′-diepoxyzeaxanthin) were extracted from fresh nettle leaves and purified chromatographically on Kieselgel plates (Merck) with solvent system benzene/ethyl acetate/methanol (75 : 20 : 5, v/v) [10].

Oriented multilayers were prepared in the following way. A lipid-pigment solution in chloroform was evaporated on the glass plate. The calculated solution concentration and volume was used in order to obtain 300 or 3000 oriented bilayers. The plates with adsorbed lipid-pigment layers were kept overnight under the pressure reduced to 10^{-2} mbar in order to remove any solvent residues. Afterwards the samples were incubated in darkness for a period of three days at 18°C under hydrated nitrogen. Several temperature changes through DMPC phase transition point were done during incubation in order to equilibrate the samples.

Diffractometric measurements were carried out with the DRON 2.0 diffractometer (U.S.S.R.) with Cu Kα-radiation ($\lambda = 1.54$ Å). Spectrophotometrical measurements were carried out at 30°C with the Specord UV-VIS spectrophotometer (Carl Zeiss, Jena) equipped with polaroids. All spectrophotometrical measurements of the pigmented multilayers were followed by the measurements of the pure lipid multilayers of the same thickness at the identical experimental conditions. The difference between both the obtained absorbances measured at 441 nm for violaxanthin and at 445 nm for zeaxanthin was used in calculations of the dichroic ratios.

Results and Discussion

1. Diffractometric measurements

Fig. 1 presents a typical diffractogram of oriented multilayers of pure DMPC, where distinct peaks can be seen starting from the second order maximum. The most intensive peak of the fourth order had been registrated on a 10-fold bigger abscissa scale than that shown in Fig. 1 and was used in calculations of the periodicity for all investigated multilayers. In Fig. 2 the periodicity of DMPC multilayers is shown as a function

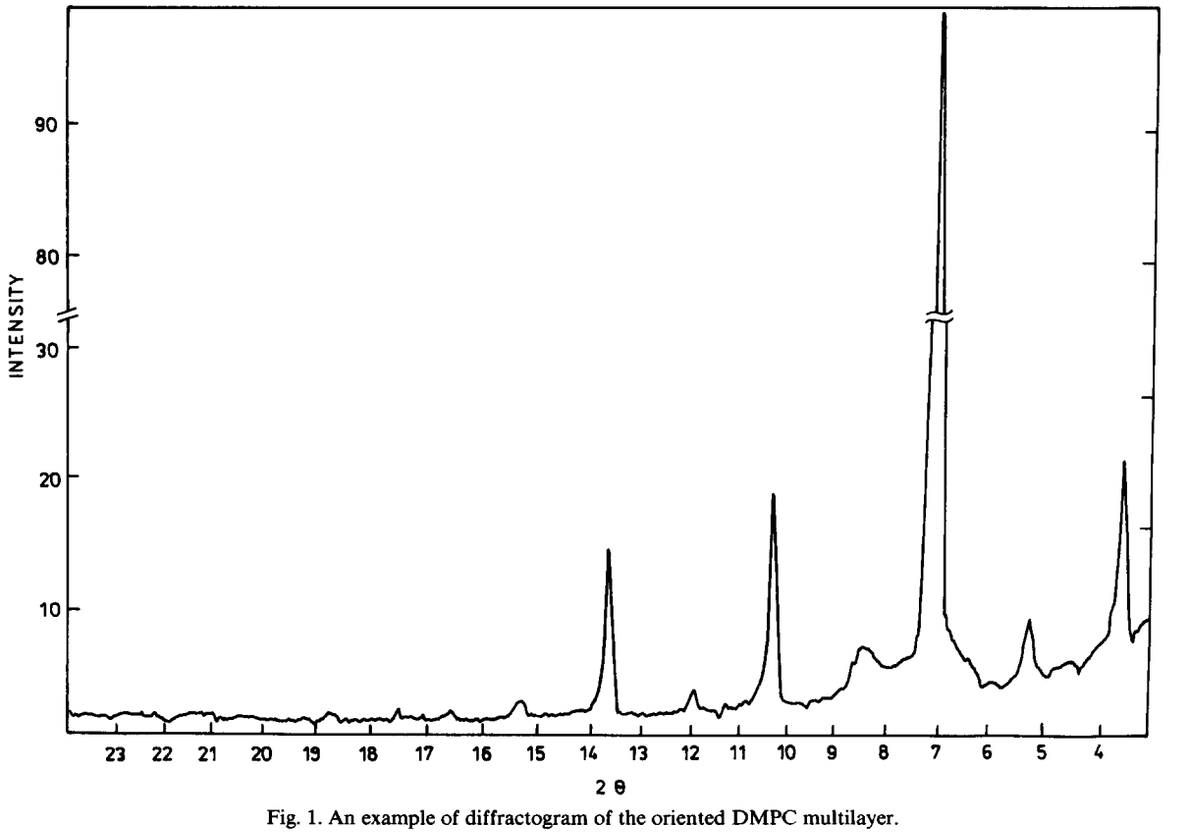

Fig. 1. An example of diffractogram of the oriented DMPC multilayer.

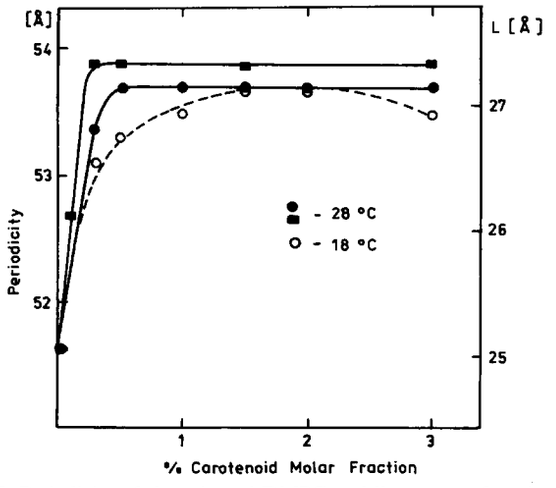

Fig. 2. Periodicity of the oriented DMPC multilayers as a function of molar fraction of the added xanthophyll; ■, zeaxanthin; ●, ○, violaxanthin; L is the calculated thickness of the hydrophobic core of bilayer.

of carotenoid molar fraction. As can be seen in Fig. 2, addition of violaxanthin or zeaxanthin to the DMPC increases the value of periodicity. According to Milon et al. [11], zeaxanthin is located in the hydrophobic core of lipid bilayer. The same is true for violaxanthin. Fig. 3 presents the correlation between the maximum absorption position of violaxanthin and the refraction index of the solvent. The point corresponding to the absorption maximum for violaxanthin in DMPC liposomes, marked by arrows, lies exactly on the correlation straight line obtained for the six solvents. So, we can conclude that violaxanthin is located in the lipid phase and rests in a monomeric form. Consequently the increase of periodicity caused by the addition of violaxanthin or zeaxanthin (Fig. 2) should be interpreted as an increase of the thickness of hydrophobic portion of the DMPC bilayers. The comparison of our values of periodicity with

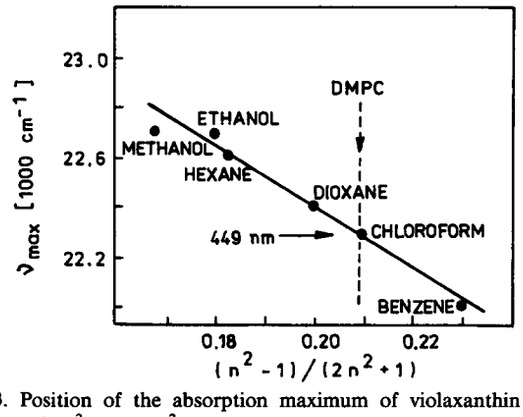

Fig. 3. Position of the absorption maximum of violaxanthin as a function of $(n^2-1)/(2n^2+1)$, where n is the refraction index of the solvent. For DMPC $n=1.445$ [14] was accepted. Absorption maximum of violaxanthin in DMPC liposomes is placed at 449 nm (22 272 cm^{-1}).

the data of Janiak [12] suggests that the examined multilayers contained about 20 weight per cent of water. Such a quantity of water is closely associated with polar heads of DMPC [12]. The thickness of hydrophobic core of a single bilayer in this type of pure DMPC multilayer has been evaluated to be 25.39 Å (Appendix I). On the other hand, the distance between two hydroxyl groups in positions 3 and 3′ is equal to 30.2 Å [11]. These two values suggest that xanthophyll molecules should be inclined in respect to the normal to the bilayer, whereas increase of the periodicity in the presence of xanthophylls demonstrates their tendency to be oriented more close to the normal. Our discussion is illustrated by Fig. 4. In this figure, carotenoid molecules are represented by broken lines containing carbon atoms 3-6-6′-3′ in the projection of β-carotene molecule on the crystallographic plane (1,0,0) [13]. If we assume that the direction of transition dipole of the pigment molecule is parallel to the axis 6-6′, then the straight line joining hydroxyls (3-3′) and the dipole transition moment form an angle β of about 11°. Diffractometric studies do not give unequivocally the orientation of pigment molecules in the lipid bilayer. The matching of the hydrophobic core thickness and the distance between polar groups of xanthophyll is consistent with many orientations of the transition dipole. But we can calculate the angle α between the axis 3-3′ and the normal to the bilayer plane. This angle equals 24.91° for violaxanthin and 24.0° for zeaxanthin. The transition dipole can have any orientation on the cone around the axis 3-3′. The two extreme orientations of those possible are shown in Fig. 4. The angle between the transition dipole and the normal to the bilayer plane is equal to $\alpha-\beta$ in the case a and $\alpha+\beta$ in the case b. Up to now we have not considered the influence of epoxy-groups of violaxanthin on its orientation in the lipid bilayer, but it is clear that their presence should prefer orientations closer rather to the case a than to the case b.

2. Linear dichroism measurements

Linear dichroism measurements do not give full information about the orientation of pigment molecules. In order to determine the orientation of the transition dipoles it is necessary to use experimental data and some additional assumptions. In the case of samples like our multilayers, it is usually presumed that the normal to the plane of layers (axis z in Fig. 5) is an axis of symmetry and transition dipole moments form with this axis the constant angle ν. Such an assumption leads to true mean value of $\langle\cos^2\nu\rangle$ but it does not mean that all of dipoles form this angle with z-axis. As a rule it is also accepted that all directions in the plane of layer (x, y) are equivalent. We could not accept such an assumption, since we observed non-zero linear dichroism at normal incidence of light.

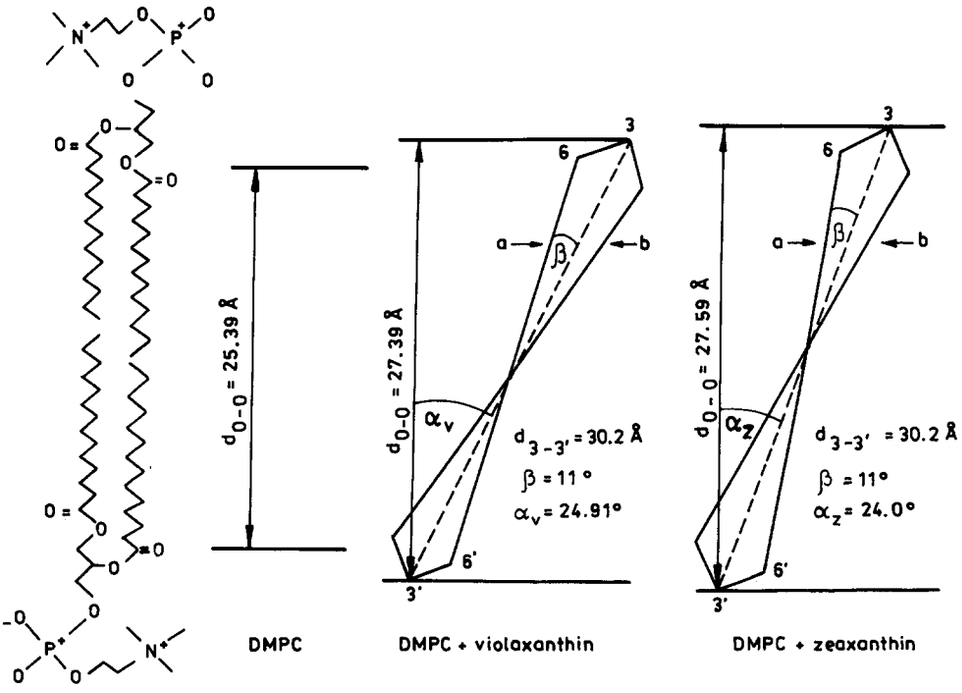

Fig. 4. Illustration of the discussion of carotenoid molecules orientation in DMPC bilayer. See the text for explanations.

The dichroic ratio r can be expressed in the following way:

$$r = \frac{A_\perp}{A_\parallel} = \frac{\tan^2 \nu \langle \sin^2\phi \rangle}{\tan^2 \nu \langle \cos^2\varphi \rangle \cos^2\alpha + \tan \nu \langle \cos \phi \rangle \sin 2\alpha + \sin^2\alpha}, \quad (1)$$

where A is absorbance of the sample, indices $\perp$ and $\parallel$ refer to the beams with the electric vector, respectively, perpendicular and parallel to the plane of incidence, ϕ is the angle between the projection of transition dipole on the plane x, y and the axis x, α is the refraction angle, brackets $\langle\ \rangle$ denote average over all absorbing molecules. The derivation of Eqn. 1 is given in Appendix II. In the case of the homogeneous distribution of transition dipoles in respect to the angle ϕ, $\langle \cos^2\phi \rangle = \langle \sin^2\phi \rangle = 0.5$, $\langle \cos \phi \rangle = 0$ and for $\alpha = 0$ we have $A_\perp = A_\parallel$.

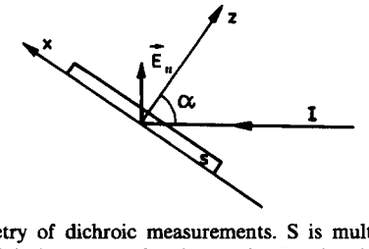

Fig. 5. Geometry of dichroic measurements. S is multilayer on the glass plate; I, light beam; α, refraction angle; $E_\parallel$, electric vector of the exciting light parallel to the plane of incidence. Z-axis is the normal to the plane of sample; X-axis is the intersection of the plane of the sample with the plane of incidence; Y-axis is in the plane of the sample and is perpendicular to the plane of incidence.

We have determined the dichroic ratio for the angles of incidence of 0, 30, and 45°. As the Eqn. 1 predicts, all of these dichroic ratios will be changed if the sample is rotated around the z-axis (see Fig. 5). In Appendix II there is shown that the calculated value of the angle ν is not affected by such rotation. So, we did not choose any special orientation of the sample in the x, y-plane. With the refraction index $n = 1.445$ [11], we obtain for the refraction angle the values of 0, 20.24, and 29.29°, respectively. The insertion of these values into Eqn. 1 gives formulas for dichroic ratios r_0, r_1, and r_2 at the angles of incidence 0, 30, and 45° (see Appendix II), from where it can be inferred that

$$\langle \cos^2\phi \rangle = \frac{1}{1 + r_0} \quad (2)$$

$$\langle \cos \phi \rangle = \frac{(r_0 - 0.8803 r_1) \tan^2\nu - 0.1197 r_1 (1 + r_0)}{0.6493 r_1 (1 + r_0) \tan \nu} \quad (3)$$

$$\tan^2\nu = \frac{0.0822(1 + r_0)}{\frac{r_0}{r_2} + 0.3966 - 1.3145 \frac{r_0}{r_1}} \quad (4)$$

Fig. 6 presents an exemplary series of the absorption spectra of pure phosphatidylcholine multilayers and those containing carotenoid. The spectra in Fig. 6 refer to the item 5 in Table I. In the same table the experimental values of dichroic ratios and the calculated values of $\langle \cos^2\phi \rangle$, $\langle \cos \phi \rangle$, and ν are shown. The values of the angle ν have been determined with an error not

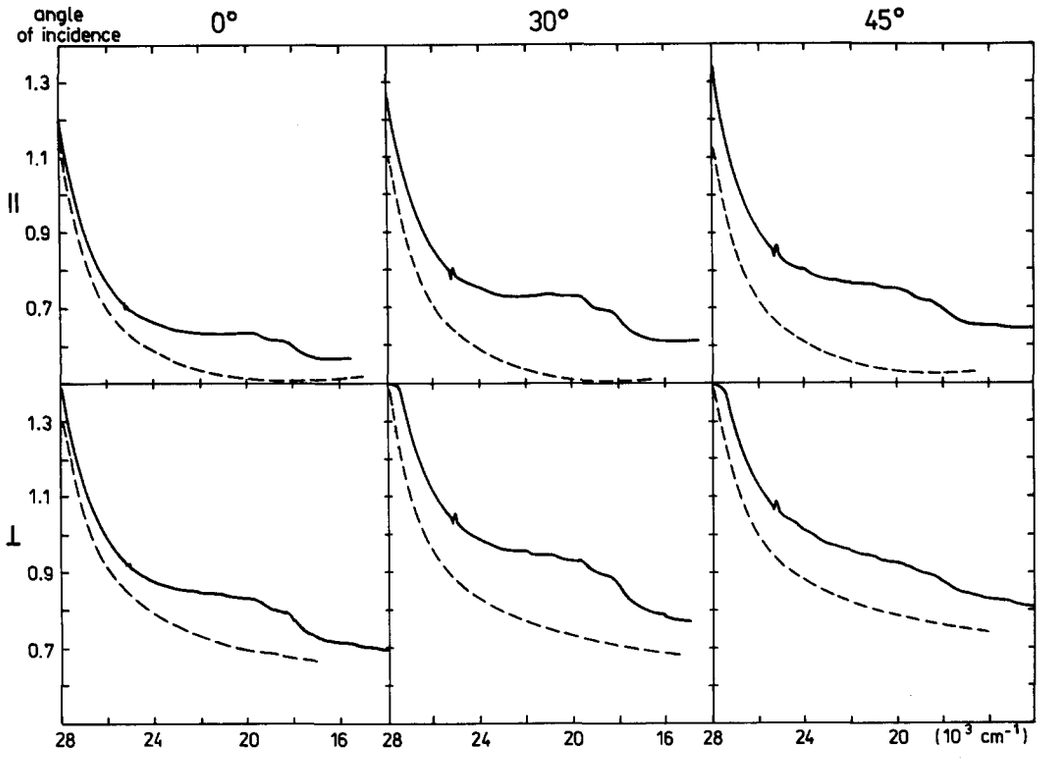

Fig. 6. Absorption spectra of multilayers containing phosphatidylcholine (— — —) and phosphatidylcholine with zeaxanthin (———). The symbols || and ⊥ refer to the light electric vector parallel and perpendicular, respectively, to the plane of incidence.

bigger than 3–4°. Therefore, the values of the angle between xanthophyll molecules and the normal to the plane of bilayer obtained from dichroic measurements (20–27°) remain in good agreement with those expected from the diffractometric experiments (24–25 ± 11°) (see Fig. 4). It is easy to find the distribution of the transition dipoles in respect to the angle ϕ, which gives the mean values of $\langle\cos^2\phi\rangle$ and $\langle\cos\phi\rangle$ cited in Table I. For example, such distribution can be represented by

$$f(\phi)\,d\phi = \frac{1}{\pi}(0.5 + a_1\cos\phi + a_2\cos 2\phi)\,d\phi \qquad (5)$$

where $a_1 = \langle\cos\phi\rangle$ and $a_2 = 2\langle\cos^2\phi\rangle - 1$.

Of course, we cannot assert that the function (5) describes the real distribution of transition dipoles in examined samples. It is a distribution which is the closest to the homogeneous one and gives expected values of $\langle\cos\phi\rangle$ and $\langle\cos^2\phi\rangle$ and is therefore consistent with experimental values of the dichroic ratios. In principle, any function with period 2π can be expanded in Fourier series. The function (5) contains only three first terms of the even part of the series. In general, such truncated series could become negative for some values of ϕ, which would be inconsistent with the physical meaning of the distribution function. However, with the values of $\langle\cos\phi\rangle$ and $\langle\cos^2\phi\rangle$ shown in Table I, the function $f(\phi)$ is positive for all ϕ, and in this

TABLE I

Results of the measurements of linear dichroism of DMPC multilayers containing violaxanthin (V) or zeaxanthin (Z).

No.	Pigment	r_0	r_1	r_2	$\langle\cos^2\phi\rangle$	$\langle\cos\phi\rangle$	ν	σ_f [c]
1 [a]	V	0.50	1.00	0.40	0.6667	−0.6601	19.4°	1.0
2 [b]		0.92	0.80	0.50	0.52	−0.2929	25.0°	0.4
3 [a]	Z	0.75	1.00	0.50	0.5714	−0.5095	21.7°	0.7
4 [a]		0.60	0.75	0.50	0.625	−0.4133	26.2°	0.6
5 [b]		1.21	1.00	0.65	0.4525	−0.232	27.6°	0.3

[a] 300 bilayers; molar fraction of carotenoid 1%.

[b] 3000 bilayers; molar fraction of carotenoids 0.1%.

[c] $\sigma_f = \dfrac{\langle(f - \langle f\rangle)^2\rangle^{0.5}}{\langle f\rangle} = [2(a_1^2 + a_2^2)]^{0.5}$, see Eqn. 5.

respect is acceptable as a distribution function. If some terms of the shape $a_n \cos n\phi$ ($n \geq 3$) or $b_n \sin n\phi$ ($n \geq 1$) are added to the function (5) neither mean values of $\langle \cos \phi \rangle$ and $\langle \cos^2 \phi \rangle$ nor the normalization of the function will be changed. But it would change mean deviation of the function from its mean value $(2\pi)^{-1}$. Such deviations, which are given in the last column of Table I, suggest that at a higher number of bilayers and lower molar fraction of carotenoids the distribution of transition dipoles is more homogeneous.

What is the reason for the nonhomogeneous orientation of xanthophyll molecules in the plane of bilayers? We cannot exclude the possibility of some imperfections of our samples. But such a possibility is not very probable, since the diffractometric results suggest rather good ordering of planes (the lack of the wide angle maxima in Fig. 1). The deviation of $f(\phi)$ from homogeneity can also be explained by the assumption that the xanthophyll molecule exerts an ordering influence on the DMPC molecules, which can determine the orientation of another xanthophyll molecule. Such ordering influence should have a relatively long-distance range, i.e. several lipid molecules in a single monolayer, since it is observable at the carotenoid molar fraction of 0.1% (items 2 and 5 in Table I). At this molar fraction there are 1000 DMPC molecules per one carotenoid molecule in the bilayer. It makes 500 DMPC molecules per one carotenoid molecule in the monolayer, and we can expect that the neighbour pigment molecules are separated with $\sqrt{500} = 22$ lipid molecules. Apart from this, the ordering should be transferred from one bilayer to the other. Although the existence of the ordering influence of carotenoids needs more reliable evidence, it is quite probable that it is observable only with the carotenoids having polar groups at both ends of molecules and long enough to span the lipid bilayer. Such a suggestion is in agreement with a hypothesis of Rohmer et al. [15]. According to them, carotenoids in biological membranes can play a role analogical to that of cholesterol.

The comparison of our values of the angle between the long axis of xanthophyll molecules and the normal to the bilayer plane with other authors' data suggest that the orientation of carotenoids in lipid layers depends on the composition of the sample and/or on the method of preparation. N'Soukpoe-Kossi et al. [16] have found that lutein molecules in lecithin monolayer are oriented at the angle of about 42° in respect to the normal to the plane of film. This is an angle wider than our value of ν (25°), though both the values are smaller than the magic angle of 54.7°. In our opinion, this discrepancy can be explained by the fact that the monolayer, in contrast to the bilayer has only one hydrophilic surface.

Van de Ven and coauthors [1] examined crocetin in lipid multilayers which contained 30% of water. They found that crocetin molecules form with the normal to the plane of layers an angle of 47°, the angle narrower than the magic angle. There are not any essential differences between the conclusions of Ourisson's team [11] and our results. We only differ in the evaluation of the thickness of the bilayer hydrophobic core. Essentially different results were obtained by Schmidt and Reich [17]. They investigated lutein in Langmuir-Blodget films of cadmium arachidate and obtained for the angle ν the value of 66°. In this case, the thickness of the hydrophobic core of the bilayer was probably bigger than the distance between hydroxyl groups in lutein molecules which could not span the arachidate bilayer.

We conclude that our result of the linear dichroism measurements give the angle between the long axis of xanthophyll molecules and the normal to the plane of bilayer narrower than the magic angle. The values of the mentioned angle are approximately equal to 22° for violaxanthin and 25° for zexanthin and are consistent with the data on multilayer structure obtained from X-ray diffraction experiments. There are some differences between the results for violaxanthin and zeaxanthin. The differences in the values of angle ν are contained in the limits of experimental error, but the differences in the values of the multilayer periodicity are rather certain. They can be essential for the understanding of differences in the photoprotective activity of the two xanthophylls in phosphatidylcholine liposomes [9] and, indirectly, for the physiological role of the xanthophyll cycle.

Appendix I

Thickness of the hydrophobic core of DMPC bilayer

We define the thickness of the hydrophobic layer in the DMPC bilayer as the shortest distance between C = O groups in two DMPC molecules situated at opposite sides of the bilayer. Calculations were performed for the water content of 20 weight per cent and for the temperature of 37° C. The molecular area of DMPC in the bilayer at this temperature is well-known ($S = 60$ Å^2) [12]. The volume corresponding to the cited area of the bilayer can be calculated as a sum of volumes of 48 CH_2 groups and 4 CH_3 groups (two DMPC molecules). According to Nagle and Wilkinson [18] these volumes amount to 27.2 Å^3 for the CH_2 group and 54.4 Å^3 for the CH_3 group. From the above data we obtain 1523.2 Å^3 as the volume of a cylinder with the cross-section of 60 Å^2. Therefore the cylinder should have a height of 25.4 Å. The last value is equal to the thickness of the hydrophobic core of the DMPC bilayer.

Appendix II

Derivation of the formula for dichroic ratio

Let us denote by $E_{\parallel}$ and $E_{\perp}$ unit vector parallel to electric vectors of the incident light, respectively, paral-

lel and perpendicular to the plane of incidence. In the coordinates shown in Fig. 5, these vectors have the following components:

$$E_{\parallel} = (\cos\alpha, 0, \sin\alpha) \quad \text{(AII-1)}$$

$$E_{\perp} = (0, 1, 0) \quad \text{(AII-2)}$$

On the other hand, the unit vector parallel to the transition dipole of a carotenoid molecule has the components:

$$M = (\sin\nu\cos\phi, \sin\nu\sin\phi, \cos\nu) \quad \text{(AII-3)}$$

where ν is the angle between the dipole and z-axis and ϕ is the angle between the projection of dipole on the x, y-plane and the x-axis. The cosine of the angle between M and $E_{\parallel}$ as well as that between M and $E_{\perp}$ can be calculated as scalar products of the respective unit vectors:

$$\cos\gamma_{\parallel} = ME_{\parallel} = \sin\nu\cos\phi\cos\alpha + \cos\nu\sin\alpha \quad \text{(AII-4)}$$

and

$$\cos\gamma_{\perp} = ME_{\perp} = \sin\nu\sin\phi \quad \text{(AII-5)}$$

The absorbance of the sample is proportional to $\langle\cos^2\gamma_{\parallel}\rangle$ and $\langle\cos^2\gamma_{\perp}\rangle$, where the brackets $\langle\ \rangle$ denote averaging above all the pigment molecules. From Eqns. AII-4 and AII-5) it follows that:

$$\langle\cos^2\gamma_{\parallel}\rangle = \sin^2\nu\langle\cos^2\phi\rangle\cos^2\alpha + \sin\nu\cos\nu\langle\cos\phi\rangle\sin 2\alpha + \cos^2\nu\sin^2\alpha \quad \text{(AII-6)}$$

$$\langle\cos^2\gamma_{\perp}\rangle = \sin^2\nu\langle\sin^2\phi\rangle, \quad \text{(AII-7)}$$

and

$$r = \frac{A_{\perp}}{A_{\parallel}} = \frac{\sin^2\nu\langle\sin^2\phi\rangle}{\sin^2\nu\langle\cos^2\phi\rangle\cos^2\alpha + \sin\nu\cos\nu\langle\cos\phi\rangle\sin 2\alpha + \cos^2\nu\sin^2\alpha} \quad \text{(AII = 8)}$$

After the division of numerator and denominator in the last formula by $\cos^2\nu$, we obtain Eqn. 1. Next we calculate angles α_0, α_1, and α_2 on the basis of the refraction law: $\sin\alpha_0 = \sin 0/n = 0$, $\sin\alpha_1 = \sin 30°/1.445$, $\sin\alpha_2 = \sin 45°/1.445$. The insertion of this values of α into Eqn. 1 in the text gives expressions for the dichroic ratio at different angles of incidence:

$$r_0 = \frac{\langle\sin^2\phi\rangle}{\langle\cos^2\phi\rangle} \quad \text{(AII-9)}$$

$$r_1 = \frac{\tan^2\nu\langle\sin^2\phi\rangle}{0.8803\tan^2\nu\langle\cos^2\phi\rangle + 0.6493\tan\nu\langle\cos\phi\rangle + 0.1197} \quad \text{(AII-10)}$$

$$r_2 = \frac{\tan^2\nu\langle\sin^2\phi\rangle}{0.7605\tan^2\nu\langle\cos^2\phi\rangle + 0.8535\tan\nu\langle\cos\phi\rangle + 0.2395} \quad \text{(AII-11)}$$

At any distribution of the transition dipoles with respect to the angle ϕ, the following equation is satisfied;

$$\langle\sin^2\phi\rangle + \langle\cos^2\phi\rangle = 1 \quad \text{(AII-12)}$$

The Eqns. 2–4 in the text, used in calculations, provide a solution of the Eqns. AII-9 to AII-12.

As can be seen from the Eqns. AII-9–AII-11, all the measured dichroic ratios depend on the angle ϕ and consequently they will change if the sample is rotated around the z-axis (Fig. 5). However, such rotation does not influence the value of the angle ν calculated according to the Eqn. 4. Let us presume that the sample has been turned around the z-axis by an angle δ and see how this operation influences the value of ν. We can write new formulas for r_0, r_1 and r_2 inserting into Eqns. AII-9–AII-11) $\phi + \delta$ instead of ϕ. It is easy to check that in this case we will obtain:

$$1 + r_0 = \frac{1}{\langle\cos^2(\phi+\delta)\rangle} \quad \text{(AII-13)}$$

$$\frac{r_0}{r_1} = 0.8803 + \frac{0.6493\tan\nu\langle\cos(\phi+\delta)\rangle + 0.1197}{\tan^2\nu\langle\cos^2(\phi+\delta)\rangle} \quad \text{(AII-14)}$$

$$\frac{r_0}{r_2} = 0.7605 + \frac{0.8535\tan\nu\langle\cos(\phi+\delta)\rangle + 0.2395}{\tan^2\nu\langle\cos^2(\phi+\delta)\rangle} \quad \text{(AII-15)}$$

The above relations give, for the numerator (N) and denominator (D) of the right hand part of Eqn. 4, respectively:

$$N = \frac{0.0822}{\langle\cos^2(\phi+\delta)\rangle} \quad \text{(AII-16)}$$

and

$$D = \frac{0.0822}{\tan^2\nu\langle\cos^2(\phi+\delta)\rangle} \quad \text{(AII-17)}$$

Thus, the right hand part of Eqn. 4 depends on neither ϕ nor δ, and we obtain the same value of ν.

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