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Organisation of xanthophyll pigments lutein and zeaxanthin in lipid membranes formed with dipalmitoylphosphatidylcholine

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Abstract

Carotenoid pigments and in particular xanthophylls play several physiological functions in plant and animal membranes. Xanthophylls are present in biological membranes in the form of pigment-protein complexes but also as direct components of lipid phase. The biological activity of carotenoids in membranes depends on a molecular organisation of pigments in lipid bilayers, in particular the localisation, orientation and aggregational state. In the present work the organisation of lutein- and zeaxanthin-containing lipid membranes was analysed with the application of electronic absorption spectroscopy. Both xanthophyll pigments incorporated to the dipalmitoylphosphatidylcholine (DPPC) unilamellar liposomes form H-type molecular aggregates, manifested by the hypsochromic shift of the main absorption band of carotenoids. The aggregation of lutein and zeaxanthin in DPPC membranes was observed even at relatively low concentrations of a pigment in the lipid phase (1–5 mol%). Gaussian analysis of the absorption spectra of lutein and zeaxanthin in DPPC membranes in terms of the exciton splitting theory revealed the formation of different molecular structures of pigments interpreted as dimers, trimers, tetramers and large aggregates. The fraction of lutein and zeaxanthin in the monomeric form was found to depend on the physical state of the lipid phase. Pronounced monomerisation of lutein and zeaxanthin was observed as accompanying the transition from the P_{β}' phase to the L_{α} phase of DPPC, mostly at the expense of the trimeric and tetrameric forms. The fraction of monomers of lutein is always lower by 10–30% than that of zeaxanthin under the same experimental conditions. Different organisational forms of lutein and zeaxanthin in the model system studied are discussed in terms of possible physiological functions of these pigments in the membranes of the retina: zeaxanthin in the protection of the lipid phase against oxidative damage and lutein in absorbing short wavelength radiation penetrating retina membranes.

Keywords: Carotenoid; Xanthophyll pigment; Lipid membrane; Molecular aggregate; Retina

1. Introduction

More than 600 naturally occurring carotenoid pigments have been identified [1]. Some of them, such as β-carotene, violaxanthin, neoxanthin, lutein and zeaxanthin are found in most plants [2] but also in blood serum and animal tissues [3]. The physiological role of carotenoids in plants is ascribed mostly to absorbing light quanta and transferring energy to chlorophyll pigments to drive photosynthesis (light harvesting) or stabilising structure of photosynthetic

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proteins [4]. The other important physiological function of carotenoid pigments in plants seems to be common also for the animal kingdom. This function is related to protection against attack by active oxygen species, leading to destruction of biological structures [5]. This type of protection is realised mostly via quenching of triplet-excited molecules of sensitisers, quenching of singlet oxygen and scavenging of free radicals. Protection by carotenoids is important, in particular for the case of biomembranes rich in polyunsaturated fatty acids and therefore susceptible to free radical-induced damage. The incorporation of polar carotenoid pigments into lipid membranes was found to increase the penetration barrier to oxygen, potentially harmful to the unsaturated lipid membrane core [6]. Two polar carotenoid pigments, lutein and zeaxanthin, are found in the membranes of the retina in the eyes of primates [7–9]. In particular the fovea, the part of the retina localised in the proximity of the optical axis of an eye, appears to be yellow owing to the enrichment in lutein and zeaxanthin and therefore is named the macula lutea [7–9]. The physiological function of lutein and zeaxanthin in the macular membranes is not fully understood at present. Most probably xanthophylls serve in an eye as photoprotectors but other possible functions such as filtering short wavelength radiation are also discussed [9,10]. Both biological functions proposed for lutein and zeaxanthin in the retina membranes of an eye require special arrangement of xanthophyll pigment molecules within the membranes. Monomeric pigments dispersed in a lipid phase seem to be better suited to function as free radical scavenger while aggregated pigment molecules seem to be suitable to absorb short wavelength radiation, owing to the aggregation-related hypsochromic shift of the main absorption band. In the present work we analyse the organisation of lutein and zeaxanthin molecules in model lipid membranes – liposomes, formed with dipalmitoylphosphatidylcholine by means of electronic absorption spectroscopy.

2. Materials and methods

Dipalmitoylphosphatidylcholine (DPPC) for liposomes was purchased from Sigma. Synthetic, crystalline xanthophyll pigments, lutein ((3*R*,3′*R*,6′*R*)-β,ε-caroten-3,3′-diol) and zeaxanthin ((3*R*,3′*R*)-β,β-caroten-3,3′-diol), were a generous gift from Hoffmann-La Roche (Basel). Pigments were stored under argon in a deepfreeze. Directly before use the pigments were repurified by means of HPLC technique (column length 250 mm, internal diameter 4.6 mm, filled with Nucleosil C-18). The solvent mixture acetonitrile:methanol:water (72:8:3, v/v/v) was used as a mobile phase. To avoid any organic contamination the water used to prepare buffers was double distilled and distilled for the third time with $KMnO_4$ added. The molar concentration of used xanthophyll pigments was evaluated spectrophotometrically using molar extinction coefficients from the literature [11]. Small unilamellar liposomes were prepared following the general procedure below. Briefly, a vacuum-dried thin film of DPPC in a glass tube containing 0, 1, 2, 4 or 5 mol% lutein or zeaxanthin was hydrated with the tricine buffer (1.5 mg/ml DPPC, the buffer was 0.1 M, pH 7.6) and vortexed vigorously for 10 min. The homogeneous suspension was then subjected to sonication for 2 min with a 20 kHz ultrasonic disintegration (model Unipan) with a titanium probe at a temperature of 45°C. The small liposome suspensions were subjected to centrifugation at $15\,000 \times g$ to eliminate possible pigment residuals not incorporated to the liposomes. No pigment microcrystals were detected in the pellet.

Electronic absorption spectra were recorded with a double beam Shimadzu spectrophotometer, model 160A-PC UV-VIS, equipped with thermostatted cell holders. All the experiments were carried out in the temperature range from 5°C to 50°C. A constant temperature during the recording of the spectra was controlled with a Poly Science thermostatted circulator. The temperature was monitored with a NiCl-NiAl thermocouple placed directly in the cell. The liposome suspension was stirred during the experiments with a magnetic microstirrer. Non-pigmented DPPC liposomes were placed in the optical way of the reference beam. Absorption spectra were recorded every 5°C except the region of the main phase transition (41°C [12]) in which spectra were recorded every 1°C. Gaussian deconvolution of the absorption spectra was performed with the PEAKFIT software from Jandel Scientific Software and Grams 32 from the Galactic Industries.

3. Results and discussion

Xanthophyll pigments dissolved in hydrated organic solvents such as ethanol or acetone form aggregated molecular structures [13–15]. Pigment aggregation is demonstrated by a hypsochromic shift of the main absorption spectrum ($1A_g^1 \rightarrow 1B_u^1$) indicative of formation of H-type molecular structures, named also card pack aggregates [16–18]. Fig. 1 presents absorption spectra of zeaxanthin and lutein in ethanol (monomeric form) and in hydrated ethanol (aggregated form). The main absorption band (the 0-0 transition around 450 nm) is shifted owing to the molecular aggregation by approx. 70 nm and appears at 380 nm. The same process of formation of the aggregated forms of xanthophyll pigments was also observed in lipid membranes [15,19,20] (see also [21] for a review). In general, aggregation of xanthophylls in a lipid phase depends on the pigment concentration and the physical state of the lipid phase [21]. Fig. 2 presents the absorption spectra of zeaxanthin incorporated at different concentrations to small unilamellar liposomes formed with DPPC.

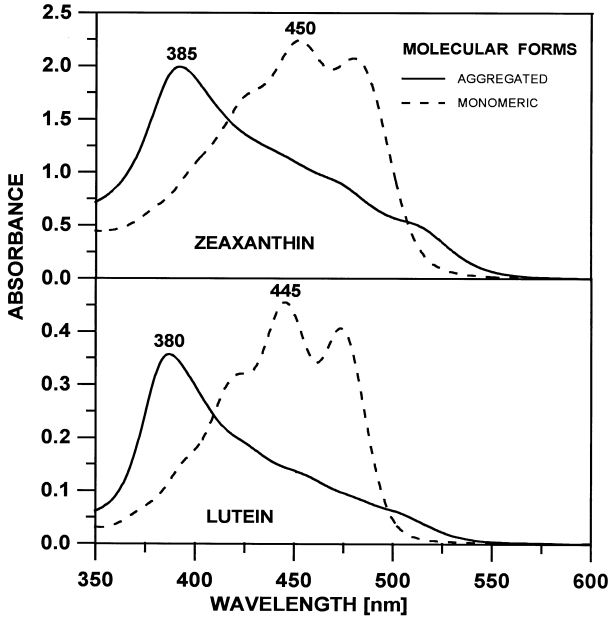

Fig. 1. Absorption spectra of the monomeric and aggregated forms (indicated) of zeaxanthin (upper panel) and lutein (lower panel). The absorption spectra were recorded in ethanol in the case of monomeric pigments, and in an ethanol:water mixture (1:9, v/v) in the case of aggregates.

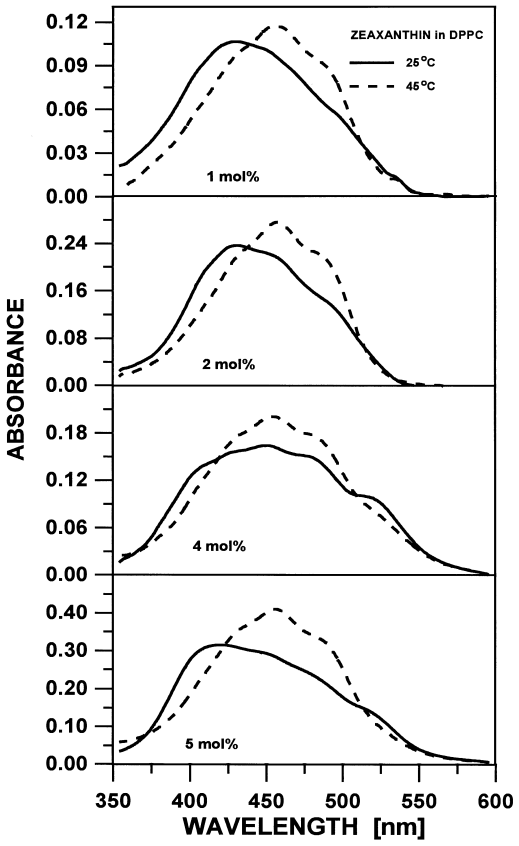

Fig. 2. Exemplary absorption spectra of a suspension of DPPC liposomes containing 1, 2, 4 or 5 mol% of zeaxanthin recorded at 25°C and 45°C, as indicated. The non-pigmented liposome suspension was recorded as a reference. Optical path 1 cm.

The spectra presented were recorded at two temperatures, at 25°C in the L_β phase of DPPC membranes, and at 45°C above the main phase transition in the L_α phase. The absorption spectra recorded for lutein under the same conditions are presented in Fig. 3. As can be seen, the aggregation level of both xanthophyll pigments depends strongly on both the concentration and fluidity of the lipid phase. The temperature-dependent reorganisation of xanthophyll molecular structures can be clearly observed from the temperature profiles presented in Fig. 4 for 5 mol% zeaxanthin in DPPC. Clearly the xanthophyll monomerisation takes place in the temperature region corresponding to the main phase transition $P_\beta' \rightarrow L_\alpha$. Gaussian deconvolution of the absorption spectra was applied in order to analyse quantitatively the aggregation of lutein and zeaxanthin in the DPPC membranes. Figs. 5 and 6 present exemplary

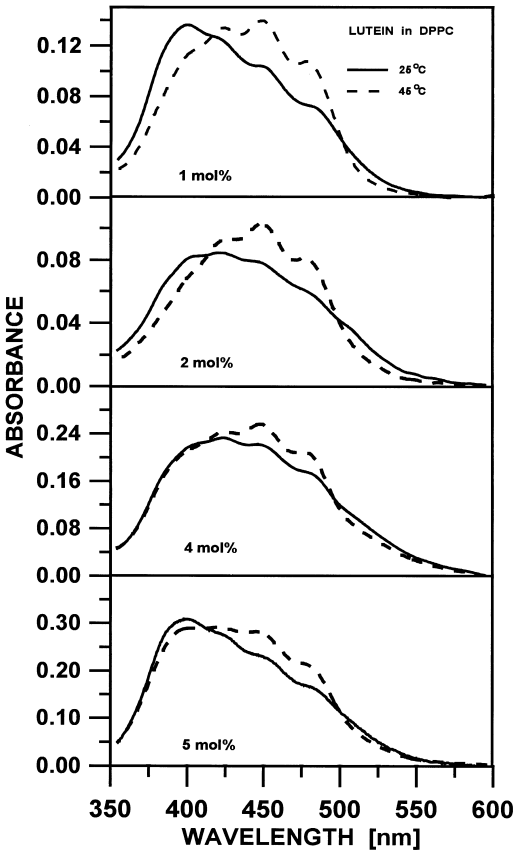

Fig. 3. The same as for Fig. 2 except that lutein was present in DPPC liposomes. Molecular percentage of lutein is indicated.

electronic absorption spectra with the main gaussian bands corresponding to the 0-0, 0-1 and 0-2 vibronic transition of a pigment in its monomeric form and other distinct bands which can be ascribed to the aggregated forms. Satisfactory deconvolution of the absorption spectra of both pigments required the application of the additional red-shifted bands, indicative of J-type aggregates or aggregates in which the chromophore axes are tilted with respect to the axis connecting the centres of molecules [16–18]. For example, the gaussian components centred at 507 nm and 522 nm correspond very well to the bathochromically shifted ($\Delta\nu = -1792$ cm^{-1}) 0-0 and 0-1 vibronic transitions of the monomeric forms of zeaxanthin, owing to the formation of molecular dimers. The spectral shift of absorption spectra of the pigment molecules undergoing aggregation is well described by the exciton splitting theory [16–18,22]. Eq. 1 describes a change of transition energies for the mth exciton state following pigment aggregation [22]:

$$\nu(N) = \nu_{\mathrm{mon}} + 2\beta \cos\left(\frac{m\pi}{N+1}\right) \quad (1)$$

where m runs over all N exciton coupled states of the aggregate and β is the coupling matrix element expressed as:

$$\beta = \frac{|\mu_{\mathrm{mon}}|^2}{4\pi\varepsilon_0 n^2 R^3}(1-3\cos\theta) \quad (2)$$

In Eq. 2, ε_0 is the permittivity of free space, μ_{mon} is the transition moment of the monomer, n is the refractive index of the medium, R is the distance between nearest neighbours in the aggregate, and θ is the angle between monomer transition moment vectors in the aggregate. One can substitute the parameter N in the place of the parameter m in Eq. 1 in the case of the highest exciton state corresponding to the H-type aggregate. The relatively high number of pigment molecules in the aggregate results in the maximum spectral shift that is practically insensitive to further increase in the number of molecules, owing to the dependence of the cosine in Eq. 1 on the $N/(N+1)$ factor. Following this assumption it is possible to calculate a value of parameter β for lutein and zeaxanthin in DPPC membranes on the basis of the position of the maximum of the spectral band corresponding to the N-aggregated form (most blue-shifted gaussian component, 385 nm in the case of zeaxanthin and 380 nm in the case of lutein). The parameters 2β were found to be −3752 cm^{-1} for zeaxanthin and −3844 cm^{-1} for lutein. The substitution of the values of these parameters allows the gaussian bands centred at 415 nm in the case of

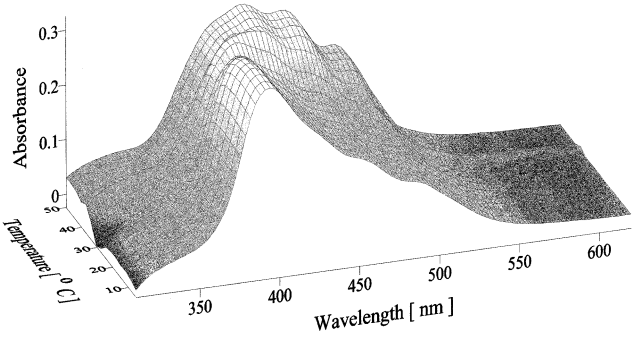

Fig. 4. Temperature dependences of the absorption spectra of zeaxanthin-containing (5 mol%) DPPC liposomes.

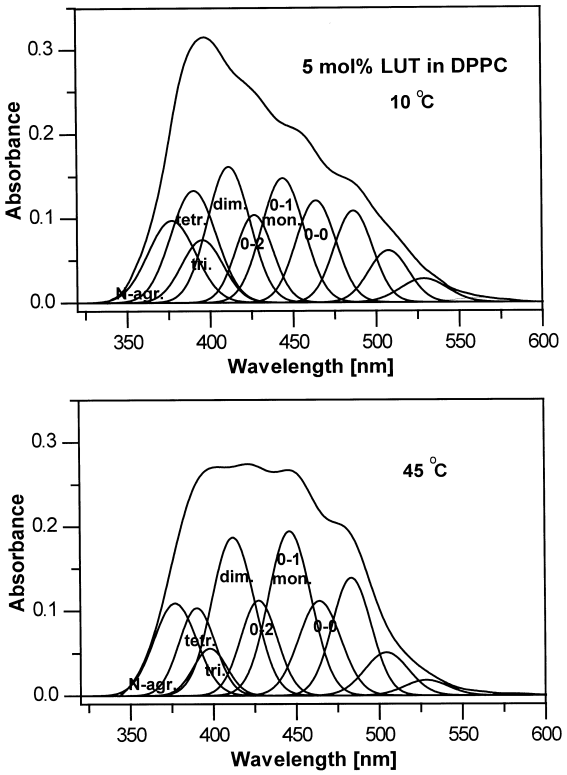

Fig. 5. Gaussian deconvolution of the absorption spectra of lutein-containing (5 mol%) DPPC liposomes recorded at 10°C (upper panel) and 45°C (lower panel). The three main vibronic maxima of the monomeric form of lutein are indicated as 0-0, 0-1, and 0-2. The gaussian components representing other forms are indicated as dim. for dimers, tri. for trimers, tetr. for tetramers and N-agr. for large aggregates.

zeaxanthin and 410 nm in the case of lutein to dimers ($N=2$, Eq. 1, the shift calculated from the main absorption band of the monomeric form of zeaxanthin 450 nm and lutein 445 nm), centred at 402 nm in the case of zeaxanthin and at 397 nm in the case of lutein for trimers, and centred at 396 nm in the case of zeaxanthin and at 391 nm in the case of lutein for tetramers. We were not able to resolve the aggregated structures comprising higher number of molecules by this method ($N=5$, 6, ...) since absorption bands, if present, overlap with the absorption band corresponding to the *N*-aggregate. All these forms will be analysed as *N*-aggregates. The gaussian components corresponding to the trimeric and tetrameric structures also appear very close to each other. Interestingly, the intensity of the components corresponding to the trimeric forms were always very low, in particular in the case of lutein. On the one hand, this may express low energy of binding in the case of a molecular assembly composed of three molecules and stabilised by van der Waals interactions. On the other hand, the same phenomenon may represent the technical effect of the deconvolution with application of two relatively closely located gaussian components. In fact, we found some dependence of a balance between an intensity of bands corresponding to the trimeric and tetrameric forms on initial conditions, while testing parameters of deconvolution. In order to avoid any uncertainty regarding this effect, trimeric and tetrameric structures of the xanthophylls were analysed as one pool. Fig. 7 presents the temperature profile of the molecular forms of lutein in DPPC liposomes and Fig. 8 presents the corresponding temperature profiles for zeaxanthin. The fractions of all forms were calculated on the basis of the intensity of the gaussian components corresponding to each spectral form. In principle, the dipole transition moment of a molecule in an aggregate depends on a number of molecules forming this structure [22]. Owing to the fact that molar extinction coefficients of

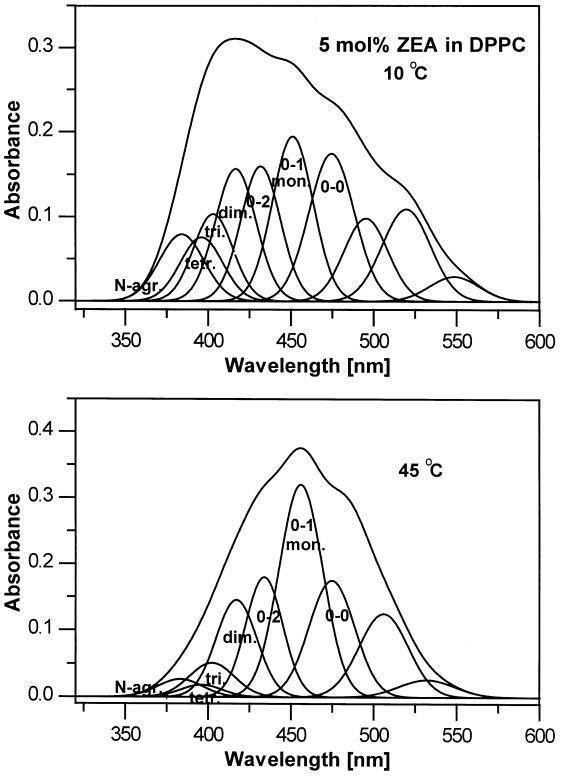

Fig. 6. The same as for Fig. 5 except that the DPPC liposomes were pigmented with 5 mol% zeaxanthin.

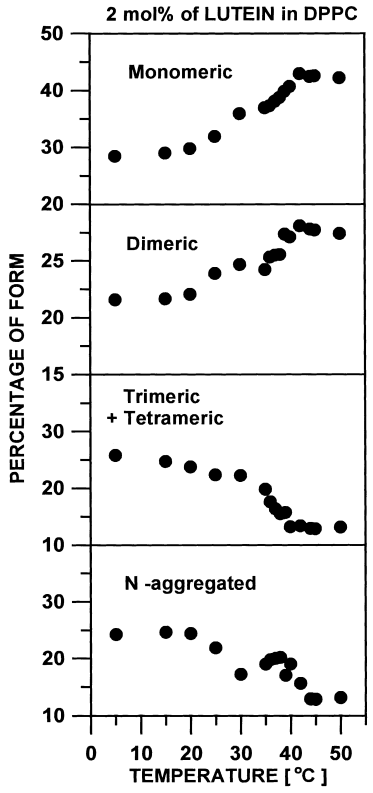

Fig. 7. Temperature profiles of the percentage of monomeric, dimeric, trimeric, tetrameric and *N*-aggregated forms of lutein in DPPC liposomes at a concentration of 2 mol% pigment with respect to lipid. The analysis is based on the intensities of the gaussian components corresponding to each form.

monomeric and aggregated lutein and zeaxanthin have been found not to differ significantly (Fig. 1) a concentration of each form was approximated by the intensity of the appropriate gaussian component. As may be seen from Figs. 7 and 8, the level of monomers increases by approx. 20% in both cases in response to the main phase transition, despite different concentrations of lutein and zeaxanthin in the lipid phase. In general, the fraction of lutein in the monomeric form is always lower than the fraction of monomeric zeaxanthin at the same concentration and at the same temperature (by 10–30%, see Figs. 9 and 10). Both in the case of lutein and zeaxanthin an increase in the temperature results in a decrease in the level of molecules in the trimeric and tetrameric form (Figs. 7 and 8). The most pronounced effect may be observed between the phase pretransition (35°C) and the main phase transition temperature (41°C). Interestingly, the temperature profile of dimers of zeaxanthin shows a local maximum in the temperature region corresponding to the P_β' phase of DPPC. A local decrease in the number of molecules of this xanthophyll pigment in the trimeric, tetrameric and *N*-aggregated forms can be observed in the same temperature region. These two effects analysed together indicate that aggregated forms of zeaxanthin are preferentially formed in a lamellar structure of DPPC (L_β' and L_α) and the ripple phase of DPPC affects molecular organisation of the large aggregates of zeaxanthin. Similarly, the disassembly of the *N*-aggregated structures of lutein is associated with the increase in the fraction of dimers but no local maximum corresponding to the ripple phase of the membrane was observed. A decrease in the concentration of large aggregates accompanies an increase in the concentration of monomers but also of the dimers in the case of lutein. Such a mechanism is consistent with the concept according to which the large molecular aggregates of lutein and zeaxanthin are composed of pigment dimers in which

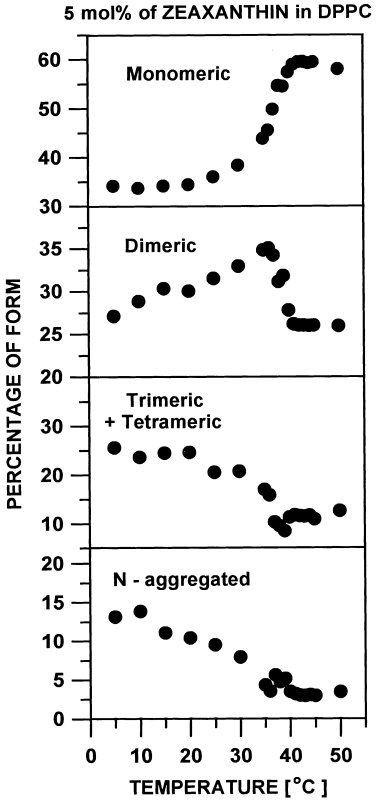

Fig. 8. The same as for Fig. 7 except that the DPPC liposomes were pigmented with 5 mol% zeaxanthin.

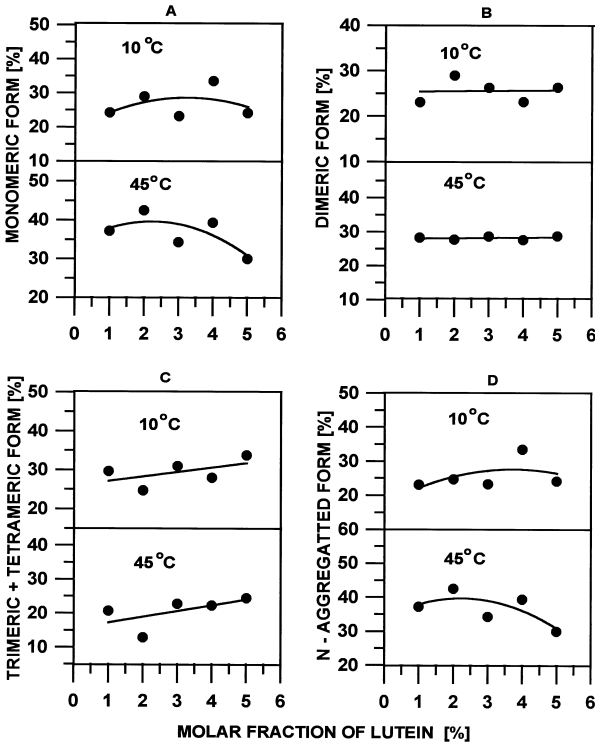

Fig. 9. Concentration dependences of the molecular forms of lutein in DPPC liposomes at 10°C and 45°C, as indicated. (A) Monomers; (B) dimers; (C) trimers and tetramers; (D) *N*-aggregates.

the pigments are bound to each other more tightly than the dimers in the aggregate. The molecular aggregates of xanthophyll pigments are stabilised by the van der Waals interaction and therefore it is very likely that the molecular dimers are stabilised by hydrogen bonding between the hydroxyl groups located at the 3 and 3′ positions of the carotenoid molecules. The formation of dimers was particularly observed in the case of lutein, most probably due to the rotational freedom of the entire ε ring about the C6′-C7′ bond [10] which is not possible in the case of zeaxanthin, as will be further discussed below. Interestingly, a phenomenon very similar to the formation of zeaxanthin dimers in DPPC with the local maximum corresponding to the P_{β}' phase was observed in our theoretical analysis of organisation of pigmented DPPC membranes [23]. The shapes of the temperature profiles show that pigment monomerisation in the course of the membrane fluidisation takes place mostly at the expense of dissociation of the large aggregated molecular forms. It may be expected that an increase in the lutein and zeaxanthin concentration in the lipid phase will result in a decrease in the number of molecules in the monomeric form. As can be seen from Figs. 9A and 10A this is not the case in both lutein- and zeaxanthin-containing membranes. In particular, in the case of zeaxanthin the maximum aggregation is observed at 2–4 mol% pigment in the lipid phase (Fig. 10D). The significantly higher tendency of lutein to form aggregated structures may possibly be related to more effective hydrogen bonding owing to the rotational freedom around the C6′-C7′ bond. This effect is particularly pronounced in the case of lutein in the liquid crystalline phase of DPPC. The fractions of monomeric lutein in different concentrations and at all temperatures analysed are distinctly less populated than the fractions of monomeric zeaxanthin under the same conditions (see Fig. 10A). The fact that solubility of lutein in the lipid phase is always lower than that of zeaxanthin may have implications in all physiological functions that these two xanthophylls exert in lipid membranes. Both lutein and zeaxanthin suppress formation of lipid peroxidation products while present

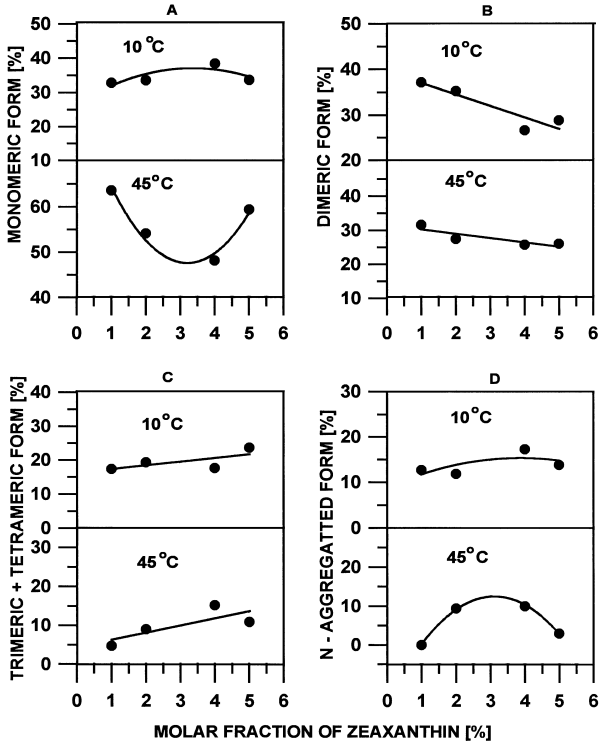

Fig. 10. Concentration dependences of the molecular forms of zeaxanthin in DPPC liposomes at 10°C and 45°C, as indicated. (A) Monomers; (B) dimers; (C) trimers and tetramers; (D) *N*-aggregates.

in the lipid phase of egg yolk phosphatidylcholine but the antioxidant activity of these xanthophyll pigments depends on how lipid peroxidation was initiated [10,24]. The antioxidant efficacies of lutein and zeaxanthin were found to be comparable in the case in which lipid peroxidation was initiated by the water-soluble free radical generator AAPH [10]. On the other hand, zeaxanthin was found to be a better membrane antioxidant in the case in which the membrane oxidation was initiated by the lipid-soluble free radical generator AMVN [24]. It is very likely that the organisation of both xanthophyll pigments in the lipid phase is responsible for the differences observed. Zeaxanthin whose distribution in the membrane is more homogeneous (in the monomeric form) provides better protection to the lipid phase by scavenging reactive oxygen species generated inside the membranes (by AMVN [24]). This is not the case in the free radical attack from the outside of the membrane, as observed with the reaction initiated by AAPH [10]. Lutein itself has been found to be susceptible to a higher degree than zeaxanthin to the oxidative damage in the membranes induced by UV radiation [10]. Such a finding was explained in terms of different distribution of the lutein and zeaxanthin molecules in the membrane. The molecules of zeaxanthin span the lipid bilayer with the hydroxyl groups anchored in the opposite polar zones of the membrane [21]. The results of the linear dichroism analysis of the lutein orientation in the lipid bilayers are consistent with the model according to which one fraction of this xanthophyll pigment (roughly 50%) is oriented following the pattern of zeaxanthin while the remaining fraction represents the pigment molecules oriented parallel with respect to the plane of the membrane [10,21]. Such an orientation of lutein is most probably possible due to the ability of the rotation of the entire ε ring around the C6′-C7′ bond which allows an interaction of two polar groups located at the opposite ends of the molecule with the same polar surface [25]. The same orientation in the case of zeaxanthin is not possible for steric reasons. The differences in organisation of lutein-containing and zeaxanthin-containing lipid membranes point toward different putative physiological functions of these two xanthophyll pigments in biomembranes and in particular in the membranes of the retina of an eye. Both xanthophylls play the role of protectors against oxidative destruction of lipids. In addition, lutein was postulated to be better suited to functioning as a filter of blue light penetrating a membrane. The results presented above show that lutein forms molecular aggregates while present within the lipid phase of the membrane. The xanthophyll aggregation is associated with the formation of the spectral forms absorbing light in the short wavelength region of the spectrum. It seems that the absorption of short wavelength radiation by lutein aggregates is important in realising the light filter function of this carotenoid pigment in biomembranes. The absorption spectra of macular xanthophylls reported were recorded in the spectral range above 420 nm [26,27], which does not allow to verify directly such a hypothesis. On the other hand, the absorption spectra reported are supported in the long wavelength region and in the short wavelength region with respect to the absorption spectra of monomeric lutein and zeaxanthin, which may be consistent with the formation of some molecular aggregates in situ. The aggregation properties of lutein and zeaxanthin in a lipid phase may also be relevant for the discussion concerning the canthaxanthin retinopathy that consists in formation of crystal-like molecular deposits in the retina by this xanthophyll pigment [28]. We plan to address the problem of canthaxanthin organisation in a lipid phase in our future research.

Acknowledgements

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