

Organisation of Xanthophyll-Lipid Membranes Studied by Means of Specific Pigment Antisera, Spectrophotometry and Monomolecular Layer Technique Lutein versus Zeaxanthin

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The structure of the xanthophyll pigments lutein and zeaxanthin differs in the position of one double bond and refers to one of the ionon rings. Specific antibodies to zeaxanthin were used to analyse the localisation and orientation of these two xanthophyll pigments in lipid membranes formed with egg yolk lecithin. Bimolecular and monomolecular layers were used. Antibody-antigen interaction was demonstrated and analysed by the bathochromic shift of the absorption spectra of both pigments and by the increase of light-scattering of the pigmented liposome suspension. It appeared that the extent of the spectral effects accompanying the interaction of the antiserum to zeaxanthin, injected to the liposome suspension which was pigmented with either zeaxanthin or lutein, was different in spite of their similar molecular structures. The results are interpreted in terms of a localisation and distribution of lutein, in the hydrophobic phase of liposomes within two essentially different pigment pools, one oriented horizontally and the other vertically with respect to the membrane plane. This interpretation is supported by the analysis of isotherms of the compression of monomolecular layers of lutein and zeaxanthin formed at the air-water interface and of mixed xanthophyll-lipid monolayers as well as by analysis of the penetration of antibody proteins dissolved in the subphase into the mixed xanthophyll-lipid films.

Introduction

Carotenoids are yellow and red pigments widely present in living organisms including prokaryotes, plants, animals and humans (Bramley and Mackenzie, 1988). The physiological importance of carotenoid pigments in photosynthesis is very well documented and it is generally accepted that carotenoids play a role in harvesting light energy as accessory antenna pigments (Siefermann-Harms, 1985), in protection of the photosynthetic apparatus against photo-damage (Krinsky, 1989; Sandman *et al.*, 1993; Wloch and Wieckowski, 1982) and in stabilisation of a native conformation of functional pigment-proteins (Kühlbrandt *et al.*, 1994; Moskalenko and Karapetyan, 1996). On the other hand there are several indications that pho-

tosynthetic carotenoids also play a physiological role directly within the lipid phase of the thylakoid membrane (Gruszecki and Strzalka, 1991; Gruszecki, 1995; Strzalka and Gruszecki, 1997). The physiological importance of carotenoid pigments in the lipid phase of biomembranes of prokaryotes is seen as rigidifying agents (Rohmer *et al.*, 1979), and in the macular fiber membranes of the eye (Bone and Landrum, 1984; Bone *et al.*, 1992) as photo-protectors against lipid photo-degradation. The effect of carotenoid pigments on structural and dynamic properties of lipid membranes was the subject of numerous recent investigations (Gruszecki and Siewewiesiuk, 1990, 1991; Subczynski *et al.*, 1992; Jezowska *et al.*, 1994; Strzalka and Gruszecki, 1994; Gabrielska and Gruszecki, 1996; Yin and Subczynski, 1996). According to the general picture emerging from this research, polar carotenoids with the polar groups localised at the two opposite ends of the pigment molecule are an-

Abbreviations: EYPC, egg yolk phosphatidylcholine; LUT, lutein; ZEA, zeaxanthin.

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chored in two hydrophilic zones of the lipid bilayer. This pigment localisation and the molecular dimension of carotenoids determine their orientation with respect to the lipid bilayer which is roughly vertical with respect to the plane of the membrane. Such a localisation and orientation of polar carotenoids has a pronounced effect on the structural properties and molecular dynamics of lipid membranes in contrast to the apolar β -carotene. Despite the considerable progress in the research on carotenoid-containing lipid membranes there are still unsolved, basic problems. One of these open problems is the effect of violaxanthin deepoxidation leading to zeaxanthin formation in the thylakoid membrane under overexcitation conditions. There are recent experimental indications that zeaxanthin is at least temporarily directly present in the lipid phase of chloroplast membranes (Gruszecki, 1999). Another problem concerns the organisation of the two xanthophyll pigments lutein and zeaxanthin in the macular membranes of the eye. In the present work we use immunological techniques to study localisation and orientation of lutein in comparison to zeaxanthin in model lipid membranes formed with egg yolk lecithin.

Materials and Methods

Egg yolk phosphatidylcholine (EYPC) was purchased from Sigma Chem. Co. Synthetic zeaxanthin (ZEA) was a generous gift from Hoffmann-La Roche, Basel and lutein (LUT) was isolated from fresh *Urtica dioica* L. leaves. Xanthophyll pigments were stored under argon atmosphere and recrystallised directly before the experiments. In the case of monomolecular layer technique experiments, xanthophyll pigments were additionally separated from possible degradation products by means of HPLC. The chromatographic column (4.6 mm \times 250 mm) filled with Nucleosil (C-18 coated) was applied with the solvent system acetonitrile : methanol : water (72 : 8 : 3, v/v) as mobile phase.

The monospecific polyclonal antiserum to zeaxanthin and the control serum was prepared according to the method described previously (Lehmann-Kirk *et al.*, 1979; Schmid *et al.*, 1993). An IgG fraction of the specific antiserum and the control serum was used in all experiments.

Small unilamellar liposomes, 0.2 g/l EYPC, were formed in 10 mM N-tris [Hydroxymethyl]methylglycine (Tricine) buffer, pH 7.6 according to the following procedure: drying the lipid or lipid/pigment film under a stream of dry nitrogen in a glass test tube, vortexing it with buffer followed by 3 min sonication at 4 °C at 50 W. Two carotenoid concentrations were applied, 3 mol% and 5 mol% with respect to lipid. These concentrations were shown to lie within the miscibility range and to be low enough not to lead to a massive pigment aggregation within the lipid phase (Gruszecki and Siewiewski, 1990, 1991; Subczynski *et al.*, 1992; Jezowska *et al.*, 1994; Strzalka and Gruszecki, 1994; Gabrielska and Gruszecki, 1996; Gruszecki, 1999).

The effect of antibodies on the aggregation of xanthophyll-containing liposome suspensions was measured as turbidity change. To a 2 ml sample, containing liposomes with zeaxanthin or lutein, different amounts of anti-zeaxanthin IgG were added. As a control the identical sample with the respective amount of control IgG was used. After 10 min incubation both samples were shaken and the difference in their turbidity was measured at 500 nm (outside the main absorption band of carotenoids) using a double beam Uvikon 930 spectrophotometer. Electronic absorption spectra of the liposome suspension were also recorded at 23 °C with the *Uvikon 930 Spectrophotometer* from Kontron Instruments or with the *UV-160A-PC Spectrophotometer* from Shimadzu. The spectra of liposome suspensions supplemented with IgG were recorded after shaking the liposome and antibody mixture for 5 min. Monomolecular layers at the air-water interface were formed in a 4 cm \times 40 cm Teflon trough. Double distilled water, used for the monolayer experiments, was distilled before use a third time with KMnO_4 in order to remove eventual organic impurities. Lipid- and carotenoid monolayers or mixed monolayers were deposited by spreading their solution in benzene : ethanol (9 : 1, v/v). After 15 min (required for solvent evaporation) the monolayer was compressed along the long side of the trough with the rate 0.5 cm/min. Surface pressure was monitored with a Nima Technology tensiometer. The process of monolayer compressing, and data acquisition was controlled by an on-line computer.

Results and Discussion

Fig. 1 shows the chemical structure of the two carotenoid pigments studied together with their absorption spectra when incorporated in egg yolk phosphatidylcholine (EYPC) liposomes. As it is seen, the only difference between these two xanthophylls is the position of one double bond in the terminal ionon ring. This difference is distinguished by the shape of the absorption spectra and the position of the main absorption maxima (Fig. 1). A second difference visible in the absorption spectra is a larger light scattering signal in

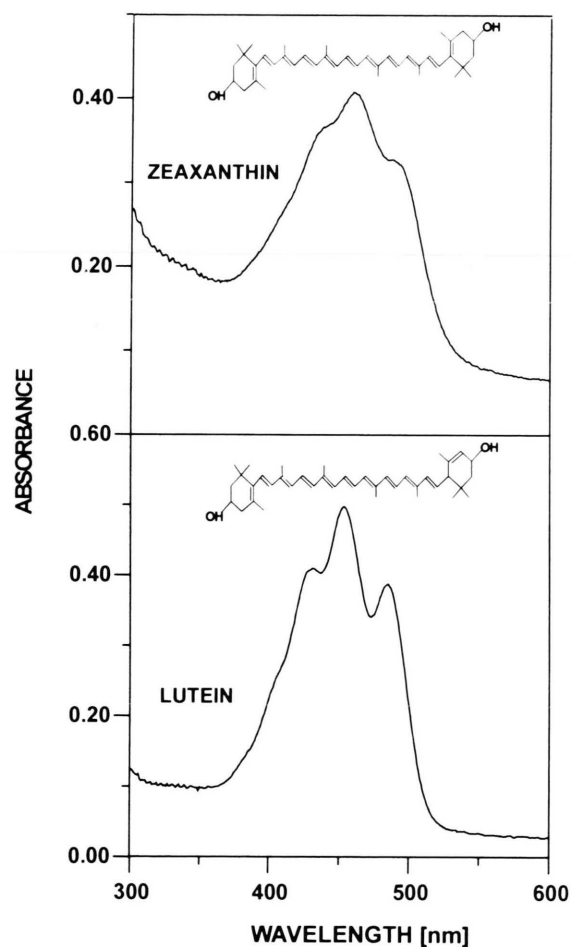


Fig. 1. Absorption spectra of a suspension of EYPC (Egg yolk phosphatidylcholine) liposomes containing 3 mol% zeaxanthin (upper curve) or lutein (lower curve). Above each curve the chemical structure of the respective carotenoid is shown. Optical path 1 cm. Measured against the non-pigmented liposome suspension.

zeaxanthin-pigmented liposome suspension, observable in the short-wavelength spectral region. This effect, indicative of the dimension of light-scattering particles, is the first direct indication of a difference in the organisation of zeaxanthin- and lutein-containing lipid membranes. The effect of zeaxanthin on the size of the liposomes formed with EYPC was also concluded on the basis of experiments carried out with NMR technique (Gabrielska and Gruszecki, 1996).

The influence of zeaxanthin antibodies on suspensions of liposomes containing zeaxanthin or lutein manifested itself as a dose-dependent rise in turbidity (not shown), up to the apparent absorbance of approx. 0.04 at 500 nm. This was valid for lutein- and zeaxanthin-containing EYPC-liposomes after addition 180 μ l of antiserum to zeaxanthin. This indicates an increase in size of the light dispersing structures. The effect may be explained as the result of binding of the antibodies to the investigated carotenoids. Measuring light scattering is a very sensitive method to follow changes in the dimension of liposomes, since it depends on the power of 4 of the radius of the light scattering particle (for particles not larger than 1/10 of the wavelength of light). The increase of size of the light scattering particles in the present experiments can be directly attributed to the binding of antibodies to the liposomes. On the other hand, antibody-mediated agglutination of vesicles has to be taken into account, as discussed above (when two pigmented liposomes interact with two active centres of the same antibody).

According to these experiments, antigenic determinants of both carotenoids (localized in the lipid membrane) are accessible to specific antibodies from the external water phase. This is valid for both zeaxanthin and lutein, having both one antigenic determinant in common.

The supplementation of a pigmented liposome suspension with antibodies to zeaxanthin leads to antibody-antigen interaction which expresses itself by a bathochromic shift of the main electronic absorption band in the absorption spectra of xanthophylls (Fig. 2). The antibody-related bathochromic shift of the absorption spectrum of zeaxanthin is expressed by the large negative band in the difference spectrum in the region of 400 nm corresponding to the short-wavelength edge of the pigmented liposome absorption and a positive band in the dif-

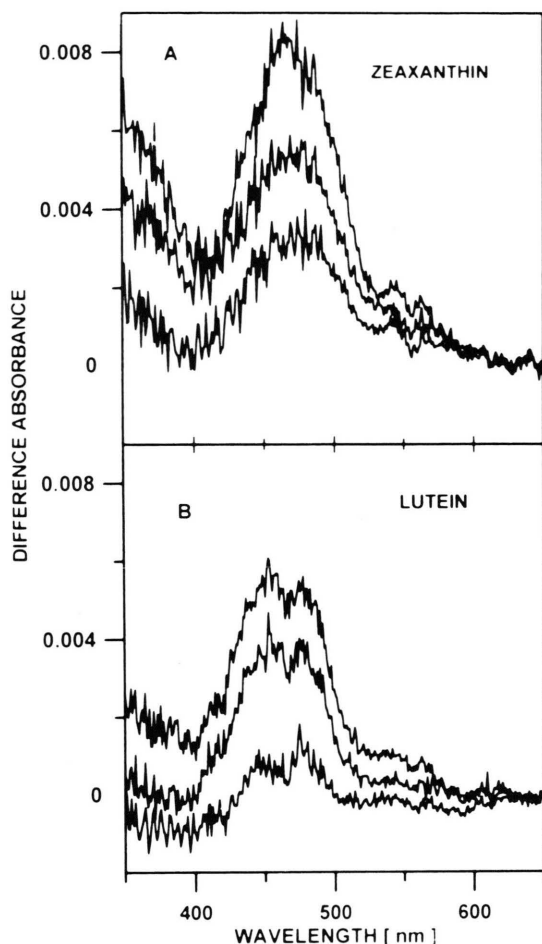


Fig. 2. Difference absorption spectra representing the effect upon addition of 60 μ l (bottom curve), 160 μ l (middle curve) and 300 μ l (upper curve) of IgG fraction of the zeaxanthin antiserum added to 2 ml of a suspension of EYPC liposomes containing 5 mol% zeaxanthin (A) or 5 mol% lutein (B). The same liposome solution was present in both spectrophotometer beams, namely the measuring and the control beam. The active IgG-fraction was added to the sample whereas the control sample contained the same amount of an IgG-fraction from control serum. Spectra were corrected for light scattering due to the addition of the active and control antibody preparation to a non-pigmented liposome suspension. All experiments were repeated 4 times. The main uncertainty comes from the level of the noise signal which is about 10% of the recorded spectral changes. The spectra in B correspond to the addition of 60 μ l (lower curve), 220 μ l (middle curve) and 300 μ l (upper curve).

ference spectrum corresponding to the long-wavelength edge in the pigmented liposome absorption. One can attribute this effect to the perturbation

of a chromophore environment although, certain changes in the oscillatory substructure of the absorption spectra are also observable. The second spectral effect accompanying the interaction of the specific IgG proteins with liposomes is the increase of light scattering which confirms the data on turbidity discussed above. The intensity of the absorption bands in the difference spectra resulting from the interaction with antibodies depends also on differences in the extinction coefficients of the same pigment in two environments, namely in the lipid phase and bound to a protein environment.

Spectral effects very similar to those observed for liposomes containing zeaxanthin were observed in lutein-containing liposomes, as shown in Fig. 2B. The extent of the spectral changes corresponding to the presence of IgGs to zeaxanthin in the membrane systems pigmented with zeaxanthin and with lutein are depicted in Fig. 3. Antibodies to zeaxanthin are also active with lutein. This is due to the fact that these xanthophyll pigments are stereochemically very similar and have one terminal ionon ring in common, which is crucial for the recognition of both membrane-bound xanthophylls by the antibody (Schmid *et al.*, 1993). The excess amount of carotenoid molecules in comparison to the amount of antibody molecules, in particular at the initial stage of the experiment, should result in very similar effects of the antisera in zeaxanthin-containing and lutein-containing liposomes. However, as seen from the analysis of the antiserum-related spectral changes in both systems, the extent of the spectral changes is much lower in the case of lutein than the effect observed for zeaxanthin. The effect for lutein-containing liposomes was only about 25% of the effect observed for zeaxanthin at the initial, linear phase of the experiment in which at low antibody concentrations the most selective interaction is expected. A possible explanation of this effect could be the existence of two distinctly different pools of lutein in the lipid membrane with interaction of antibodies with only one of them. Only the reacting pool would give spectral changes similar to those observed for zeaxanthin. The two different hypothetical pools of lutein are represented schematically in Fig. 4. The picture shows two vertically oriented molecules oriented like zeaxanthin (Gruszecki and Siewiesiuk, 1990, 1991) and two molecules located in a position par-

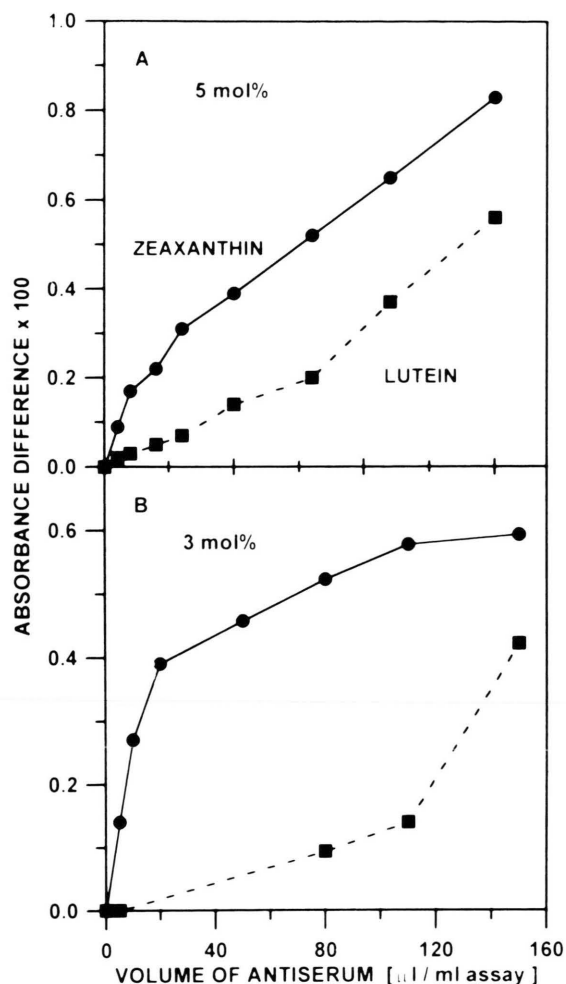


Fig. 3. Difference spectra amplitudes measured at 453 nm for lutein and at 470 nm for zeaxanthin as a function of the antiserum volume added to 2 ml of pigmented liposome suspension. The xanthophyll concentration in the liposomes was 5 mol% in panel A and 3 mol% in panel B.

allel with respect to the plane of the membrane, being localised at the external and internal surface of the liposomal membrane. Only one orientation of lutein, namely the vertical one with the (ionon ring exposed to the bulk water phase of the liposome suspension) is accessible to the antibody to zeaxanthin. The planar orientation of the xanthophyll pigment with respect to the plane of the membrane would require the simultaneous interaction of two hydroxyl groups located at opposite sides of the molecule (3 and 3' positions) with

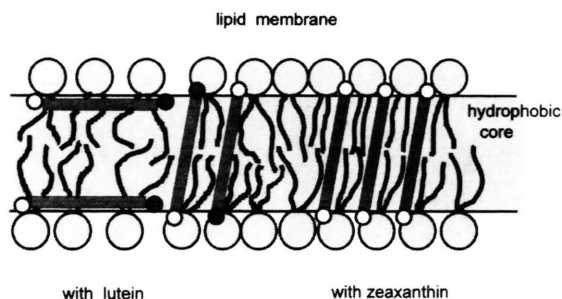


Fig. 4. A schematic model of a hydrophobic core of the lipid bilayer containing zeaxanthin and lutein. The pigment molecules are represented by bars.

the same polar head group region of the lipid bilayer. This simultaneous interaction is for stereochemical reasons not possible in the case of zeaxanthin but seems to be probable in the case of lutein, taking into consideration the relative rotational freedom of the terminal ring along the C6'-C7' bond. The possibility that lutein might adopt a planar orientation with respect to the lipid bilayer was discussed by Gruszecki (1999) in order to explain differences in linear dichroism data concerning the orientation of lutein and zeaxanthin in lipid multibilayers. The relatively large orientation angle of lutein with respect to the axis normal to the plane of the membrane formed of egg yolk phosphatidylcholine (67 deg.) in comparison to zeaxanthin (44 deg.) was interpreted as being due to the distribution of lutein among the two differently oriented pools. The first pool was supposed to follow the orientation pattern of zeaxanthin (54%) and the second was thought to be parallel to the plane of the membrane (46%).

The possibility that lutein is able to contact with its two polar ends the same polar surface is further supported by results from monomolecular layer experiments carried out at the air-water interface. Fig. 5 shows isotherms of compression of monomolecular layers formed with zeaxanthin and lutein. The same molecular dimensions of both pigments are represented by very similar molecular specific areas that are occupied at the air-water interface, and which are calculated by extrapolation of the linear portions of the isotherms of compression to the zero surface pressure, as indicated in Fig 5. Values of $0.38 \text{ nm}^2 \pm 0.04 \text{ nm}^2$ and $0.42 \text{ nm}^2 \pm 0.04 \text{ nm}^2$ obtained in this study for zeaxanthin and lutein, respectively, correspond

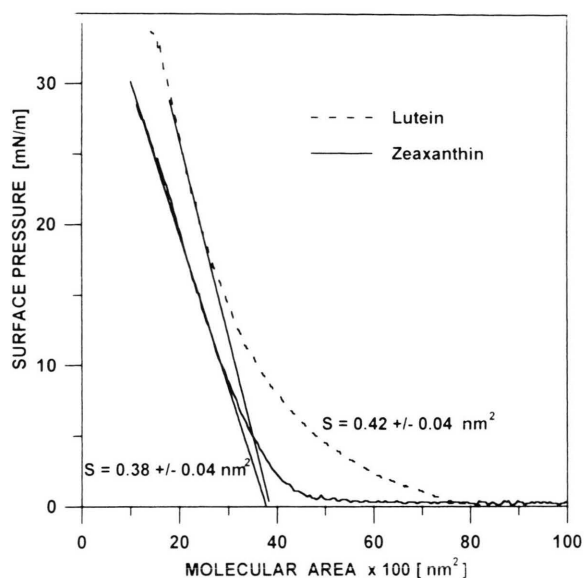


Fig. 5. Isotherms of the compression of monomolecular layers of lutein and zeaxanthin at the air-water interface. Specific molecular areas (S) are found by extrapolation of the linear parts of the isotherms to zero surface pressure. Specific molecular areas for lutein and zeaxanthin (S), presented, are the average of three experiments \pm S. D.

well to the specific molecular areas found under similar conditions (Chifu *et al.*, 1983; N'soukpoe-Kossi *et al.*, 1988). As shown in Fig. 5, despite very close specific molecular areas of both xanthophyll pigments the isotherms of compression differ considerably. Lutein occupies always a larger area at the air-water interface than zeaxanthin at the same surface pressure values. This behaviour of lutein is particularly pronounced at low surface pressure values, namely before the pigment molecules are forced to adopt a vertical orientation when compressing the film. This is a clear indication for a pool of lutein molecules which despite the increasing surface pressure in the course of compressing occupies a planar orientation, presumably as the result of binding to the water surface by their two polar ends. As can be seen, compression of the lutein monolayer to the same average molecular areas as the zeaxanthin monolayer always leads to higher surface pressure values. Fig. 6 shows the isotherms of the compression of monomolecular layers formed with EYPC and mixed layers formed with EYPC and 5 mol% lutein or zeaxanthin. In the case of the two-component films the

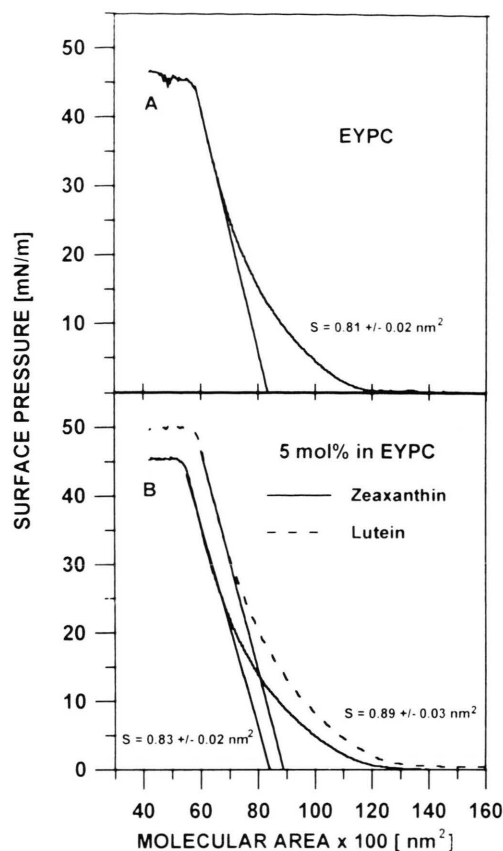


Fig. 6. Isotherms of the compression of monomolecular layers of egg yolk phosphatidylcholine and mixed monolayers of EYPC containing 5 mol% zeaxanthin and lutein as indicated. Other specifications as Fig. 5.

mean molecular areas are larger in monolayers containing lutein than those containing zeaxanthin. This finding along with the effect of the increased surface pressure for the collapse of the film can be interpreted in terms of a lateral orientation of a lutein pool even at high surface pressures which correspond to the surface pressure of natural biomembranes (~ 30 mN/m). Most probably, laterally oriented lutein stabilises the monolayer at high surface pressures via the interaction with polar groups of different lipid molecules. According to a simple estimation following the additivity rule and assuming the specific molecular area of laterally oriented lutein to be 1.5 nm², the difference of 0.05 nm² between the EYPC films containing 5 mol% lutein and zeaxanthin corresponds to a fraction of 27% of lutein molecules ori-

ented vertically and 73% oriented in the plane of the lipid-pigment monolayer.

Fig. 7 shows the effect of the antiserum to zeaxanthin injected into the subphase on a mean molecular area of the lutein- and zeaxanthin-containing EYPC monolayer at the air-water interface, stabilised at relatively low surface pressure and at the surface pressure of biomembranes. At the pressure of 10 mN/m the mean area occupied by a statistical molecule decreases, indicating that water-soluble antibodies extract pigment molecules from the interface. The rate of this extraction is higher in the case of zeaxanthin and lower in the case of lutein, which is characteristic for the structural differences between lutein and zeaxanthin and the exposition of lutein β -ionon rings to the subphase. At the relatively high surface pressure pigment extraction out of the lipid-pigment

monolayer can not be expected. Under such conditions antibodies to zeaxanthin have an opposite effect on monolayers containing lutein and zeaxanthin. The pronounced increase in the mean molecular area observed in the case of zeaxanthin may be interpreted in terms of the penetration of the monolayer with large protein molecules. This is not the case for the lutein-containing film. In this system the decrease in mean molecular area is most probably an effect of antibody-related reorientation of the carotenoid molecules. This reorientation might go from the lateral to the vertical position. The idea of lutein reorientation in response to antibody binding might explain the complexity of this response (Figs. 3A and B).

The above presented studies conducted by spectrophotometry of pigmented-liposome suspensions supplemented with specific antibodies to zeaxanthin and the studies by monolayer technique applied to mono-component xanthophyll films and mixed xanthophyll-lipid films support the model of organisation of a xanthophyll-lipid membrane according to which lutein is distributed in two essentially different pools: one oriented vertically and the other laterally with respect to the membrane plane. The model is different to earlier ones which implied that all polar carotenoid pigments had to span the lipid membrane and would always adopt a more or less vertical orientation (Gruszecki and Strzalka, 1991; Gruszecki, 1995; Strzalka and Gruszecki, 1997; Rohmer *et al.*, 1979; Bone and Landrum, 1984; Bone *et al.*, 1992; Gruszecki and Siewiesiuk, 1990, 1991; Subczynski *et al.*, 1992; Jezowska *et al.*, 1994; Strzalka and Gruszecki, 1994; Gabrielska and Gruszecki, 1996). The present model seems particularly interesting with respect to the physiological role of membrane carotenoids for the absorption and dissipation of excess radiation. The presence of large amounts of lutein in the membranes of insects and particularly so in the membranes of the fovea of the eye is directly linked to this physiological function (Rohmer *et al.*, 1979; Bone *et al.*, 1992). The presence of lutein in two perpendicularly oriented fractions will provide a membrane with an efficient screening system: A light beam which illuminates the surface of the membrane (along the axis normal to the membrane) will not be efficiently absorbed by the pigment which is oriented vertically but will on the other hand be efficiently ab-

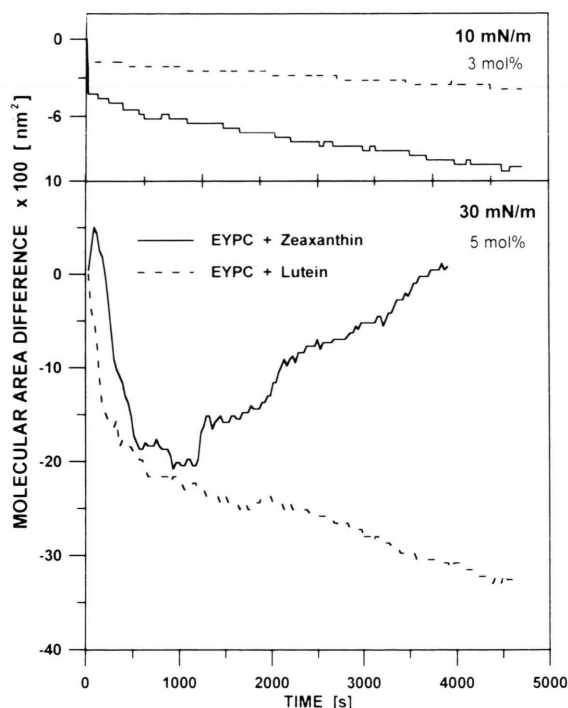


Fig. 7. Differences of the average molecular area of EYPC monolayers formed at the air-water interface and containing lutein or zeaxanthin after injection of 1 ml of antiserum to zeaxanthin to the subphase (130 ml). Monolayers were stabilised at 10 mN/m or 30 mN/m as indicated. The traces give the effects of the active antibodies corrected for the effect of the control serum. The subphase was thermostated at 23 °C and magnetically stirred during experiments.

sorbed by chromophores oriented perpendicularly to the direction of light propagation, that is in the membrane plane. This is a direct consequence of the dependence of light absorption on the square of the cosine of the angle formed by the dipole transition moment of the chromophore and the electric vector of the electromagnetic wave. On the other hand zeaxanthin was reported to be very efficient in the protection against active oxygen species-related membrane degradation (Sielewiesiuk *et al.*, 1997; Woodall *et al.*, 1994, 1998). It seems that it is the localisation and vertical orientation of zeaxanthin, which is the reason for its protective efficiency (Gruszecki, 1999; Woodall *et al.*, 1998). The synergistic effect of lutein present

in the same lipid membrane together with zeaxanthin or other carotenoid pigments in protecting against free radical-induced oxidation of membrane lipids was recently reported (Stahl *et al.*, 1998). This finding corroborates the concept presented in this report on the presence of lutein in two pools.

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