

Incorporation of Lutein and Zeaxanthin into membranes in comparison to other carotenoids and under consideration of their abundance in Retinal Epithelial Cells

PhD Dissertation submitted to Faculty of Physics and
Electrotechnics at



Bremen University, Germany

**In fulfillment of the requirement for the academic degree
of a Doktor der Naturwissenschaften**

-[Dr.rer.nat.]-

By

Medhat Wahba Ismail Shafaa

[Egypt]

December 2004

Referee 1: Prof. Dr. Horst Diehl

Referee 2: Prof. Dr. Carmen Socaciu

Date of oral examination: 17-2-2005

Table of Content

ACKNOWLEDGMENTS	6
LIST OF ABBREVIATIONS.....	7
SUMMARY	8
1.INTRODUCTION	10
2.THEORETICAL BACKGROUND AND LITERATURE REVIEW	13
2.1-ANATOMY OF THE EYE.....	13
2.1.1-SIMPLE ANATOMY OF THE RETINA.....	15
2.2-POTENTIAL ROLE OF LUTEIN AND ZEAXANTHIN IN PROTECTING THE EYE.....	17
2.2.1-SPECTROSCOPIC PROPERTIES OF THE MACULAR CAROTENOIDS	19
2.2.2-THE YELLOW COLOR OF LUTEIN AND ZEAXANTHIN, THE MECHANISTIC BASIS.....	21
2.2.3-APPROXIMATE LOCATION AND DISTRIBUTION OF LUTEIN AND ZEAXANTHIN IN THE RETINA AND MACULA.....	22
2.2.4-POSSIBLE CONTRIBUTION OF LUTEIN AND ZEAXANTHIN TO REDUCE THE RISK FOR AGE- RELATED MACULAR DEGENERATION	24
2.2.4.1-AGE-RELATED MACULAR DEGENERATION [AMD].....	24
2.2.4.2-ETIOLOGY OF AMD AND ANTIOXIDATIVE PROPERTIES OF LUTEIN AND ZEAXANTHIN.....	25
2.2.5-THE PHYSICOCHEMICAL BASIS FOR THE ROLE OF CAROTENOIDS IN PREVENTING AND LIMITING PHOTOCHEMICAL DAMAGE	27
2.2.6-NUTRITIONAL ASPECTS	30
2.3-CAROTENOIDS AS MODULATORS OF MOLECULAR MEMBRANE DYNAMICS.....	31

2.3.1-MEMBRANE STRUCTURE AND FUNCTION	31
2.3.2-PHYSICAL PROPERTIES OF CAROTENOIDS IN RELATION TO MEMBRANE STRUCTURE AND DYNAMICS	36
2.3.3-RELEVANT EXPERIMENTAL DATA REGARDING THE INTERACTION OF CAROTENOIDS WITH MEMBRANES	38
2.3.3.1-CAROTENOIDS IN LIPOSOMES	40
2.3.3.2-CAROTENOIDS IN MICROSOMES	49
2.3.3.3-CAROTENOIDS IN RETINAL PIGMENT EPITHELIUM [RPE] CELLS AS “ IN VITRO MODEL ”	51
2.4-INTRODUCTION TO FLUORESCENCE TECHNIQUES	52
3.EXPERIMENTAL	57
3.1. CHEMICALS	57
3.2. INCORPORATION OF CAROTENOIDS INTO LIPOSOMES	59
3.3. EVALUATION OF CAROTENOID INCORPORATION AND CAROTENOID STABILITY IN LIPOSOMES BY UV-VIS SPECTROMETRY	60
3.4. EVALUATION OF PHOSPHOLIPID CONTENT BY STEWART ASSAY	61
3.5. INCORPORATION OF CAROTENOIDS INTO LIVER MICROSOMES	63
3.6. EVALUATION OF CAROTENOID INCORPORATION AND CAROTENOID STABILITY IN MICROSOMES BY UV-VIS SPECTROMETRY	65
3.7. FLUORESCENT ASSAYS FOR LIPOSOMES AND MICROSOMES	66
3.8. EVALUATION OF CAROTENOID UPTAKE BY RPE CELLS, TYPE D407	68
3.9. FLUORESCENCE STUDIES ON D407 RPE CELLS	70
4.RESULTS AND DISCUSSION	72

4.1. CAROTENOIDS INCORPORATED INTO LIPOSOMES.....	72
4.1.1. EFFECTIVE CONCENTRATIONS, INCORPORATION YIELDS AND STABILITY OF CAROTENOIDS IN LIPOSOMES	72
4.1.2. EFFECTS OF CAROTENOIDS ON MEMBRANE PROPERTIES, EVALUATED BY FLUORESCENCE SPECTROMETRY OF LAURDAN - AND DPH - LABELED LIPOSOMES	77
4.2. CAROTENOIDS INCORPORATED INTO MICROSOMES.....	86
4.2.1. EFFECTIVE CONCENTRATIONS, INCORPORATION YIELDS AND STABILITY OF CAROTENOIDS IN MICROSOMES	86
4.2.2. EFFECTS OF CAROTENOIDS ON MEMBRANE PROPERTIES, EVALUATED BY FLUORESCENCE SPECTROMETRY OF LAURDAN - AND DPH - LABELED MICROSOMES	91
4.3. CAROTENOIDS INCORPORATED INTO D407 RPE CELLS	99
4.3.1. EFFECTIVE CONCENTRATIONS AND INCORPORATION YIELDS OF CAROTENOIDS IN CELLS	99
4.3.2. EFFECTS OF CAROTENOIDS ON MEMBRANE PROPERTIES, EVALUATED BY FLUORESCENCE SPECTROMETRY OF LAURDAN-LABELED CELLS	105
5. CONCLUSION	110
LITERATURE CITED.....	116

Acknowledgments

I wish to express my deep gratitude and respect to **Prof. Dr. Horst Diehl**, Biophysics Institute, Bremen University, Germany, for giving me the opportunity to work in his laboratory, support and the great hospitality he offered me. I am thankful for his encouragement and guidance he gave me during the course of this investigation. I would like to express my gratitude for his patience, understanding, and constructively critical eye. I appreciate his vast knowledge and skill in many areas and his assistance in writing this thesis.

My deep and sincere thanks and gratitude go out to **Prof. Dr. Carmen Socaciu**, Dept. of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, for giving me a closer guidance, experience and suggestions throughout the completion of this work. I am deeply grateful to her tender encouragement, constructive advices, endless support and scientific attitude that I owe a great deal.

My appreciation and thanks to **Christina Kenst**, Biophysics Institute, Bremen University, Germany, for her valuable technical assistance.

I am deeply grateful to *the Egyptian Government* for the financial support that allowed me to prepare this PhD Dissertation and to have this international experience.

Very special thanks to **Prof. Dr. Aleya Khattab**, Egyptian Cultural Counselor and the Head of Educational Missions, Berlin, Germany for her generous assistance, follow up and advice. She was gracious and generous with her time which enabled me to accomplish this work.

I would also like to thank **my parents** and **my wife** for the tremendous support they provided me through my entire life, without whose encouragement and guidelines, I would not have finished this work.

List of Abbreviations

AMD	Age Macular degeneration	EXC	Excitation
AAPH	2,2'-azobis[2-amidinopropane hydrochloride]	EYPC	Egg yolk phosphatidylcholine
BC	β -Carotene	FCS	Fetal calf serum
BHT	Di- <i>t</i> - buthyl- <i>p</i> - cresol	GP	Generalized Polarization
C₆-NBD-PC	Hexanoyl [7-nitro-2,1,3-benzoxadiazol-4-yl] 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine	HPLC	High performance liquid chromatography
CTX	Canthaxanthin	IC	Incubated concentration
DiI-C₁₈	1,1-Dioctadecyl-3,3,3,3'-tetramethylindocarbo-cyanine perchlorate	IY	Incorporation yield
DMEM	Dulbecco Modified Eagle Medium	Laurdan	6-Dodecanoyl-2-dimethylamine-naphthalene
DMF	Dimethyl formamide	LUT	Lutein
DMPC	Dimyristoylphosphatidylcholine	LUV	Large unilamellar vesicles
DPH	1,6-Diphenyl-1,3,5-hexatriene	MLV	Multilamellar vesicles
DPPC	Dipalmitoylphosphatidylcholine	NMR	Nuclear magnetic resonance
DPPE	1,2-Dipalmitoyl phosphatidyletanolamine	PBS	Phosphate buffer saline
DPPS	1,2-Dipalmitoyl diphosphatidylserine	PC	Phosphatidylcholine
DSC	Differential scanning calorimetry	ROS	Reactive oxygen species
EC	Effective concentration	RPE	Retinal Pigment Epithelium
EM	Emission	SUV	Small unilamellar vesicles
EPR	Electron paramagnetic resonance	THF	Tetrahydrofuran
EtOH	Ethanol	ZEA	Zeaxanthin

Summary

Lutein and zeaxanthin are two carotenoids among hundreds that exist in nature. These two substances are unique because they are selectively accumulated in the macular membranes of the eye and provide what is known as the macular pigment. The regular distribution pattern of these carotenoids within the human macula indicates that their deposition is actively controlled in this tissue. The incorporation yields of four structurally different carotenoids : β -carotene, canthaxanthin, lutein and zeaxanthin in a model [small unilamellar liposomes] and natural membranes [microsomes and retinal pigment epithelium] were comparatively investigated. We applied and quantified two methods of preparations to incorporate carotenoids into liposomes and microsomes: *Preparation I* where a fixed incubated concentration [IC] was used for each carotenoid and then the effective concentration [EC] and incorporation yield [IY] values were monitored. *Preparation II* where we used different IC, based on the informations obtained from *Preparation I*, in order to achieve a similar or close EC values of carotenoids inside membrane.

Moreover, we used retinal pigment epithelium cells to incorporate the same carotenoids using as well two different procedures. By *Procedure I* carotenoids were delivered to cells in fetal calf serum as vehicle at equal IC and then the EC and IY values were monitored. Alternatively by *Procedure II* the carotenoids were delivered to cells in tetrahydrofuran as vehicle at equal IC and then the EC and IY values were monitored. The effects of carotenoid intercalation into these membranes were evaluated by UV-Vis spectrophotometry.

The UV-Visible study showed that the membrane structure was affected very differently by incorporating polar carotenoids [lutein and zeaxanthin] or β -carotene. The incorporation of β -carotene at different concentrations resulted in a very small miscibility of β -carotene with the phospholipid bilayer while a high miscibility was seen for lutein and

zeaxanthin. Canthaxanthin exhibited an intermediate miscibility. The stability of carotenoid formulations in liposomes and microsomes under storage was measured by monitoring the shape of UV-Vis spectra and the percentage of carotenoid content which remained incorporated into the membrane. The stability test revealed that lutein was the most stable carotenoid upon storage, followed by zeaxanthin. β -carotene and canthaxanthin decayed rapidly in liposomes and gradually in microsomes. The slow degradation of lutein and zeaxanthin may be one explanation for the prevalence of these carotenoids in the retina of man and other species. The quenching ability of carotenoids incorporated into membrane systems simultaneously loaded with the fluorescent dyes Laurdan and DPH has been investigated. Lutein and zeaxanthin were the strongest quenchers of fluorescence intensity compared to other carotenoids. The Laurdan quenching even offers a method to determine the carotenoid contents intrinsically from membranes. The inclusion of lutein and zeaxanthin into the lipid bilayer is associated with increased rigidity of the membrane [as estimated by a generalized polarization parameter derived from Laurdan emission spectra] which is not observed in membranes prepared with β -carotene. Such differences are discussed in terms of carotenoid structure and its influence on the physical properties of the lipid membrane.

1. Introduction

Carotenoid pigments are widely present in living organisms: plants, animals, and humans [Britton *et al.*, 1995]. Physiological functions of plant carotenoids are directly related to the place they appear. In the case of the photosynthetic apparatus, carotenoids play the role of accessory pigments and photoprotectors against oxidative damage. This protection, necessary to maintain the physiological activity of chloroplasts subjected to illumination under the condition of accompanying oxygen evolution, is realized via two main physical mechanisms. These mechanisms are the quenching of the triplet state of chlorophyll, which is a very efficient photosensitizer, and the quenching of active oxygen species: singlet oxygen and free radicals. Basically, the same protective mechanisms of carotenoid pigments are recognized to play a major role in other tissues of living organisms, including humans. Interestingly, the macular membranes of human retina contain principally two polar carotenoid pigments, lutein and zeaxanthin. Two main physiological functions are ascribed to the macular xanthophylls: protection against photoinduced damage of the membrane system and a shielding effect protecting the system against potentially harmful, short-wavelength radiation. The question still open is why the macular membranes always contain both, lutein and zeaxanthin, and not just one pigment and not other carotenoids? What role[s] could the macular pigment play in possibly protecting the retina in general, and the macula in particular? First and foremost, these are colored compounds, and as such, absorb visible light. Even though the structures are very similar, the β,ϵ -structure of lutein means that there are 10 conjugated double bonds in the molecule, whereas zeaxanthin, with the β,ϵ -structure, has 11 conjugated double bonds [fig.2-6]. As such, lutein absorbs at slightly shorter wavelengths than does zeaxanthin. This difference is depicted in [fig.2-7]. Both lutein and zeaxanthin are effective in

filtering blue light [400–475 nm], but zeaxanthin is much more effective in absorbing blue-green light at 500 nm and slightly above. This ability to filter out blue light on entering the retinal tissue has the effect of decreasing the chromatic aberration associated with the lower wavelengths of visible light, i.e., the blue and blue-green region of the visible spectrum. This action could therefore explain the presence of coloured carotenoids in the macular region, but it does not explain why the two xanthophylls, lutein and zeaxanthin, have been selected from the more than 20 carotenoids present in human plasma [*Khachik et al., 1997*]. β -Carotene, lycopene or β -cryptoxanthin, also present in human plasma at concentrations equivalent to that of lutein and zeaxanthin [*Krinsky et al., 1990*], would also serve as effective filters of blue light, but they have not been selected for this action. What are the distinguishing characteristics of the macular carotenoids, lutein and zeaxanthin, that account for their virtually exclusive accumulation within the primate macula? What makes them functionally unique? There must be some specific property of these xanthophylls that might help explain their presence in the primate retina. One such property is their disposition in biological membranes. Both biological functions proposed for lutein and zeaxanthin in the retinal membranes of an eye require a special arrangement of xanthophyll pigment molecules within the membranes. The two hydroxyl groups, at either end of the retinal carotenoids may have special importance. Zeaxanthin was found to adopt a roughly perpendicular orientation to the plane of the membrane, whereas lutein appeared to exist in two distinct pools in these membranes. One pool followed the orientation of zeaxanthin, whereas the second pool was parallel with respect to the membrane. Such a localization and orientation of lutein and zeaxanthin has a pronounced effect on the structural properties and molecular dynamics of lipid membranes in contrast to the apolar β -carotene. β -Carotene, lacking hydrophilic substitutes, remains entirely within the hydrophobic core of the membrane and it

retains a substantial degree of mobility which suggests a less orderly integration into the membrane structure. Fluorophores designed to localize within a specific region of a biological specimen easily share membrane locations with carotenoids. In addition to the high detection sensitivity, the fluorescence process is sensitive to subtle changes in molecular environment. Therefore, the use of fluorescent probes is a powerful method to obtain information about the structure, function, and health of cells. The fluorescent dyes Laurdan and DPH have been used to label model membranes. Special fluorescence characteristics obtained from model membranes which have been doped with carotenoids, were evaluated in order to interpret mechanisms involved. Our purpose in this study is to clarify definitive differences at molecular level between carotenoids with a polar group [lutein and zeaxanthin], the less polar carotenoid canthaxanthin and β -carotene lacking a polar group with regard to their distribution and positioning and/or orientation in the membranes. We have examined [1] the miscibility or incorporation yield of the carotenoid into model or native membranes using a spectrophotometer technique, [2] the stability of carotenoids in membranes upon storage using spectrometry, and furthermore, [3] the quenching abilities of the main dietary carotenoids incorporated into a membrane to fluorescent dyes. Several aspects resulting from these examinations can be potentially analyzed in order to understand better the physiological importance of lutein and zeaxanthin in the vision apparatus.

2.Theoretical background and Literature Review

2.1-Anatomy of the Eye

The eye is a complex organ composed of many parts. Good vision depends on the way in which those parts work together. The basic structures of the eye are explained in [fig.2-1] as follows:

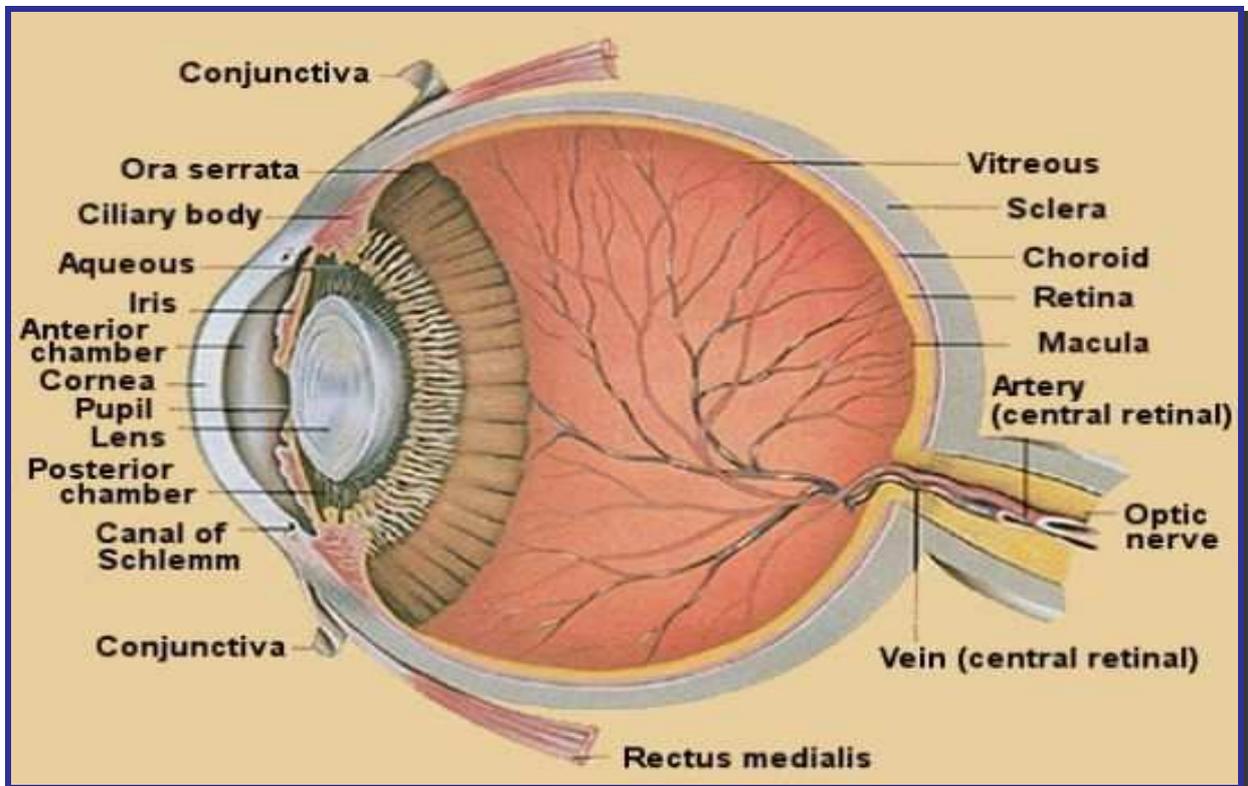


Fig.2-1.: A simple diagram of the eye.

- The **anterior chamber** is an aqueous area bound in front by the cornea and in back by the lens.
- The **aqueous** area contains a clear, watery solution in both anterior and posterior chambers.
- The **artery** is the vessel supplying blood to the eye.

- The **canal of Schlemm** is the passage way for the aqueous fluid to leave the eye.
- The **choroid**, which carries blood vessels, is the inner coat between the sclera and the retina.
- The **ciliary body** is a hidden part of the iris, which together with the ora serrata forms the uveal tract.
- The **conjunctiva** is a clear membrane covering the white of the eye [sclera].
- The **cornea** is a clear, transparent part of the outer coat of the eyeball through which light passes to the lens.
- The **iris** gives our eyes color and it functions like the aperture of a camera, enlarging in dim light and contracting in bright light. The aperture itself is known as the pupil.
- The **lens** focuses light on the retina.
- The **macula** is a small area in the retina that provides our most central, acute vision.
- The **optic nerve** conducts visual impulses from the retina to the brain.
- The **ora serrata** and the ciliary body form the uveal tract, hidden part of the iris.
- The **posterior chamber** is the area behind the iris, in front of the lens, which is filled with aqueous solution.
- The **pupil** is the opening, or aperture, of the iris.
- The **rectus medialis** is one of the six muscles of the eye.
- The **retina** is the innermost coat of the back of the eye, formed of light-sensitive nerve endings that carry the visual impulse to the optic nerve. The retina may be compared to the photo-sensitive film of a camera.
- The **sclera** is the white of the eye.
- The **vein** is the vessel that carries blood away from the eye.

- The **vitreous** is a transparent, colorless mass of soft, gelatinous material filling the eyeball behind the lens [*webvision, October 2003*].

2.1.1-Simple anatomy of the retina

When an ophthalmologist uses an ophthalmoscope to look into a eye he sees the following view of the retina [fig.2-2].

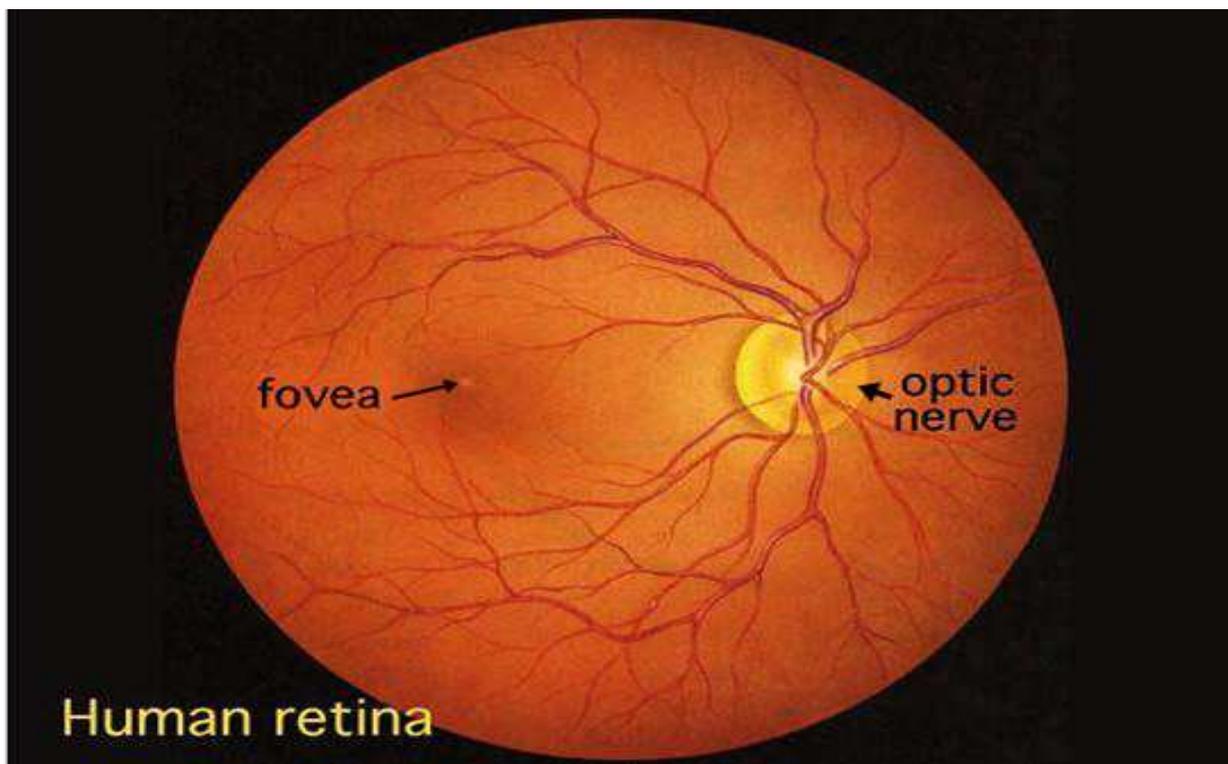


Fig.2-2.: Human retina as seen through an ophthalmoscope.

In the center of the retina is the optic nerve, a circular to oval white yellowish area measuring about 2 x 1.5 mm across. From the center of the optic nerve radiate the major blood vessels of the retina. Approximately 17 degrees [4.5-5 mm], or two and half disc diameters to the left of the disc, can be seen the slightly oval-shaped, blood vessel-free reddish spot, the fovea, which is at the center of the area known as the macula by ophthalmologists. Familiar to ophthalmologists is a yellow

pigmentation to the macular area known as the macula lutea [yellow around fovea]. This pigmentation is the reflection from yellow screening pigments, the xanthophyllic carotenoids, zeaxanthin and lutein. The physiological significance of this selective accumulation of xanthophylls is based on filtration of potentially damaging blue light, on their quenching of photochemically-induced reactive oxygen species, attenuation of chromatic aberration, and inhibition of apoptosis. It is believed that via these mechanisms, lutein and zeaxanthin can contribute to reduce the risk for age related macular degeneration, the leading cause of irreversible loss of vision in ageing populations [*webvision, October 2003*].

The yellow pigment that forms the macula lutea in the fovea can be clearly demonstrated by viewing a section of the fovea in the microscope with blue light [fig.2-3]. The dark pattern in the foveal pit extending out to the edge of the foveal slope is caused by the macular pigment distribution indicating an absorption of blue light by the macular pigment took place [*Snodderly et al., 1984*].

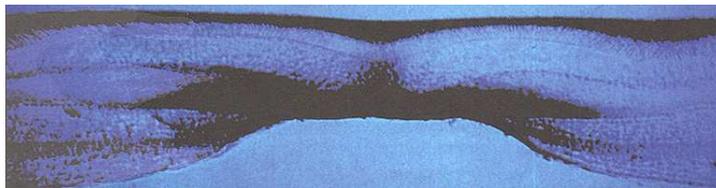


Fig.2-3.: Vertical section through the monkey fovea to show the distribution of the macula lutea [black].

The retina is composed of several layers with different roles [fig.2-4]. The first layer encountered by light is called the nerve fiber layer. Here, the nerve cells travel from all the parts of the retina to the optic nerve. Under this layer are found most of the retinal blood vessels. They are responsible for nourishing the inner parts of the retina. The outermost layer is the photoreceptor layer. The

photoreceptor layer, composed of cones for fine and color vision, and rods for vision in dim light, consists of the cells that actually convert light into nerve impulses. There are approximately 120 million rods and 6 million cones in a human retina. Most of the cones are located in the macula. The photoreceptor cells lie on top of a layer of cells called the retinal pigment epithelium or RPE. The RPE is responsible for keeping the photoreceptors healthy and functioning well. Under the RPE is the retina's second set of blood vessels which are in a layer called the choroid. The RPE, fed by the blood vessels of the choroid, nourish the photoreceptors. The macular carotenoids are most densely localized between the incoming light and the photoreceptors where they could efficiently act as a blue light filter, shielding the most delicate functional structures of the retina including the photoreceptors, retinal pigment epithelium and the underlying choriocapillaris from this radiation. At the same time, however, carotenoids are close enough to the photoreceptors to allow a direct chemical quenching [Kirschfeld, 1982].

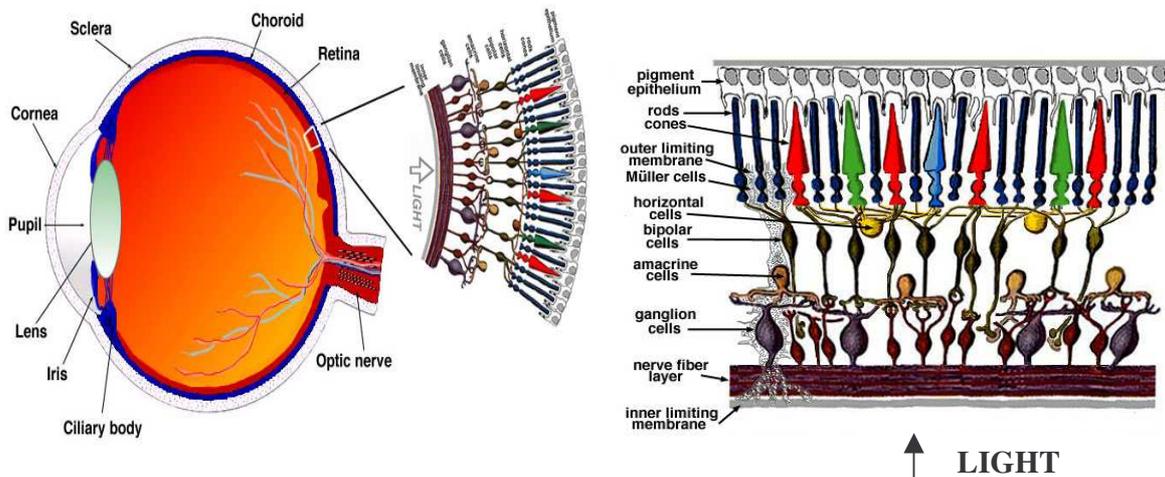


Fig.2-4.: Simple diagram of the organization of the retina.

2.2-Potential role of Lutein and Zeaxanthin in protecting the eye

The carotenoids are among the most common pigments in nature and are natural lipid soluble antioxidants [Machlin and Bendich, 1987]. β -carotene is the

best studied carotenoid because of its importance as a vitamin A precursor. However, it is only one of the approximately 600 naturally occurring carotenoids [Erdman, 1988]. In addition to β -carotene, α -carotene, lutein and lycopene are important carotenoid components of the human diet [Micozzi *et al.*, 1990].

The xanthophylls, lutein and zeaxanthin, may play a critical function for maintenance of normal visual function. These polar compounds are the predominant carotenoids in the macula, while other non-polar carotenoids, including β -carotene and lycopene are absent [Bone *et al.*, 1988 ; Handelman *et al.*, 1988]. The high concentrations of these carotenoids are responsible for the yellowish color of this region of the retina designated as the macula lutea or “yellow spot”. Like in the macula, the predominant carotenoids in the lens are lutein and zeaxanthin, while β -carotene and lycopene have not been detected in human lenses [fig.2-5] [Cognis website, May 2003].

● Lutein/Zeaxanthin

● Lycopene

● α -Carotene

● β -Carotene

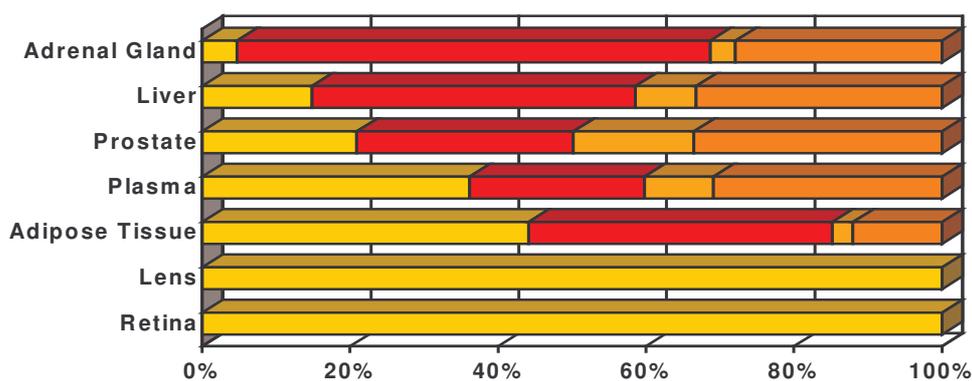


Fig.2-5.: Distribution of Lutein and Zeaxanthin in different tissues.

The hydroxy groups of the polar carotenoids may allow them to incorporate into cell membranes in an ordered orientation that stabilizes the membrane [fig.2-6] [Snodderly, 1995]. A membrane-spanning orientation of a typical xanthophyll molecule seems to be a direct consequence of reasonably good matching of the thickness of the hydrophobic core of biomembranes [about 3 nm] and the distance between opposite polar groups [3 to 3.2] nm depending on the exact location of oxygen atoms in the carotenoid molecule [Milon *et al.*, 1986a]. The condition of matching the distance between polar groups and the thickness of the membrane hydrophobic core leads to relatively good predictions of a carotenoid orientation, in particular in the case of fluid membranes formed with unsaturated lipids [egg yolk phosphatidylcholine] [Gruszecki, 1999].

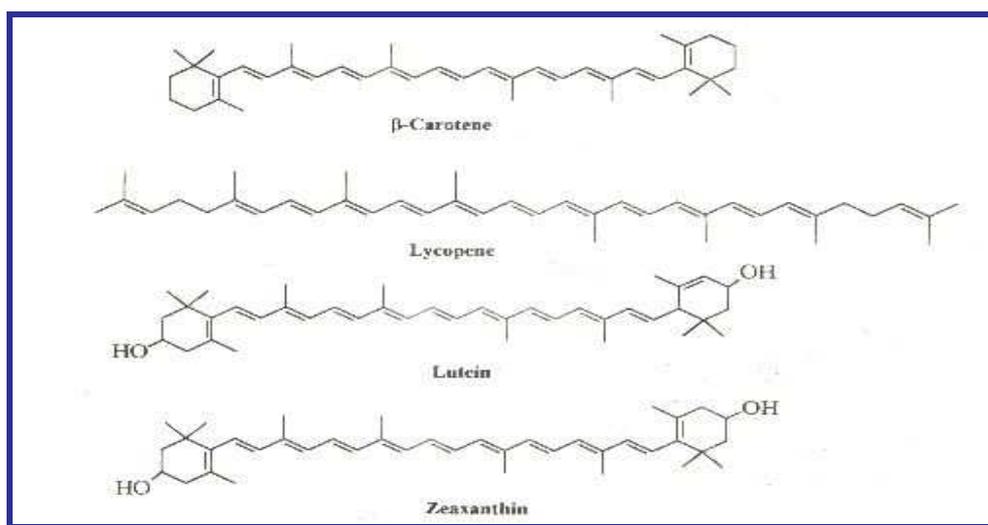


Fig.2-6.:Chemical structure of β -carotene and lycopene [not found in the retina], compared with the structures of zeaxanthin and lutein, the retinal carotenoids that form the macular pigment.

2.2.1-Spectroscopic properties of the macular carotenoids

The most obvious characteristic of all carotenoids is their intense colouration, as a result of the extensive π - π conjugation in the polyene chain leading to absorption in the visible specific region above 400 nm [Britton, 1995].

The colour differences of carotenoids arise from the differences in the number of conjugated bonds: more extended conjugation [more double bonds] determine absorption at higher wavelengths [bathochromic effect]. Lutein and zeaxanthin differ very slightly in colour. Purified zeaxanthin typically has a rosy appearance, which is not observed in lutein [deep yellow]. In both of these carotenoids the number of fully conjugated double bonds in the polyene chain is $n = 9$. The extent of conjugation in the zeaxanthin molecule is longer [β,β -ring] [consisting of 11 double bonds] than that of lutein [β,ϵ -ring, 10 double bonds] explaining the higher λ_{\max} [451 nm] for zeaxanthin than for lutein [445 nm].

The shape of the UV-Visible spectra of lutein and zeaxanthin, in ethanol, are almost the same. They have a typical carotenoid fine structure of three peaks [fig.2-7]. A central maximum [II] is flanked at longer wavelengths by a secondary maximum [III] and a distinct shoulder at shorter wavelengths [I]. These transitions are numbered I, II, and III from short to long wavelength [Britton, 1995]. The presence of the added interaction of the second β -ring double bond in zeaxanthin with the extended conjugation of the polyene chain, very slightly lowers the energy separation between the ground state and the excited state. This small effect results in a roughly 6-nm red-shift in the absorption maximum of zeaxanthin [$\lambda_{\max} = 451$ nm in ethanol] when compared to that of lutein [$\lambda_{\max} = 445$ nm] [fig.2-7]. The other optically significant difference in these two carotenoids is the distance between peaks II and III, illustrated by the intervening minimum, and the ratio of their intensities. For zeaxanthin, peak II and III are both somewhat broader and the spacing between them is smaller, resulting in a less distinct maximum for III with a more shallow minimum between peaks II and III. For zeaxanthin, $\lambda_{\text{III}} - \lambda_{\text{II}}$ is 26 nm and the II/III ratio is 38%. The corresponding spacing for lutein is 29 nm and a distinct minimum between II and III is observed in well-purified samples. The lutein II/III ratio is 60%. For lutein, peak I is a fully distinct maximum, not a

shoulder. Both lutein and zeaxanthin are effective in filtering blue light [400-475 nm], but zeaxanthin is much more effective in absorbing blue-green light at 500 nm and slightly above [fig.2-7].

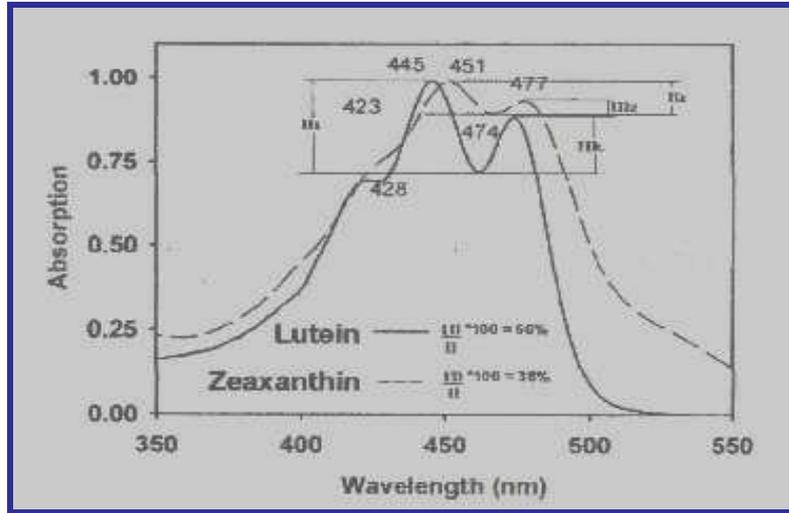


Fig.2-7.: Spectra of lutein and zeaxanthin, in ethanol, illustrate the characteristic differences in the absorption properties of the two carotenoids [adapted from *Landrum and Bone, 2001*].

2.2.2-The yellow color of lutein and zeaxanthin, the mechanistic basis

Carotenoids appear yellow because they absorb blue light [blue being the complementary color of yellow]. On the other hand, blue light can damage the retina [*Ham and Müller, 1989; Gottsch et al., 1990*], and this property of carotenoids is one basis for their physiological action in the retina. The relationship between the wavelength of blue light and its potential to induce damage in the retina is expressed by the “Blue light hazard function” [*Ham and Müller, 1989*]. This function is maximized at around 450 nm, the wavelength at which lutein and zeaxanthin absorb light is shown in [fig.2-8]. Thus, these carotenoids can absorb blue light before it initiate a damaging reactions in the photoreceptors. Their location just in front of the photoreceptors, is appropriate and efficient to their filter action and also explains the classical function of a macular pigment, namely the

attenuation of chromatic aberration [a defect in a lens system in which different wavelengths of light are focused at different distances because they are refracted through different angles. It produces a blurred image with colored fringes].

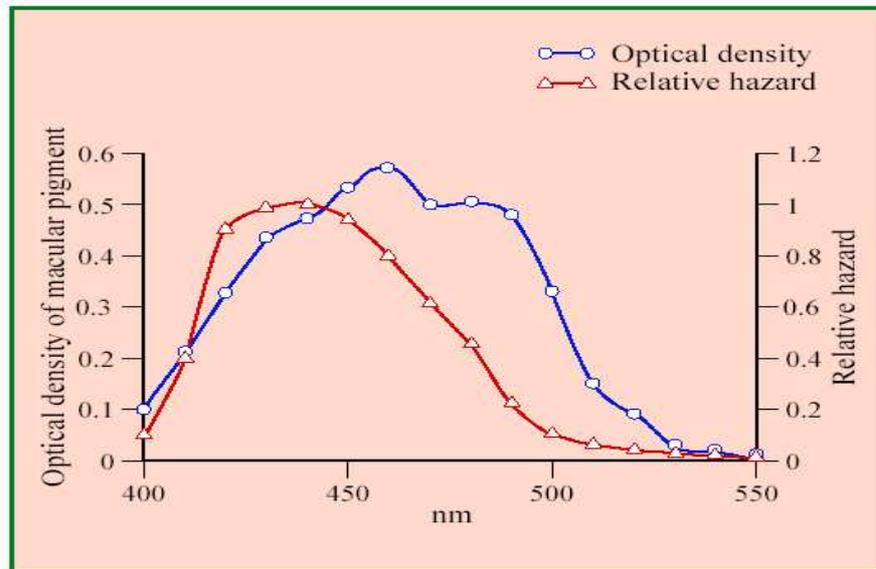


Fig.2-8.: Blue light hazard function and absorption spectrum of macular pigment [adapted from *Ham and Müller, 1989*].

2.2.3-Approximate location and distribution of lutein and zeaxanthin in the retina and macula

Fig.[2-9] is a schematic representation of the localization of the macular pigment within the retina, relative to the incoming light and photoreceptors. While the visibility of the yellow color marks just the regions of highest concentration of the macular pigment, carotenoids can also occur in other regions of the retina as indicated recently [*Sommerburg et al.,1999;Rapp et al.,2000*].

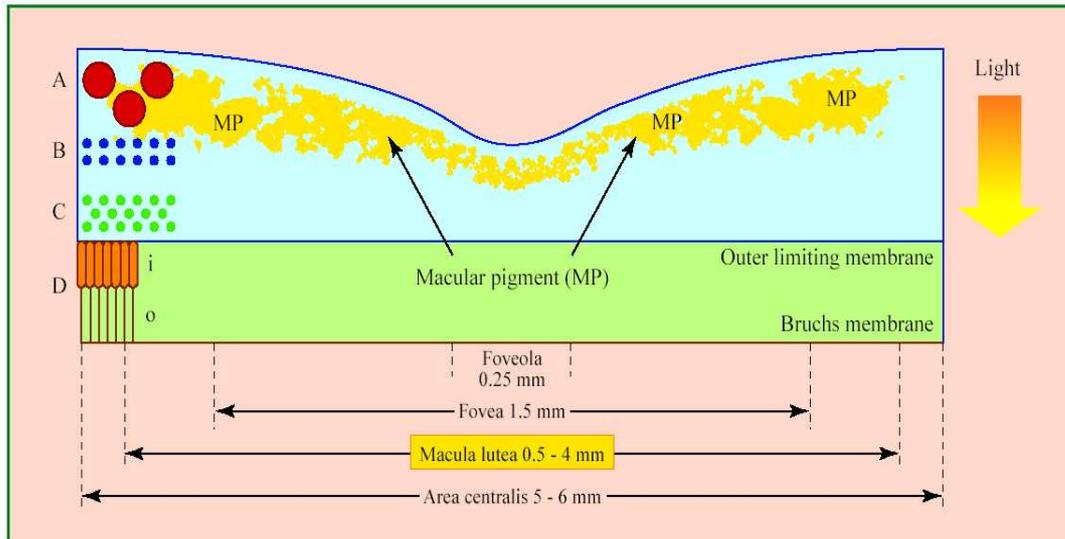


Fig.2-9.:Diagram of approximate location of the macular pigment in the retina [adapted from *Schalch, 2000*]. The outermost layer [farthest from the incoming light] contains the rods and cones. The middle layer contains the bipolar neurons. The inner layer [closest to incoming light] contains the ganglion cells. The axons of the ganglion cells make up the optic nerve, [see fig.2-4 for more details]. Rods and cones consist of outer segment and inner segment.

Bone & Landrum carried out the first chromatographic characterization of the macular pigment using high performance liquid chromatography [HPLC] analysis to demonstrate that there are actually two xanthophylls present in the macula, namely lutein and zeaxanthin [*Bone et al., 1985 and Bone et al., 1988*]. Shortly thereafter, a different ratio between lutein and zeaxanthin in the central fovea and the more peripheral regions was reported, more zeaxanthin than lutein being present in the center of the macula. Zeaxanthin declines very rapidly towards the edge of the macula, so that lutein becomes dominant carotenoid at the periphery. The ratio of lutein to total zeaxanthin is about 0.8 in the center of the macula and about 2.4 in the peripheral retina, whereas in plasma, lutein-to-zeaxanthin ratios from 7 to 4 are observed. Thus, it appears that nature by still unknown mechanisms not only facilitates the accumulation of the lutein and zeaxanthin specifically in the macula, but also reverses the ratio of lutein to zeaxanthin found in plasma, making

zeaxanthin dominant in the center of the macula and lutein dominant in the peripheral retina.

Further evidence that lutein predominates peripherally was provided by the finding that subretinal fluid extracts from patients with rhegmatogenous retinal detachment*, a disease that primarily involves the peripheral retina, contain retinol and lutein, but no zeaxanthin, as determined by HPLC analysis [Chan *et al.*, 1998].

It is important to note that the non-polar carotenoids, β - carotene and lycopene, two major carotenoids in human plasma, are not found in the human retina [Handelman *et al.*, 1988]. This indicates that the polarity of a molecule may determine its potential to have access to the retina. Interestingly, the apolar β -carotene was not detected in a postmortem retina from a subject who has been taking very high doses of a combination of β - carotene and canthaxanthin, whereas the less polar carotenoid canthaxanthin was present in substantial amounts [Daicker *et al.*, 1987].

2.2.4-Possible contribution of lutein and zeaxanthin to reduce the risk for age-related macular degeneration

2.2.4.1-Age-related Macular Degeneration [AMD]

AMD is the major cause of severe irreversible vision loss in the western world among persons older than 50 [Bressler *et al.*, 1988]. The majority of authors [Bressler *et al.*, 1988] understand AMD as a complex clinical picture which in the presence of some degree of visual loss is manifested by drusen, yellowish-white, elevated, and often confluent nodules consisting of abnormal glycoproteins and glycolipids at the base of the RPE cells [Frank, 1989]. This induces a regionally localized atrophy of the RPE and retinal changes associated with

* Retinal separation associated with a break, a hole, or a tear in the sensory retina.

choroidal neovascularization [fig.2-10]. Drusen have unusually high lipid content and it has been suggested that they form when lipofuscin builds up in the RPE and just below it. Lipofuscin is a fluorescent catabolic product of lipid peroxidation resulting from the lifelong shedding of photoreceptor. It has been postulated that exposure of the retina to excessive blue light may increase the rate of lipofuscin formation.

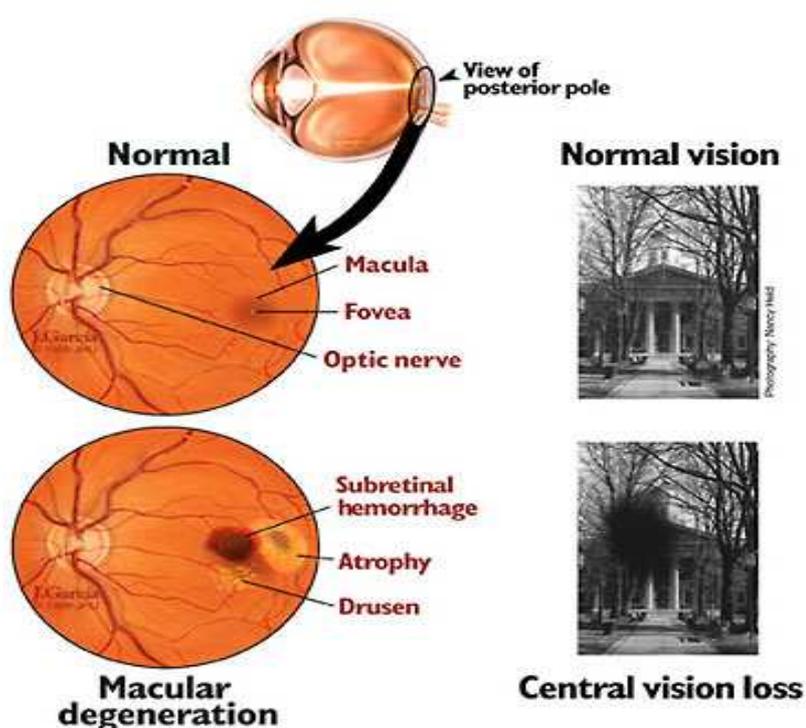


Fig.2-10.: Fundus photograph of 2 persons normal or patients with AMD and the same scene as it might be viewed by these persons.

2.2.4.2-Etiology of AMD and antioxidative properties of lutein and zeaxanthin

The etiology of AMD is only poorly understood and both, genetic and environmental factors have been hypothesized to play a role. One environmental factor seems to be ocular exposure to sunlight [McCarty and Taylor, 1999], in particular a history of exposure to blue light in the preceding 20 years [Taylor et

al., 1992]. In the presence of photosensitizers, light can induce oxidative damage. However, oxidative damage can also be mediated independently of light by endogenous metabolic processes. The retina is highly active metabolically and has a much higher blood flow than other tissues. In such an environment, characterized by simultaneous presence of light and oxygen, numerous reactive oxygen species [ROS], including singlet oxygen and the superoxide radicals, can be generated [Schalch, 1992]. The ROS induce peroxidation of polyunsaturated fatty acids. Through such damage, the integrity of the complex of photoreceptors and the retinal pigment epithelium is impaired, as well as the cyclic process of photoreceptor phagocytosis and renewal. Ultimately, this can lead to the accumulation of cell debris and lipofuscin in Bruch's membrane, drusen formation, and finally neovascularization and retinal detachment. Effective treatments for the neovascular form of the disease other than Laser and/or Verteporfin are not known [Fine, 1999].

The screening effect of carotenoids, described above, attenuates blue light and thus indirectly limits the photochemical generation of reactive oxygen species mediated via endogenous or exogenous photosensitizers. However, carotenoids in general, and lutein and zeaxanthin in particular, have intrinsic properties that result in direct quenching of these potentially damaging reactive entities. This quenching capability of carotenoids depends on the number of conjugated double bonds. As can be seen from the chemical structure of lutein and zeaxanthin [fig.2-6], lutein has 10 conjugated double bonds, while zeaxanthin contain 11 bonds and it is indeed a better singlet oxygen quencher than lutein [Conn *et al.*, 1991]. This may explain the preponderance of zeaxanthin over lutein in the macular center [fig.2-11], where the incident light is most intense and the formation of reactive oxygen species is most intensive. Given that much of the retinal zeaxanthin has a chiral isomer [meso-zeaxanthin] which is not present in the blood [Bone *et al.*, 1993], it has been

suggested that lutein may be the substrate converted into meso-zeaxanthin by the retina [Bone *et al.*, 1993], because lutein is more available from food and is found in blood in higher concentrations than zeaxanthin [Gross, 1987,1991]. Such a conversion could be possible by a shift of the double bond in the lutein molecule. This conversion has the advantage of giving the retina a broader array of sources for its local zeaxanthin [better singlet oxygen quencher].

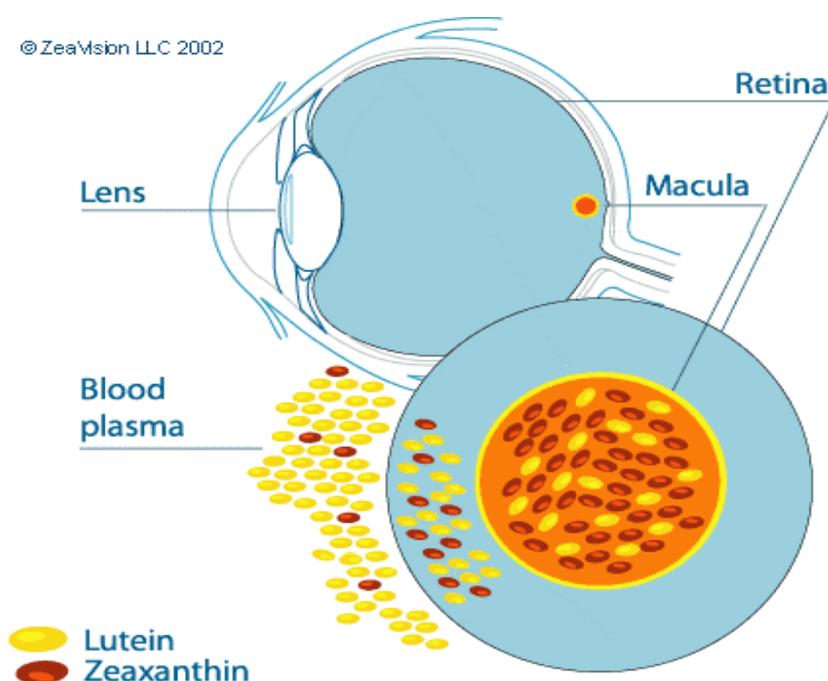


Fig.2-11.: Macular preference for zeaxanthin. Adapted from www.zeavision.com

2.2.5-The physicochemical basis for the role of carotenoids in preventing and limiting photochemical damage

If light of appropriate wavelength is absorbed by a sensitizer, this molecule can be excited to its first excited singlet state [1S]. This singlet state has a very short lifetime [$< 10^{-7}$ sec] and dissipates its energy either by interacting with the solvent, emitting a photon in the process of fluorescence or by a radiationless transition to

its lowest excited triplet state [3S]. This triplet state is of central importance for photochemical reactions. The efficiency of any photosensitizer is determined by its ability to form a long-living triplet state with a high quantum yield [fig.2-12]. The retina contains a large number of molecules which absorb visible or near-ultraviolet light and therefore can act as photosensitizers [Dayhaw-Barker, 1986].

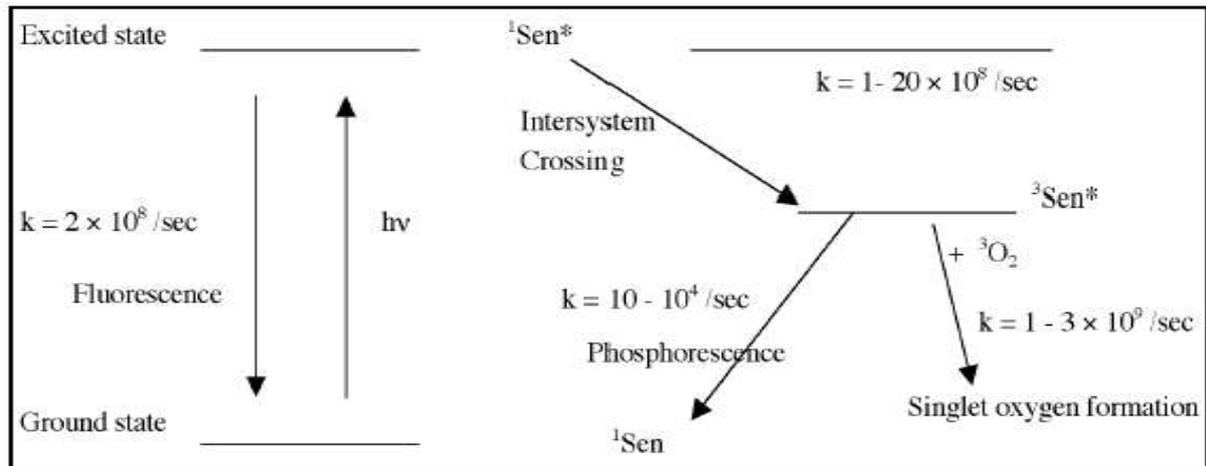
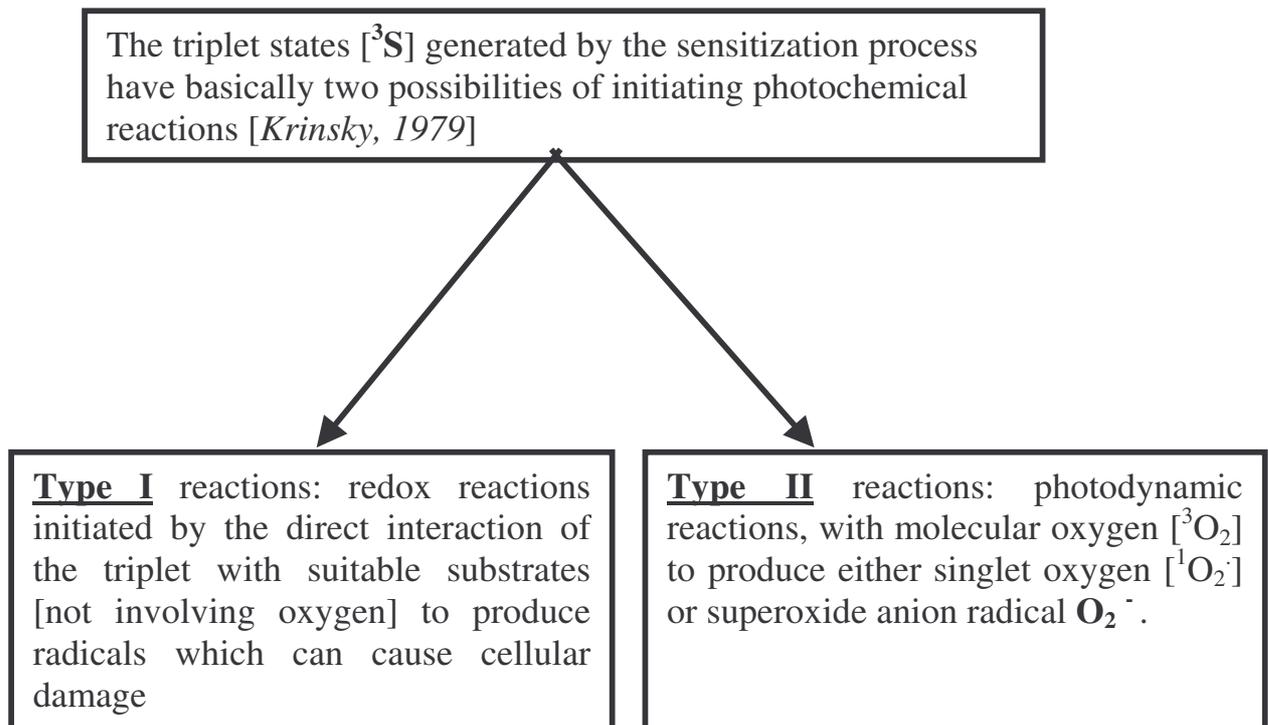


Fig.2-12.: The chemical mechanism for the formation of singlet oxygen in the presence of a sensitizer and light. The reaction with a triplet oxygen induces the excitation and deactivation of the photosensitizer, resulting in a singlet oxygen.

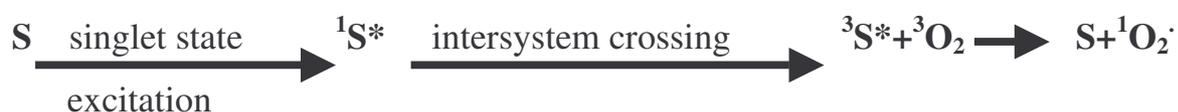


In both, **Type I** or **Type II** reactions, the net result is the production of active free radicals, while Type II reactions predominantly yield singlet oxygen. All these reactive molecules have the potential of causing temporary or permanent damage to retinal tissue [Andley, 1987].

Theoretically, there are two ways by which the yellow carotenoids can interfere with the initiation of photochemical reactions: by physical absorption of blue light thus preventing light from reaching the sensitizing molecules [1] or by chemical quenching of either the sensitizer triplet state or the singlet oxygen subsequently generated [2].

The quenching efficiencies of zeaxanthin and all-trans beta carotene against singlet oxygen are similar, whereas lutein, which has one conjugated double bond less than zeaxanthin, has a lower efficiency. This is consistent with data showing that the number of conjugated double bonds determines the efficiency of singlet oxygen quenching [Krinsky, 1968]

The previous mechanisms can be summarized as follow:



A triplet sensitizer ${}^3\text{S}$ can initiate both **Type I** and **Type II** photosensitized reactions. Carotenoid protection against photosensitized reactions is the ability of these pigments to quench, by an energy transfer process, either triplet sensitizers or ${}^1\text{O}_2$. A carotenoid triplet that is formed can readily lose its energy to the environment and return to its original form.



2.2.6-Nutritional aspects

As can be seen in table [2-1], the vegetables and fruits that are normally consumed in large quantities contain more lutein than zeaxanthin [*Hart and Scott, 1995; Müller, 1996; Lam K-W and But, 1999; Park et al., 1998*]. Therefore, it is not surprising to find that in human plasma the concentration of lutein is higher, by up to seven times, than that of zeaxanthin [*Khachik et al., 1997; Olmedilla et al., 1997; Ascherio et al., 1992*]. Zeaxanthin is the dominant carotenoid in red peppers and also in the small red berry *Lycium Barbarum*, “*Gou Qi Zi*” which can contain zeaxanthin in amounts of up to 5 mg/100g [*Lam K-W and But, 1999*]. This berry is commonly used in home cooking in China and is a constituent of traditional Chinese herbal medicine, in which, interestingly, it is used to improve visual acuity.

Some dietary carotenoids such as β -carotene and β -cryptoxanthin act as precursors of essential vitamin A because they can be transformed by endogenous enzymes into retinol [vitamin A], which is involved not only in the vision process but also in the maintenance of conjunctival integrity. Therefore, these carotenoids can, at least partly, ameliorate vitamin A deficiency. However, lutein and zeaxanthin do not have substantial provitamin A activity [*Weiser and Kormann, 1993*] and therefore, cannot provide retinol to the retina.

Amounts of Carotenoids in Selected Plants						
Amount of carotenoids ¹ (mg/100g) in selected plants ¹⁴⁻¹⁷						
	Lutein	Zeaxanthin	β -carotene	α -carotene	β -cryptoxanthin	Lycopene
<i>Vegetables (raw)</i>						
Spinach ¹⁵	9.5	0.4	3.3	np	np	np
Carrot	0.3	np	9.9	3.9	nd	np
Sweet corn	0.5	0.4	0.05	0.06	nd	np
Orange pepper	0.5	1.6	0.2	0.2	nd	np
Red pepper ¹⁵	np	2.2	3.3	0.5	1	0.1
Tomato	0.08	np	0.4	np	nd	1.6-5.6
<i>Fruits/berries/flowers</i>						
Mandarins	0.1	0.05	0.3	0.01	1.8	np
Peach	0.08	0.04	0.08	Trace	0.09	np
<i>Gou Qi Zi</i> ¹⁶	nr	4.3	nr	nr	nr	nr
Marigold ¹⁷	37	0.5	nr	nr	nr	nr

Abbreviations: nd = not done; np = not present; nr = not reported.

Table [2-1]: Different amounts of carotenoids [mg/100g] in some selected plants.

2.3-Carotenoids as modulators of molecular membrane dynamics

2.3.1-Membrane structure and function

1-Composition

Membranes are primarily composed of lipids and proteins with small contributions from carbohydrates. Phospholipids are major components of the cell membrane, they are similar to fats, but have only two fatty acids instead of three. The third hydroxyl group of glycerol is joined to a phosphate group, which is negatively charged. Additional small molecules, usually charged or polar [choline, serine, ethanolamine] can be linked to the phosphate group to form a variety of phospholipids [fig.2-13]. Phospholipids are described as being amphipathic, having both a hydrophobic and a hydrophilic region. Their tails, which consist of

hydrocarbons, are hydrophobic and are excluded from water. Their heads, however, which consist of the phosphate group and its attachments, are hydrophilic, and have an affinity for water [fig.2-14a]. Because of their structure, when phospholipids are added to water, they self-assemble into aggregates so that the phosphate heads are in contact with water and the hydrophobic hydrocarbon tails prefer water-free areas [fig.2-14b].

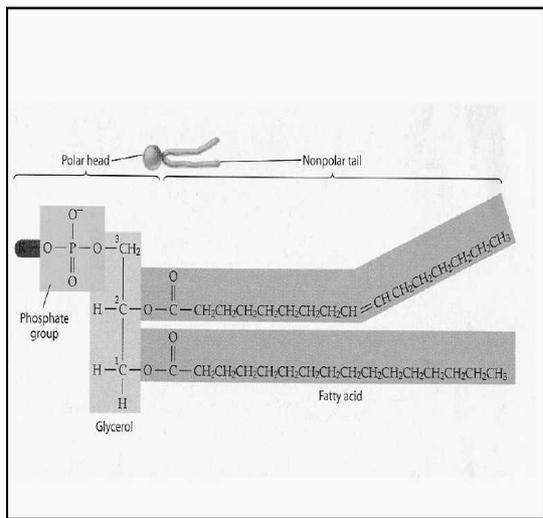


Fig.2-13.: Structure of phospholipids.

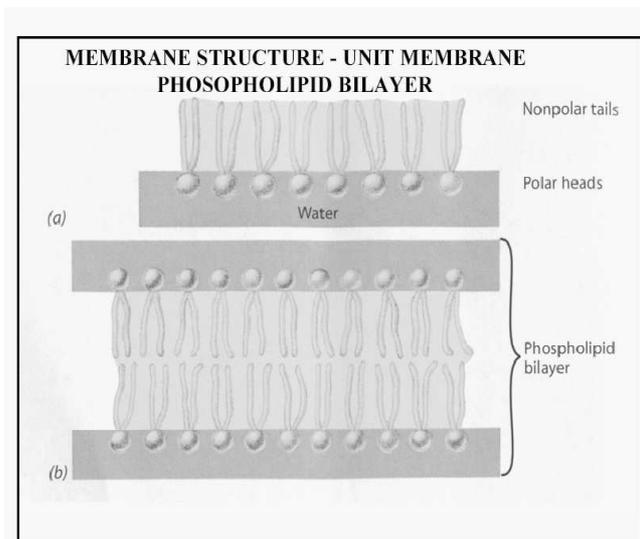


Fig.2-14.: Spontaneous assembly of phospholipids to form films and bilayers.

2-Membrane architecture

Much evidence stands to show that membrane properties are best explained by a dynamic, fluid lipid bilayer structure as in the "Fluid Mosaic" model [Singer and Nicholson, 1972].

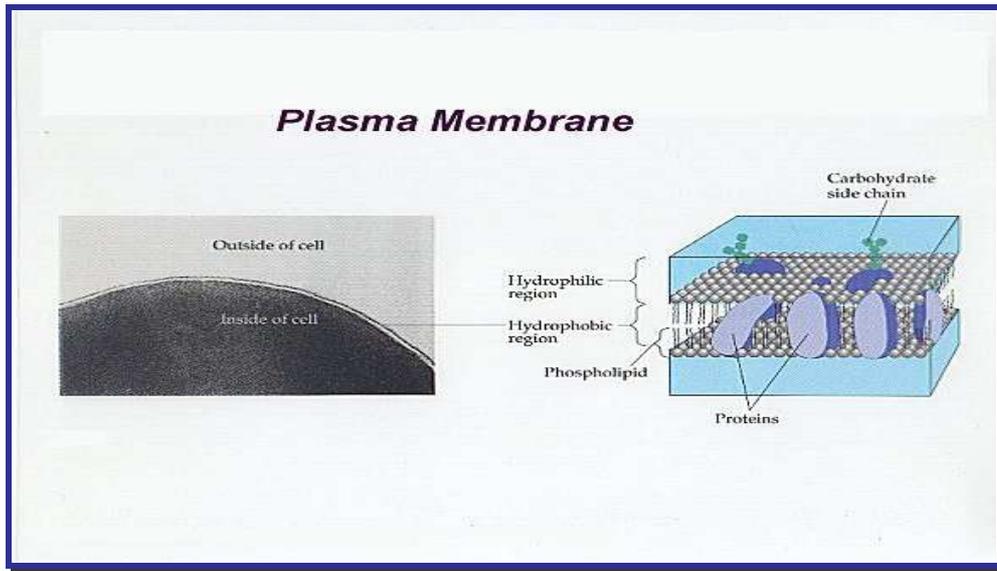


Fig.2-15.: Fluid mosaic model of a lipid bilayer membrane [Singer and Nicholson, 1972].

The fluid mosaic model of lipid bilayer membranes [fig.2-15], describes the essential features of the biological membrane. It is a two-dimensional fluid, or liquid crystal state, in which the hydrophobic components such as lipids and membrane proteins are constrained within the plane of the membrane, but are free to diffuse laterally. Using spectroscopic methods [ESR, NMR, Fluorescence, X-Ray crystallography] it is possible to study the various motions within bilayers. The lipid bilayer gives to the membranes its fluidity characteristics, and the temperature affects the packing properties of the hydrocarbons [fig.2-16]. At low temperatures, the bilayer is in a gel state and tightly packed. At higher [body] temperatures, the bilayer actually "melts" and the interior is fluid allowing the lipid molecules to move around, rotate, exchange places. This also allows movement of other components of the membrane [Wolfe, 1993].

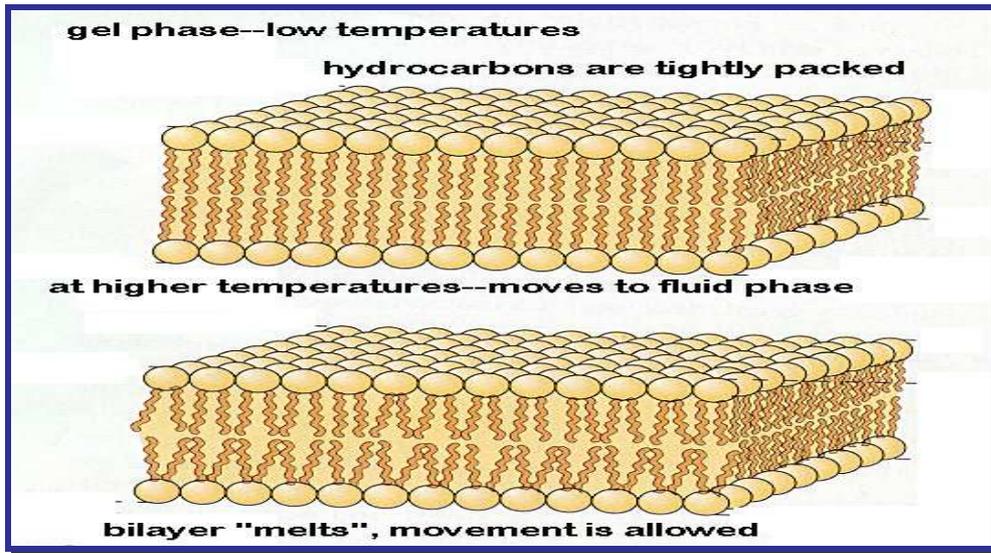


Fig.2-16.: The effect of temperature on packing properties of hydrocarbons of the lipid bilayer membrane.

3-Membrane cholesterol

Cholesterol has a rigid ring system and a short branched hydrocarbon tail [fig.2-17]. Cholesterol is largely hydrophobic, but it has one polar group, a hydroxyl, making it amphipathic. Cholesterol is an essential constituent of cell membranes. It is also the precursor for synthesis of steroid hormones & vitamin D. Cholesterol inserts into bilayer membranes with its OH oriented toward the aqueous phase and its hydrophobic ring system adjacent to fatty acid chains of phospholipids. The hydroxyl group of cholesterol forms hydrogen bonds with polar phospholipid head groups [fig.2-17].

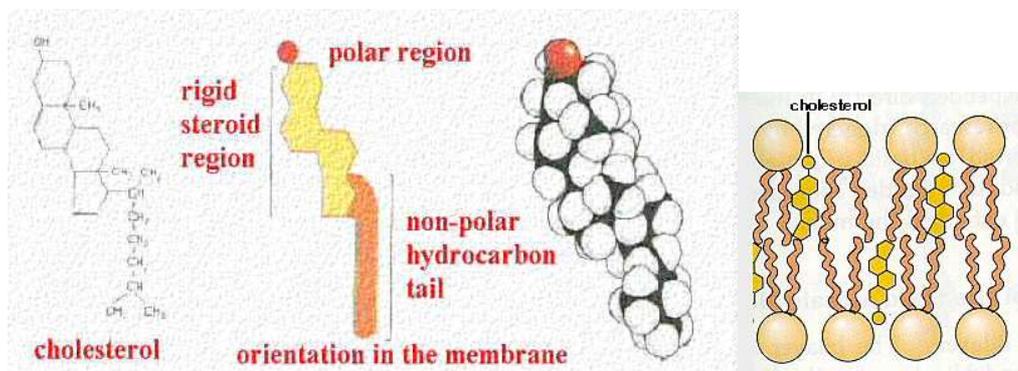


Fig.2-17.: Cholesterol intercalation between the phospholipids [adapted from Alberts et al., 1994].

Incorporation of cholesterol into a phospholipid membrane modifies the transition energy from liquid crystal to gel state. However, interaction with the relatively rigid cholesterol decreases the mobility of hydrocarbon tails of phospholipids. The membranes containing cholesterol, reach an intermediate fluidity between the liquid crystal and the gel states.

4-Membrane integral proteins

These proteins are an integral part of the membrane [fig.2-18] inserted completely in the bilayer and they perform a variety of functions. Generally protein molecules are polar, their charged portions extending into the water at the membrane surface. These proteins may act as receptors for hormones or transmitters, they may form linkages with adjacent cells or with extracellular fibers, they may act as surface enzymes of organelles [e.g., Golgi bodies, mitochondria, or endoplasmic reticulum]. They may act also as carriers and pumps, or may be structural anchors for the cytoskeletal fibers that pull cells into non-globular forms.

Pores [ion gates] are integral proteins and may have an active role in regulating movement of particular substances, such as sugar or electrolytes [*David L. Atkins, 1998*].

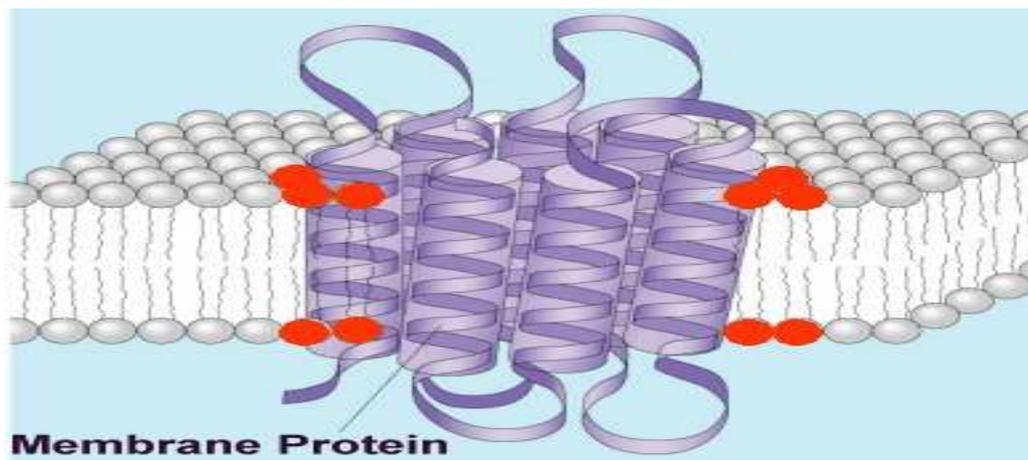


Fig.2-18.: Membrane structure showing the integral protein passage through the lipid bilayer.

2.3.2-Physical properties of carotenoids in relation to membrane structure and dynamics

Much is known about the physical and chemical properties of carotenoids in simple organic solutions. *In vivo*, however, carotenoids are part of a much more complex system-the living cell-and are in close proximity to other components such as proteins and lipids, frequently in organized and ordered structures such as membranes. The carotenoid must be able to fit into this complex system in the correct location and orientation. The overall shape, size and hydrophobicity of a carotenoid are obviously major features that determine the ability of a carotenoid to fit into subcellular structures. The structural details that characterize the individual carotenoid define the precise orientation that carotenoids can adopt as well as their interactions with surroundings.

Better understanding of the physico-chemical properties of carotenoids and their interactions with various phospholipids is needed to clarify the mechanisms of their action in biological membranes. Carotenoids are in the immediate vicinity of other molecules, such as lipids and proteins, in organized structures [e.g. light-harvesting complexes] in plant membranes and are able to fit into this complex system with correct position and orientation [Britton, 1995]. The capability of carotenoids to change membrane fluidity is essential with respect to their physiological implications as a modulator of signal transductions across membranes and membrane protein activities. The common chemical features of carotenoids are a polyisoprenoid structure, a long conjugated chain of double bonds in the central portion of the molecule, and high symmetry around the central double bond. Substantial differences in the activities of the carotenoids are afforded by certain groups attached to the terminal carbon rings. The structural details of the individual carotenoids may define the precise position and orientation in biological membranes.

The lipids comprising most biological membranes are in their liquid-crystalline state under physiological conditions, and in some cases, the lipid bilayers may be primarily in their gel state, or the mixture of the liquid-crystalline and gel states or the mixture of two liquid-crystalline states [*Mateo et al., 1995; Ten Grotenhuis et al., 1996*]. The phase state of constituent lipids plays a major role in the control of membrane-mediated functions.

The actual localization of lutein and zeaxanthin in macular membranes is still not clear and also a physiological function of these pigments is not fully understood at present. The role of these two pigments in the macula is most probably directly related to the protection against free radical attack to the membrane components and also to the attenuation of the effects of short-wavelength radiation penetrating the retina. Both the radical scavenger and the filtering efficacy of zeaxanthin and lutein in lipid membranes are potentially dependent on the organization of xanthophyll-lipid membranes. Several aspects of this organization can potentially be analyzed in order to understand better the physiological importance of lutein and zeaxanthin in the vision apparatus. The most frequently discussed aspects are the following:

- Localization of the pigments in the membranes [lipid head-group region versus hydrophobic core or protein complexes].
- Orientation of xanthophylls with respect to a membrane.
- Organization of the xanthophyll-lipid membranes [pigment aggregation].
- Effect of xanthophylls on the physical and dynamic properties of lipid membranes.

2.3.3-Relevant experimental data regarding the interaction of carotenoids with membranes

Most of recent studies are devoted to the effect of carotenoids on physical properties of membranes and are performed using model systems of lipid bilayers. Multi- and unilamellar phospholipid vesicles [liposomes] are a convenient and well defined system for studying the interaction between carotenoids and lipids. Lack of proteins and cholesterol, which are important constituents of all natural membranes, facilitates analysis but one should also consider that some conclusions drawn from such systems may not be applicable to the situations found in natural membranes, where the interaction of carotenoids with proteins or cholesterol occurs in a more complex way.

Liposomes are very useful models when used as a matrix to host foreign molecules in order to study the activity of those molecules or their influence on the bilayer, without undue interference. Liposomes are similar to cells in that they are composed of phospholipids that self-assemble in water forming an internal aqueous cavity that is isolated from an external aqueous solution [fig.2-19].

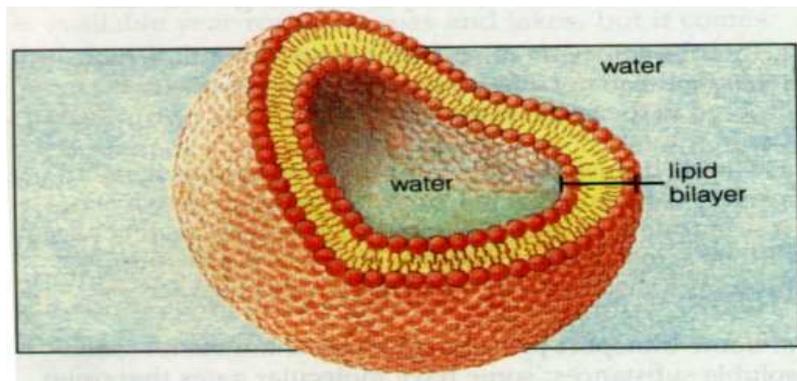


Fig.2-19.: Liposome structure.

Because of the small size of the phospholipid molecules and microspheres [50-200 nm], liposomes can cross outer cell layers and act as a carrier for the entrapped substances. It is postulated that, when they reach the outside of a living

cell membrane, they may become accepted as part of the membrane, having a similar composition. This process is shown in fig.2-20 and represents endocytosis.

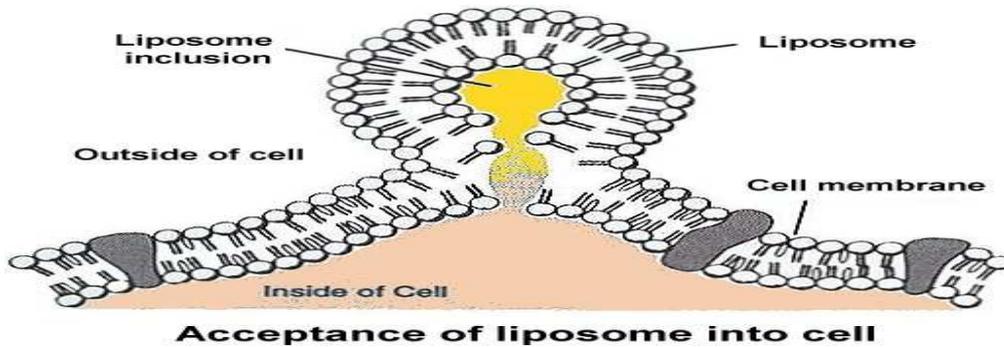


Fig.2-20.: Interaction of liposomes with cellular structures and their inclusion by endocytosis.

Carotenoids, important lipophilic constituents of many natural membranes, may modulate physical properties and molecular dynamics of such membranes. Therefore, studies on interactions between carotenoids and membrane constituents at a molecular level are very important in understanding the precondition for their activities in cells, including their disease-preventive action.

Microsomes, natural membranes separated from endoplasmic reticulum [ER], are artifactual vesicles formed spontaneously when cells are disrupted [fig.2-21]. They are isolated by different steps and finally by differential centrifugation [105000g] and are composed of three fractions: rough ER vesicles, smooth ER vesicles, and ribosomes. Numerous enzyme activities are associated with the microsomal fractions, such as monooxygenase activity. The procedure of preparing microsomes is well documented and will be described in the section of experimental [chapter 3].



Fig.2-21.: Storage form of hepatic microsomes

Retinal pigment epithelium [RPE] is a layer of cells well suited to inhibit blue light damage. Carotenoids absorb the blue light before it reaches many of the retinal elements or RPE cells [fig.2-22]. So, the study of carotenoid actions at this level is of great interest considering the location in the lipid environment of this type of cells and the possible physiological functions of these pigments in the membranes of the retina: zeaxanthin in the protection of the lipid phase against oxidation damage and lutein in absorbing short wave length radiation penetrating retina membranes.

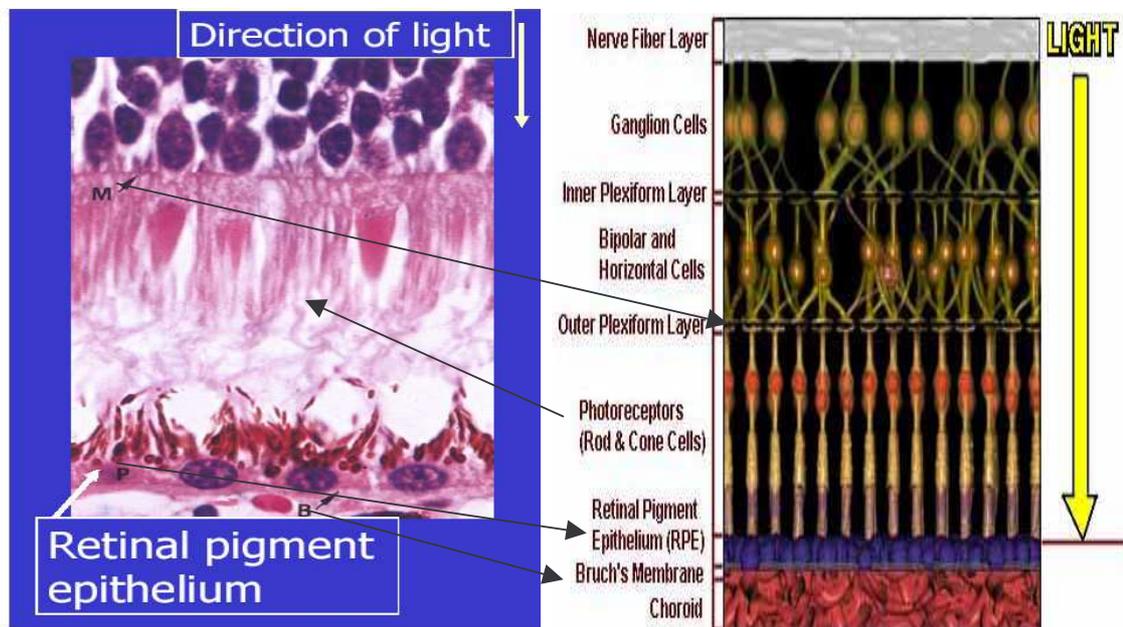


Fig.2-22.: Microscopic view of retinal pigment epithelium cells. The letter [m] at the figure on the left side is referred to Muller cells [also see fig.2-4].

2.3.3.1-Carotenoids in liposomes

The review of literature describing the influence of carotenoids on the physical properties of membranes using liposomes as model system will be summarized with a regard to different aspects:

1. *Carotenoid lipid-miscibility and effects on membrane structure*
2. *Incorporation efficiency*

3. *Antioxidant activity*
4. *Blue light filtering efficacy*
5. *Singlet oxygen quenching*

1-Carotenoid lipid-miscibility and effects on membrane structure

Spin label EPR studies revealed that β -carotene affects structural and dynamic properties of model dipalmitoylphosphatidylcholine [DPPC] membranes [multilamellar liposomes] more than the polar carotenoid lutein by: decreasing the order of crystalline state of the membrane or increasing the penetration of non-polar molecules into the membrane [*Strzalka and Gruszecki, 1994*]

It was found that β -carotene increases the motional freedom of lipid head groups as revealed by means of ^{31}P -NMR as well the motional freedom of alkyl chains forming the hydrophobic core of the membrane greater than that of a choline moiety as revealed by means of ^{13}C -NMR. In all cases the effect of β -carotene with respect to the dynamics of DPPC molecules is found to be more pronounced below the main phase transition temperature [gel state] than in the membrane's fluid state [*Jezowska et al., 1994*].

The ^1H -NMR technique was applied to study egg-yolk phosphatidylcholine [EYPC] liposomes containing two carotenoid pigments: β -carotene or zeaxanthin. A strong rigidifying effect of zeaxanthin, but not of β -carotene, with respect to the hydrophobic core of the lipid bilayer was concluded from the carotenoid-dependent broadening of the NMR lines assigned to $-\text{CH}_2-$ groups and terminal $-\text{CH}_3$ groups of lipid alkyl chains. A similar rigidifying effect of zeaxanthin with respect to polar headgroups was concluded on the basis of the effect which the pigment imposed

the shape of NMR lines attributed to-N⁺[CH₃]₃ groups. In contrast, β-carotene increases the motional freedom of lipid polar headgroups [*Gabrielska and Gruszecki, 1996*].

Since both superoxide and nitrogen monoxide, which react to form peroxynitrite, are found in the retina, *Scheidegger et al., 1998* studied the reaction of peroxynitrite with zeaxanthin in liposomes. Zeaxanthin was easily incorporated into liposomes built of the fully saturated lipid L-α dimyristoyl-phosphatidylcholine and from egg lecithin, and its absorbance spectrum in liposomes strongly resembles in shape and amplitude the free zeaxanthin spectrum in methanol. The reaction between peroxynitrite and zeaxanthin is of first-order in both substrates. The authors hypothesize that zeaxanthin plays a major role in protection of macular tissue from oxidative damage.

Incorporation of 10 mol% polar carotenoids [zeaxanthin, violaxanthin, or lutein] into the saturated phosphatidylcholine [PC] bilayer significantly increases the hydrophobicity of the inner membrane but decreases hydrophobicity [increases water penetration] in the polar headgroup region. In an unsaturated egg yolk PC membrane, the same polar carotenoids were found to increase the hydrophobicity of the inner membrane region to a higher level than in saturated PC membranes. At the membrane center, hydrophobicity reaches a level close to pure hexane [*Wisniewska and Subczynski, 1998*].

The molecular characteristics of monolayers of astaxanthin and β-carotene and their interactions in mixed carotenoid–phospholipid monolayers and the effects of carotenoids on the phase behavior of the phospholipid bilayers were examined by the monolayer technique and differential scanning calorimetry [DSC].

Astaxanthin, by its insertion into the monolayer at the hydrophobic/hydrophilic interface was more stable than β -carotene. Dimyristoylphosphatidylcholine [DMPC] as monolayer was miscible with astaxanthin in the range of 0–0.4 mol astaxanthin, but not fully miscible with β -carotene, even at low concentrations below 0.1 mol β -carotene. The DSC study showed that when a small amount of astaxanthin was added, the transition temperature of DPPC was markedly shifted to lower temperatures and that the transition peak was asymmetrically broadened, indicative of a significant depression in cooperativity of the gel to liquid–crystalline transition. On the contrary, the incorporation of β -carotene resulted in a small depression of the main transition temperature with a slight broadening of the transition peak, suggesting a small miscibility of β -carotene with the phospholipid bilayer or a formation of aggregates of β -carotene in the membranes [Shibata *et al.*, 2001].

2-Incorporation efficiency

Specific physical parameters [membrane fluidity, micropolarity and anisotropy] have been evaluated in multilamellar and unilamellar liposomes of DPPC after incorporation of carotenoids. When 1, 2.5 and 5 mol % β -carotene, lutein, zeaxanthin, canthaxanthin, or astaxanthin were incorporated into such liposomes using 4 mol% pyrene or 1 μ M 1,6-diphenyl-1,3,5-hexatriene [DPH] as fluorescent labels, no significant changes in membrane fluidity [as evaluated by the pyrene excimer method] were be found. But a change of micropolarity in the pyrene label environment has been observed from the pyrene monomer fluorescence emission fine structure after incorporation of such carotenoids. The membrane anisotropy is enhanced significantly by canthaxanthin and astaxanthin which incorporated comparatively worse into the membrane. This leads to the

hypothesis that carotenoid incorporation into membranes is governed not only by carotenoid polarity, but also by their ability to change membrane anisotropy [Socaciu *et al.*, 1999].

The incorporation yields of β -carotene, lutein, zeaxanthin, canthaxanthin and astaxanthin when incubated at 1, 2.5 and 5 mol% into phosphatidylcholine in multilamellar [MLV] and small unilamellar liposomes [SUV] were investigated. The effects of carotenoid intercalation into these vesicle were evaluated by UV-Vis spectrophotometry, light scattering and differential scanning calorimetry. Lutein revealed the highest incorporation yields in MLV and SUV, and decreased significantly the gel-to-liquid transition temperature and enthalpy, broadening the transition peak. Zeaxanthin incorporation into SUV was weaker than into MLV, suggesting its possible interlamellae distribution in MLV. β -carotene, canthaxanthin and astaxanthin showed lower incorporations into both MLV and SUV, lower saturation limits and no significant effects on the transition phase [Socaciu *et al.*, 1999]. Those measurements can offer useful information about the distribution of carotenoids in membrane bilayers and their effects on thermodynamic properties of membranes [Socaciu *et al.*, 2000a].

5 mol% dietary carotenoids [β -carotene, lutein and zeaxanthin], or cholesterol [16 and 48 mol%] in the absence or presence of 15 mol% carotenoids, respectively, were incorporated into pure DPPC or mixed DPPC:1,2-dipalmitoyl phosphatidyletanolamine [DPPE]:1,2-dipalmitoyl diphosphatidylserine [DPPS] [17:5:3] liposomes. The carotenoid incorporation yields [IY] ranged from 42% to 72% in pure or mixed phospholipid liposomes. The IY decreased significantly to 14%, in the corresponding cholesterol-doped liposomes. Highest incorporation yields were achieved by zeaxanthin and lutein in phospholipid liposomes while in

cholesterol-containing liposomes, lutein was better incorporated and β -carotene was weaker incorporated. The authors suggest a competition between carotenoids and cholesterol with respect to their modulating effect on membrane mechanics [Socaciu *et al.*, 2000b].

Two different techniques to incorporate carotenoids into liposomes have been evaluated: [i] preparation of unilamellar liposomes from mixtures of phospholipids and a carotenoid or cholesterol; [ii] insertion of carotenoids into yet prepared liposomes. A comparison of the two techniques lead to the conclusion that the “preparation from the mixture” technique disadvantages β -carotene incorporation, while lutein and zeaxanthin give more compact and richer vesicles. The fluorescent dyes Laurdan, DiI-C₁₈, C₆-NBD-PC were used to label the liposomes and to evaluate the modifications of ordering, hydrophobicity and permeability to water molecules adjacent to the bilayer in the presence of carotenoids and/or cholesterol. Zeaxanthin incorporation [up to 0.1–1 mol%] attributes to the symmetric and ordered structure of the bilayer, causing both, a strong hydrophobicity and a lower water permeability at the polar region of the membrane. The incorporation of lutein has similar effects, but its ordering effect is inferior in the polar region and superior in the non-polar region of the membrane. β -carotene, which can be incorporated at lower effective concentrations only, distributes in a more disordered way in the membrane, but locates preferentially in the non-polar region and, compared to lutein and zeaxanthin, it induces a less ordered structure, a higher hydrophobicity and a lower water permeability on the bilayer [Socaciu *et al.*, 2002].

3-Antioxidant activity

The ability of carotenoids to protect egg-yolk phosphatidylcholine EYPC lipids against oxidation by peroxy radicals generated from azo-initiators revealed that zeaxanthin and β -cryptoxanthin were more effective than β -carotene. Astaxanthin and canthaxanthin offered less protection to the liposomal lipids. Lycopene was destroyed most rapidly, so it was least effective as an antioxidant. Located in the hydrophobic inner core of the bilayer, the hydrocarbons lycopene and β -carotene are not in a position to readily intercept free-radicals entering the membrane from the aqueous phase. Carotenoids with polar end groups span the bilayer with their end groups located near the hydrophobic–hydrophilic interface where free-radical attack from 2,2'-azobis[2-amidinopropane hydrochloride] [AAPH] first occurs [Woodall *et al.*, 1997].

The antioxidant activity of carotenoids in multilamellar liposomes, assayed by inhibition of the formation of thiobarbituric acid-reactive substances, has been ranked as follows : Lycopene > α -tocopherol > α -carotene > β -cryptoxanthin > zeaxanthin = β -carotene > lutein . Mixtures of carotenoids were more effective than the single compounds. This synergistic effect was most pronounced when lycopene or lutein was present. The superior protection of mixtures may be related to the specific positioning of different carotenoids in membranes [Stahl *et al.*, 1998].

A protective effect of lutein and zeaxanthin against oxidative damage of egg yolk lecithin liposomal membranes induced by exposure to UV radiation and incubation with AAPH [a water-soluble peroxidation initiator] was found. Both, lutein and zeaxanthin were found to protect lipid membranes against free radical attack with almost the same efficacy. The UV-induced lipid oxidation was also

slowed down by lutein and zeaxanthin to a very similar rate in the initial stage of the experiments [5–15 min illumination] but zeaxanthin appeared to be a better photoprotector during the prolonged UV exposure. The decrease in time of the protective efficacy of lutein was attributed to the photooxidation of the carotenoid itself. Linear dichroism analysis of the mean orientation of the dipole transition moment of the xanthophylls incorporated to the lipid multibilayers revealed essentially different orientation of zeaxanthin and lutein in the membranes. Zeaxanthin was found to adopt roughly vertical orientation with respect to the plane of the membrane. The relatively large orientation angle between the transition dipole and the axis normal to the plane of the membrane found in the case of lutein [67° in the case of 2 mol% lutein in EYPC membranes] was interpreted as a representation of the existence of two orthogonally oriented pools of lutein, one following the orientation of zeaxanthin and the second parallel with respect to the plane of the membrane [fig.2-23]. The differences in the protective efficacy of lutein and zeaxanthin in lipid membranes were attributed to a different organization of zeaxanthin–lipid and lutein–lipid membranes [Sujak *et al.*, 1999].

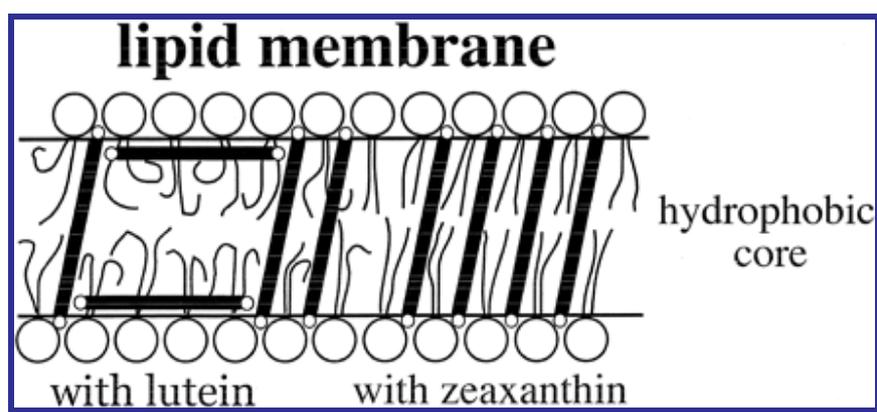


Fig.2-23. A schematic drawing of the location of lutein and zeaxanthin in egg yolk phospholipid bilayer membranes [adapted from Gabrielska and Gruszecki, 1996].

The ability of astaxanthin and canthaxanthin as chain-breaking antioxidants was studied in Cu^{2+} -initiated peroxidation of phosphatidylcholine large unilamellar

vesicles [LUVs]. Both carotenoids increased the lag period that precedes the maximum rate of lipid peroxidation. Astaxanthin showed stronger activity [*Rengel et al., 2000*].

It has been questioned whether carotenoids can act as antioxidants in biological membranes. Biological membranes can be modeled for studies of lipid peroxidation using unilamellar liposomes. Both, carotenoid depletion and lipid peroxidation were increased with increasing oxygen tension in unilamellar liposomes. Carotenoids in such liposomes were found to be very sensitive to degradation by free radicals generated by iron and AAPH, but they were not protective against lipid peroxidation. Lycopene and β -carotene were even more sensitive to free radical attack than lutein, zeaxanthin, and β -cryptoxanthin [*Chen and Djuric, 2001*].

4-Blue light filtering efficacy

The blue light filter [absorbing] efficacy of carotenoids was investigated in unilamellar liposomes loaded with the fluorescent dye Lucifer yellow, excitable by blue light. Also carotenoids were incorporated into the lipophilic membrane. The fluorescence emission from carotenoid-containing liposomes was lower than from carotenoid-free controls when exposed to blue light, indicating a filter effect. Filter efficacy was in the order lutein > zeaxanthin > β -carotene > lycopene. Some of the difference in blue light filter efficacy of carotenoids is attributable to differences in extinction coefficients, and a further contribution is suggested to be related to the orientation of the incorporated molecules in the liposomal membrane [*Junghans et al., 2001*].

5-Singlet oxygen quenching

Recently, the ability of several dietary carotenoids to quench singlet oxygen in a model membrane system [unilamellar DPPC liposomes] has been investigated. Singlet oxygen was generated in both, the aqueous and the lipid phase, and it was quenched by particular carotenoids, independent of the site of generation. However, singlet oxygen quenching is dependent on the specific carotenoid incorporated: xanthophylls are less efficient than hydrocarbon carotenoids. Lycopene and β -carotene cause the fastest singlet oxygen quenching rate constants [$2.3\text{--}2.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$] while lutein was least efficient [$1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$]. The other carotenoids, astaxanthin and canthaxanthin, had intermediate effects. Zeaxanthin exhibits anomalous behavior, the singlet oxygen quenching decreases with increasing amounts of zeaxanthin, leading to non-linear plots for the decay of singlet oxygen versus zeaxanthin concentration. Such differences are discussed in terms of the carotenoid structure and their influence on the properties of the lipid membrane. The formation of aggregates by the polar carotenoids is also proposed to be related to their ability to quench singlet oxygen [Cantrell *et al.*, 2003].

2.3.3.2-Carotenoids in microsomes

Few authors studied systematically the localization, incorporation yields and behavior of carotenoids in microsomal membranes. Mainly, the antioxidative effect of carotenoids has been proved in microsomes by the thiobarbituric acid reactive species reaction system or by inhibiting azo compound –induced lipid peroxidation [Palozza *et al.*, 1992].

When the conjugated keto-carotenoids, either astaxanthin or canthaxanthin, are added to rat liver microsomes undergoing radical-initiated lipid peroxidation

under air, they are as effective as α -tocopherol in inhibiting this process. This contrasts with the effect of β -carotene, which is a much less potent antioxidant when added to this system, without the addition of other antioxidants [*Palozza and Krinsky, 1992*].

The effects of the partial pressure of oxygen [pO_2] on antioxidant efficiency of β -carotene in inhibiting the AAPH-induced lipid peroxidation were investigated in rat liver microsomal membranes. The rate of peroxy radicals generated by thermolysis of AAPH at 37°C is markedly higher at 150 than 760 mm Hg pO_2 . At 150 mm Hg pO_2 β -carotene acts as an antioxidant, inhibiting AAPH-induced Malondialdehyde [MDA] formation, but at 760 mm Hg pO_2 , it loses its antioxidant activity and shows a prooxidant effect, increasing lipid peroxidation products. α -Tocopherol prevents the prooxidant effect of β -carotene in a dose-dependent manner. The data provide the first evidence of a prooxidant effect of β -carotene under 100% oxygen pressure in a biological membrane model and point out the existence of cooperative interactions between β -carotene and α -tocopherol [*Palozza et al., 1995*].

Liebler et al. [1997] indicated that β -carotene is a relatively ineffective antioxidant in biological membranes. Based on the measurement of thiobarbituric acid reactive species, the supplementation by β -carotene *in vitro* did not inhibit AAPH-initiated oxidation of rat liver microsomes at atmospheric pressure or at 3.8 torr O_2 . In PC liposomes, into which β -carotene was incorporated during the liposome preparation, it inhibited the AAPH-initiated lipid oxidation. In contrast, β -carotene added to pre-formed liposomes provided essentially no antioxidant activity, despite the similar content of β -carotene [0.35 mol%] in both liposome

preparations. Thus, the manner of carotenoid incorporation into a membrane affects the antioxidant activity.

Six different carotenoids β -carotene, lycopene, lutein, zeaxanthin, canthaxanthin and astaxanthin have been incorporated into pig liver microsomes. Effective incorporation concentrations in the range of about 1–6 nmol/mg microsomal protein were obtained. A stability test at room temperature revealed that after 3 hours β -carotene and lycopene had decayed totally whereas, gradually, canthaxanthin [46%], lutein [21%], astaxanthin [17%] and zeaxanthin [5%] decayed. The incorporation of carotenoids caused a small rigidification of the microsomal membrane. The membrane anisotropy seems to offer only a small tolerance for the incorporation of carotenoids and to limit the achievable incorporation concentrations of carotenoids into microsomes. The inhibition of microsomal lipid peroxidation seems also not to depend on the polarity of the carotenoid applied. Microsomes should be preferred as a membrane model to study mutual effects of carotenoids on membrane dynamics [*Socaciu et al., 2000c*].

2.3.3.3-Carotenoids in retinal pigment epithelium [RPE] cells as “in vitro model”

No data about the localization, incorporation yields and behaviour of carotenoids in retinal pigment epithelium membranes have been reported. Recently, the ability of exogenous zeaxanthin alone and in combination with vitamin E or C, to protect cultured human retinal pigment epithelium cells against oxidative stress was carried out. The survival of ARPE-19 cells, subjected to merocyanine 540-mediated photodynamic action, was determined by the yellow, water soluble dye 3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetra-zolium bromide [MTT] test and the content of lipid hydroperoxides in photosensitized cells was analyzed by HPLC

with electrochemical detection. It has been found that zeaxanthin-supplemented cells, in the presence of either α -tocopherol or ascorbic acid, were significantly more resistant to photoinduced oxidative stress. Cells with added antioxidants exhibited an increased viability and accumulated less lipid hydroperoxides than cells without the antioxidant supplementation. Such a synergistic action of zeaxanthin and vitamin E or C indicates the importance of the antioxidant interaction in efficient protection of cell membranes against oxidative damage induced by photosensitized reactions [Wrona *et al.*, 2004].

2.4-Introduction to Fluorescence Techniques

Fluorescent probes enable researchers to detect particular components of complex biomolecular assemblies, including live cells, with exquisite sensitivity and selectivity.

Fluorescence is the result of a three-stage process that occurs in certain molecules [generally polyaromatic hydrocarbons or heterocycles] called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. In addition to the high detection sensitivity, the fluorescence process is sensitive to subtle changes in molecular environment. Therefore, the use of fluorescent probes is a powerful method to obtain information about the structure, function, and health of cells.

The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram [Jablonski diagram] shown in [fig.2-24].

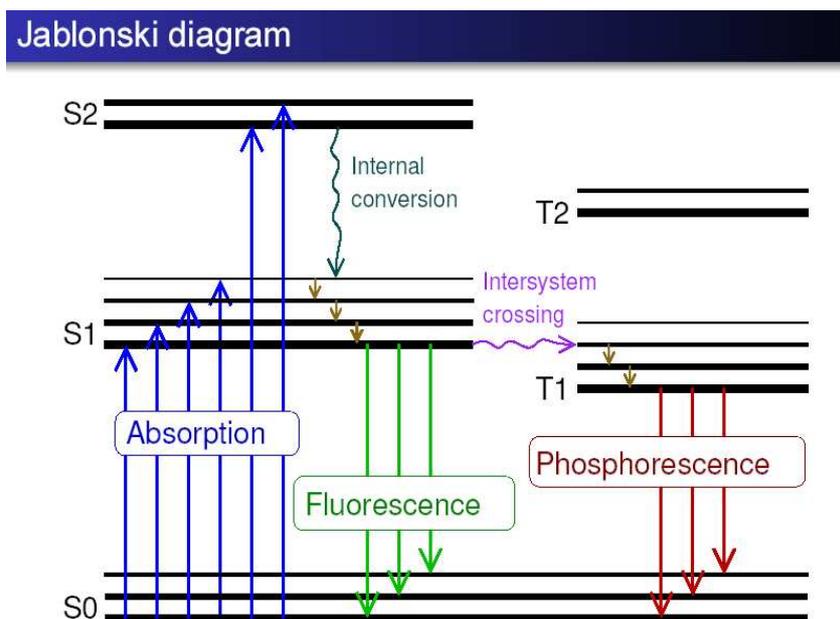


Fig.2-24: Jablonski Diagram.

Stage 1: Excitation [Absorption]

A photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state [S₂]. This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2: Excited-State Lifetime

The excited state exists for a finite time [typically 1–10 nanoseconds]. During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S₂ is partially dissipated, yielding a relaxed singlet excited state [S₁] from which fluorescence emission originates. Second, not all the molecules initially excited by absorption [Stage 1] return to the ground state [S₀] by fluorescence emission. Other processes such as collisional quenching, fluorescence energy transfer and intersystem crossing may also depopulate S₁. The fluorescence quantum yield,

which is the ratio of the number of fluorescence photons emitted [Stage 3] to the number of photons absorbed [Stage 1], is a measure of the relative extent to which these processes occur.

Stage 3: Fluorescence Emission

A photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0 . Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by $[h\nu_{EX} - h\nu_{EM}]$ is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

The observation and quantitation of coexisting gel and liquid crystalline phases in phospholipid bilayers have been attempted by using different fluorescent membrane probes. The decay of the popular probe 1,6-diphenyl-1,3,5-hexatriene [DPH] shows little spectroscopic variations in the two phospholipid phases. Moreover, the measured DPH decay in phospholipid vesicles can be equally described by the linear superposition of the properties of the two phases or by a continuous variation of the fluorescence properties along the phase transition [*Parasassi et al., 1991*]. For example, the lifetime value of DPH in dipalmitoyl-phosphatidylcholine [DPPC] gel phase vesicles is ~ 10.5 ns, in liquid crystal line phase vesicles it is ~ 7.5 ns, and at the transition midpoint it is ~ 9 ns. The life time value at the transition midpoint can arise either from the superposition of two components with equal contribution or from a unique species characterized by a life time value of 9 ns. Present instrumentation is incapable of distinguishing

between these two possibilities, due to the relatively small changes in the life time values between the two phases.

Laurdan [6-dodecanoyl-2-dimethylamine-naphthalene] is an amphiphilic fluorescence probe synthesized by *Weber and Farris, 1979* to study the effect of solvents with different polarity on the characteristics of its fluorescence emission properties. In solvents of high polarity, Laurdan shows a considerable shift of the emission spectra to higher wavelengths due to dipolar relaxation processes [*Parasassi et al., 1991*]. When the local environment of Laurdan is a phospholipid phase, the emission depends strongly on the physical state of the lipid aggregate. The dipolar relaxation in a given phase physically corresponds to the reorientation of the surrounding dipoles [water molecules] around the Laurdan excited-state dipole. At temperatures below the lipid phase transition [gel state] this process is slower than the Laurdan fluorescence lifetime and the probe emits with an emission spectra localized at high energy frequencies [blue region]. At temperatures above the phase transition [liquid crystalline state] this process is faster and comparable to the Laurdan excited state lifetime and the emission spectrum shifts about 50 nm from 440 nm to 490 nm [*Parasassi et al., 1991*].

The fluorescence steady-state parameter Generalized Polarization [*GP*] relates quantitatively these spectral changes by taking into account the relative fluorescence intensities of the blue and red edge regions of the emission and excitation spectra, respectively [fig.2-25]. For phospholipids, a characteristic *GP* value was found for the gel and liquid crystalline phases independent on the polar head group of the phospholipid. The *GP* values obtained for phospholipids undergoing phase changes are related to the different motional freedom of water molecules around the fluorescent group inserted in the lipid membrane [*Parasassi et al., 1991*].

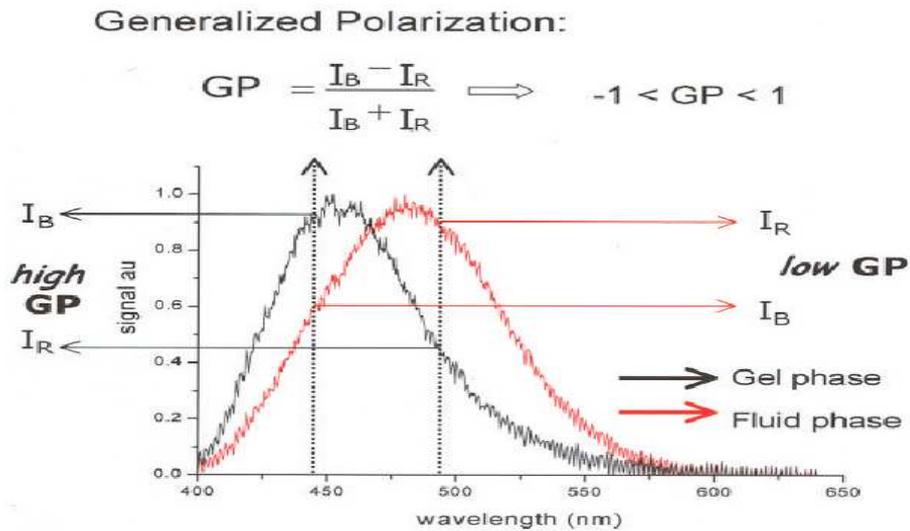


Fig.2-25: Of particular interest in our studies is the use of the so-called General Polarization [GP] of Laurdan, given by $GP = [I_B - I_R]/[I_B + I_R]$, where I_B [440 nm] and I_R [490 nm] are the Laurdan fluorescence intensities at the blue and red edges of the emission spectrum, respectively.

Laurdan can be particularly useful because it shows characteristic fluorescence spectra in the lipid gel and liquid-crystalline phases and in phase domain coexistence. Modifications of these important membrane physicochemical features are known to modulate important physiological functions. Moreover, this probe is particularly sensitive to membrane cholesterol content, which is known to modulate membrane lipid order and dynamics and to decrease hydration of phospholipid membranes [Parasassi *et al.*, 1994].

3. Experimental

3.1. Chemicals

β -carotene, lutein, and zeaxanthin were bought from by S.C. Proplanta S.A. Cluj-Napoca, Romania. They were purified from natural sources and checked for purity by HPLC. Canthaxanthin was purchased from Carl Roth [Karlsruhe, Germany]. The molecular weight [MW] of β -Carotene is 536.43, canthaxanthin is 564.40, lutein is 568.42 and zeaxanthin is 568.42.. The molecular structures of the carotenoids used are shown in fig.3-1.

High Purity Egg Yolk phosphatidylcholine [EYPC] of MW 750 was purchased from Lipoid KG [Ludwigshafen, Germany]. The lipid purity of of the preparation was higher than 99% and used without further purification.

The fluorescent probes used in the measurements were 6-dodecanoyl-2-dimethylaminonaphthalene [Laurdan] MW of 353.55 and 1,6- diphenyl-1,3,5-hexatriene [DPH] MW of 232.32. They were purchased from Molecular Probes [Eugene, OR, USA]. The molecular structure of fluorescent probes are shown in fig.3-2.

Dulbecco Modified Eagle Medium [DMEM], fetal calf serum [FCS], the antibiotic-antimycotic solution [penicillin G sodium, streptomycin sulfate, amphotericin B], glutamine and trypsin/EDTA solution were obtained from Gibco [Paisley, Scotland]. Culture flasks and 96 well culture plates from Nunc [Wiesbaden, Germany]. Trypan blue was from Sigma [Deisenhofen, Germany]. Tetrahydrofuran [THF] 99.9% was purchased from Sigma-Aldrich Corp. [St. Louis, MO, USA]. Triton X-100 was obtained from Serva [Heidelberg, Germany]. Di-*t*- buthyl-*p*- cresol [BHT], dichloromethane [DCM], Ethanol [EtOH], ammonium ferrothiocyanate, were from sigma [Deisenhofen, Germany]. Dimethyl

formaide [DMF] was obtained from ACROS [Geel, Belgium]. Tris [tris-hydroxymethyl-aminomethane] was purchased from Merck [Darmstadt, Germany]. Potassium chloride [KCL] was supplied from Fluka [Buchs, Switzerland]. All chemicals were of research grade. Solutions were prepared in de-ionized ultra pure water.

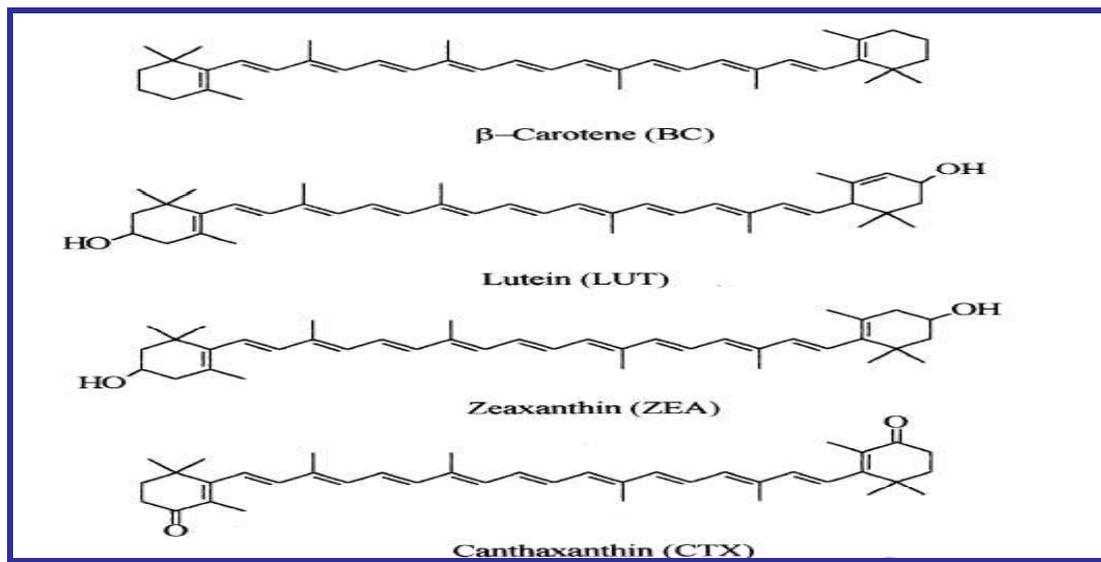


Fig.3-1. Molecular structures and abbreviations of the carotenoids used in our studies: BC- β -carotene; LUT- lutein; ZEA- zeaxanthin and CTX- canthaxanthin.

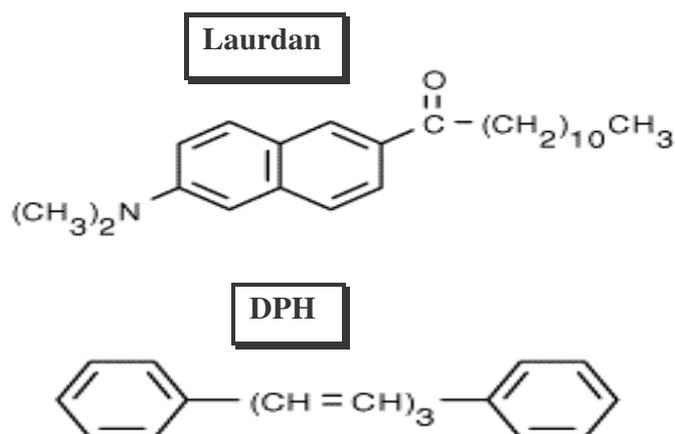


Fig.3-2. Molecular structure of the fluorescent probes used: Laurdan and DPH respectively.

3.2. Incorporation of carotenoids into liposomes

Control liposomes

Aliquots of 20 mg of EYPC were dissolved in 10 ml EtOH, then a vigorous vortexing took place to assure a complete solvation, and evaporated in the Rotavapor [Vacuum System]. The film was dried under vacuum [vacutherm, Heraeus Instr., Hannover, Germany] overnight at 42°C. This preparation was suited to be the control liposomes. The liposomes were prepared following classical methods [Deamer and Uster, 1983].

Preparation I

Parallel to the control, 20 mg of EYPC were mixed with each of 0.21 mg of β -carotene, 0.2273 mg of lutein, 0.2273 mg of zeaxanthin and 0.225 mg of canthaxanthin in round bottom flasks. 10 ml of EtOH were added to each mixture, then a vigorous vortex took place to assure a complete solvation for each mixture. The mixture was evaporated under the same conditions of control as before and the film was similarly dried. The initial concentration [IC] of carotenoids to lipids was set to be approximately 1.5 mol%.

Preparation II

In order to obtain around equal effective concentration [EC], 20 mg of EYPC were mixed with a corresponding amounts of 0.429 mg of β -carotene, 0.3 mg of canthaxanthin, 0.07575 mg of both lutein and zeaxanthin, in round bottom flasks. The initial concentration [IC] of carotenoids in this case was set to be approximately 3 mol% β -carotene, 2 mol% canthaxanthin, 0.5 mol% of both lutein and zeaxanthin. A 10 ml of EtOH also was added to each mixture, then a vigorous vortex assured a complete homogenization of each mixture. The mixtures were evaporated under the same conditions and the film was similarly dried. In all cases [control liposomes, Preparation I and Preparation II], the dried film was vortexed for 10 min at 42°C with 10 ml Tris buffer [0.1M, pH 7.4], giving a heterogenous

suspension of multilamellar vesicles [MLVs]. MLVs were sonicated using the sonicator Bandelin Sonoplus HD70 [Bandelin Electronics, Germany] at maximal power of 70-80% [cycle 30%] at 42°C, under nitrogen stream for 15 min.

After sonication the small unilamellar vesicles suspensions [SUVs] of control and doped with carotenoids were centrifuged at 6000 r.p.m for 5 minutes and the supernatant was harvested. The pellet was suspended in Tris buffer [same molarity and pH], centrifuged again for 5 minutes at 6000 r.p.m, the supernatant was collected and mixed with former supernatant. In all cases the supernatant fractions represented a heterogeneous population of unilamellar liposomes [empty ones or doped with carotenoids] while the pellet contained aggregates of non-incorporated molecules.

The unilamellar liposomes were extruded using the thermo-stabilized membrane extruder [Thermostat Membrane Extrusion Equipment, Lipex Biomembranes Inc., Vancouver, Canada] through polycarbonate filters of 0.4 and 0.2 μm . Finally, all prepared probes of control liposomes and carotenoids incorporated into liposomes were of final concentration 2 mg lipid /ml. The vesicle size was 0.2 μm , containing 0.0025% antioxidant BHT.

3.3. Evaluation of carotenoid incorporation and carotenoid stability in liposomes by UV-Vis spectrometry

To characterize the carotenoid concentration and stability before and after incorporation, UV-Vis spectra of the liposomal suspension and of the carotenoids released from the liposomes [200 μl] in an ethanol solution [800 μl] were taken on a Shimadzu UV-2102 PC Scanning Spectrophotometer. From UV-Vis spectra [300-550 nm], the following parameters were determined: initial concentration, i.e. the incubated concentration, of the carotenoid added to the phospholipid mixture [IC]; effective concentration of carotenoids encapsulated in SUVs [EC]. By the

calculation, the incorporation yield [IY] is determined as the ratio between EC and IC. IC and EC were determined using the formula [Britton *et al.*,1995] to quantify the carotenoid extracts, known volumes of a carotenoid solution were used.

$$\text{Carotenoid [mg]} = \frac{A * V * 1000}{\epsilon * 100}$$

A is the optical density recorded [its value should be between 0.2-0.8]. V is the volume of the solution containing the carotenoid. ϵ is the specific extinction coefficient of each carotenoid in the solvent. For example, ϵ of β -carotene in ethanol is 2620, for lutein is 2550, for canthaxanthin is 2200, and for zeaxanthin is 2480 [Britton *et al.*,1995].

Carotenoid stability in liposomes with respect to leakage and degradation upon storage was studied for a period of two months after preparation. The vials of liposomes containing carotenoids were stored under specified conditions of storage of 4°C, successive flushing with nitrogen, in dark, and they were closed with parafilm to resist the moisture. The stability of carotenoid formulations was confirmed by measuring the shape of UV-Vis spectra, calculating the IY. The quenching efficiency was evaluated using Laurdan as fluorescence label to display the fluorescence spectra of liposomes which incorporated carotenoids .

3.4. Evaluation of phospholipid content by Stewart assay

In the Stewart assay for phospholipids [Stewart, 1980], the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution is utilized. The advantage of this method is that the presence of inorganic phosphate does not interfere with the assay. A simple conversion factor is used to translate absorbance values into milligrams of phospholipid. This method is not

applicable to samples where mixtures of unknown phospholipids may be present. In particular, this method is especially unresponsive to phosphatidyl glycerol. In liposomes which contain only phosphatidyl choline and phosphatidyl glycerol, the Stewart assay could be used as a specific test for the former.

This assay is not recommended for the liposomes doped with carotenoids because of the absorption peak of the complex at 485 nm [near to the absorbance maxima of carotenoids around 450 nm].

Preparation of reagents

Ammonium ferrothiocyanate solution [0.1M] obtained by dissolving 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double-distilled water, and fill up to 1 liter. The solution is stable at room temperature for several months.

Preparation of standard and sample solutions

Make up 10 ml of solution of phospholipid in dichloromethane [DCM] at a concentration of 0.1 mg/ml. From both these preparations the following assays are prepared.

Assay method

- Pipette reagents and standard into 10-ml centrifuge tubes as follows:

Tube no.	Standard [ml]	DCM [ml]	Ferrothiocyanate [ml]
0	0.0	2.0	2.0
1	0.1	1.9	2.0
2	0.2	1.8	2.0
3	0.4	1.6	2.0
4	0.6	1.4	2.0
5	0.8	1.2	2.0
6	1	1.0	2.0

Prepare duplicates of each tube. Perform the same procedure with the test samples.

- Vortex contents of each tube vigorously on a whirl mixer for 30 sec.
- Spin each tube for 5 min at 5000 r.p.m in a centrifuge, remove the lower layer using a Pasteur pipette and retain it.
- Read the optical density at 485 nm for samples and standards.
- Find the concentration in the test sample solutions by comparing with the standard curve.

3.5. Incorporation of carotenoids into liver microsomes

Pig liver microsomes were prepared as follow:

All solutions and materials are on ice during work

150 g pig liver from a public slaughter house were freshly provided and washed with 0.15% [w/w] KCL. The liver was cut in small pieces and homogenised with 400 ml of buffer I [0.05M Trizma Base [Tris] + 0.15M KCL + 1mM EDTA mixed in one liter distilled water and pH adjusted to 7.4 with HCl] four times for 20 sec with 20 sec breaks. The homogenous solution obtained was centrifuged at [1000g, 4°C] for 30 min. The top layer of the solution was filtered through a cotton cloth. The solution was divided on the ultracentrifuge tubes and all tubes were filled up with buffer I and centrifuged at [105000g, 4°C] for 1 hour. A homogenous solution with sediment was obtained and potted with buffer II [0.05M Trizma Base [Tris] + 0.15M KCL + 10mM EDTA mixed in one liter distilled water and pH adjusted to 7.4 with HCl], using a potter for this purpose [400U/min, ten times up and down]. The solution was transferred to the ultracentrifuge tubes and all tubes were filled up with buffer II and centrifuged at [105000g, 4°C] for 1 hour. The sediment was used, and filled with 80 ml of buffer III* into the potter tubes and potted at 400U/min, ten times. Small portions of microsomes [fig.3-3] were transferred into

Eppendorf micro test tubes [Hamburg, Germany] and stored at -20°C [for longer storage -80°C]. Microsomal protein was determined according to [Peterson, 1977].

***Buffer III** is defined as :

A: 0.01M $\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$ [potassium phosphate, dibasic] in 20% [v/v] Glycerol

B: 0.01M KH_2PO_4 [potassium phosphate, monobasic] in 20% [v/v] Glycerol

adjust pH to 7.7 by mixing A with B

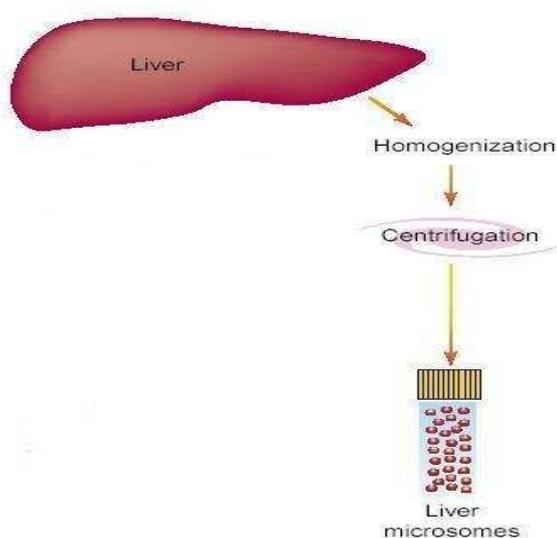


Fig.3-3. Preparation of liver microsomes.

As for liposomes, we used 2 preparations to incorporate carotenoids.

Preparation I

Aliquots of 2 ml of microsomal suspensions [24 mg protein/ml] were homogenized with 0.5 mg β -carotene or 0.54 mg canthaxanthin or 0.55 mg of lutein or zeaxanthin for 15 min on ice using a Potter-Elvehjem tube at 400U/min to incorporate the thin film of carotenoids spread at the tube's wall. All carotenoids were previously dissolved in EtOH and evaporated under a nitrogen stream to dryness in a Potter-Elvehjem tube. The IC of carotenoids was set to be approximately 1.5 mol% relative to microsome lipid content [1.5 mol

carotenoid/100 mol microsomal lipid]. With respect to the microsomal protein content, the IC was 20 nmol carotenoid/mg protein.

Preparation II

In order to achieve about the same EC in all assays, aliquots of 2 ml of microsomal suspensions were homogenized with 0.8 mg β -carotene or 0.72 mg canthaxanthin or 0.3637 mg of lutein or zeaxanthin in a Potter-Elvehjem tube at 400U/min to incorporate the thin film of carotenoids spread at the tube's wall. The IC was set to be approximately as follows:

Carotenoid	IC referred to lipid [mol% carotenoid/lipid]	IC referred to protein [nmol carotenoid/mg protein]
β -carotene	2.5	31.1
Canthaxanthin	2	26.6
Lutein	1	13.3
Zeaxanthin	1	13.3

The microsomes enriched with carotenoids were ultracentrifuged at 105000 g for 1 hour at 4°C. In all cases [control microsomes, Preparation I and Preparation II], the supernatant was collected and the pellet containing coloured microsomes was resuspended in 2 ml Tris buffer [0,1M pH 7.4] and used for all determinations.

3.6. Evaluation of carotenoid incorporation and carotenoid stability in microsomes by UV-Vis spectrometry

For the evaluation of the effective concentration [EC] of carotenoids in microsomes, immediately after incorporation, aliquots of 30 μ l of microsomal suspension were added to 970 μ l EtOH, and on a Shimadzu UV-2102 PC Scanning

Spectrophotometer the absorption spectra were displayed. From UV-Vis spectra [300-550 nm], the EC was determined using the formula [Britton *et al.*,1995]:

$$\text{Carotenoid [mg]} = \frac{A * V * 1000}{\epsilon * 100}$$

where A is the optical density recorded, [its value should be between 0.2-0.8]. V is the volume of solution containing the carotenoid. ϵ is the specific extinction coefficient of each carotenoid in EtOH. The IY was calculated as the ratio between EC and IC.

The carotenoid stability in microsomes with respect to oxidative degradations upon storage was carried out for a period of one month after preparation and seven months, respectively. The Eppendorf micro test tubes of microsomes containing carotenoids were stored under specified conditions of storage of -20°C , successive flushing with nitrogen, and in dark. The stability of carotenoid formulations was confirmed by measuring the shape of UV-Vis spectra, calculating the IY and evaluating the Laurdan quenching efficiency.

3.7. Fluorescent assays for liposomes and microsomes

For the studies performed on *liposomes*, we did steady-state fluorescence measurements with Laurdan and DPH. A stock solution of 1mM Laurdan in DMF was prepared. 100 μl of each type of liposomes [control or doped with carotenoids] was suspended in 5 ml Tris buffer [0.1M, pH 7.4]. 4 μl of 1mM Laurdan was added and incubated for 1 h at 25°C under magnetic stirring in dark. The ratio of dye to lipid was 1.5 mol %. The emission spectra were recorded between 375 and 600 nm at $\lambda_{\text{exc}} = 353$ nm, and the excitation spectra between 300 and 420 nm at

λ_{em} = 440 nm. All measurements were performed in triplicate at room temperature. From the spectroscopic data, the Laurdan emission “generalized polarization” GP_{em} was calculated from the emission spectra as follows:

$$GP_{em} = [I_{440} - I_{490}] / [I_{440} + I_{490}]$$

I_{440} and I_{490} are the intensities at the emission maxima of 440 and 490 nm, respectively.

A stock solution of 0.5 mM DPH in DMF was prepared. 100 μ l of each type of liposomes [control or doped with carotenoids] was suspended in 5 ml Tris buffer [0.1M, pH 7.4]. 8 μ l of 0.5 mM DPH was added and incubated for 1 h at 25°C under magnetic stirring in dark. The ratio of dye to lipid was 1.5 mol%. The emission spectra were recorded between 375 and 600 nm at λ_{exc} = 358 nm, and the excitation spectra between 300 and 420 nm at λ_{em} = 440 nm. All measurements were performed in triplicate at room temperature.

Fluorescence measurements were performed on a computer controlled Perkin Elmer LS-50 Luminescence Spectrometer equipped with a thermostatic cuvette [Julabo Labortechnik, Seelbach, Germany]. The scan speed was 100 nm/min and the monochromator slits were fixed at 5 nm.

For the studies performed on *microsomes*, volumes of 4 μ l Laurdan from a 1mM stock solution in DMF were added to each 50 μ l microsomal suspension of control and doped with carotenoids in 5 ml Tris buffer [0.1M, pH 7.4]. The ratio of dye to lipid was 0.25 mol%. All samples were incubated in dark at room temperature for 1 hour under magnetic stirring in order to allow the fluorescent probe to incorporate into the membrane. The emission spectra were registered between 375 and 600 nm at λ_{exc} = 353 nm, and the excitation spectra between 300 and 420 nm at λ_{em} = 440 nm. All measurements were performed in triplicate at

room temperature. From the spectroscopic data, the Laurdan emission “generalized polarization” GP_{em} was calculated from the emission spectra as follows:

$$GP_{em} = [I_{440} - I_{490}] / [I_{440} + I_{490}]$$

I_{440} and I_{490} are the intensities at the emission maxima of 440 and 490 nm, respectively.

Volumes of 8 μ l DPH from 0.5 mM stock solution in DMF were added to each 50 μ l microsomal suspension of control and doped with carotenoids in 5 ml Tris buffer [0.1M, pH 7.4]. The ratio of dye to lipid was 0.25 mol%. All samples were incubated in dark at room temperature for 1 hour under magnetic stirring in order to allow the fluorescent probe to incorporate into the membrane. The emission spectra were registered between 375 and 600 nm at λ_{exc} = 358 nm, and the excitation spectra between 300 and 420 nm at λ_{em} = 440 nm. All measurements were performed in triplicate at room temperature.

Fluorescence measurements were performed on a computer controlled Perkin Elmer LS-50 Luminescence Spectrometer equipped with a thermostatic cuvette [Julabo Labortechnik, Seelbach, Germany]. The scan speed was 100 nm/min and the monochromator slits were fixed at 5 nm.

3.8. Evaluation of carotenoid uptake by RPE cells, type D407

The human retinal pigment epithelial cell D407 retains many characteristics of these cells in vivo, including the ability to phagocytize [Davis *et al.*, 1995]. D407 cells were cultivated in high glucose DMEM containing 4.5 mg/l glucose; 4 mM glutamine; 3.7g/l sodium bicarbonate; 1mM sodium pyruvate; 1% antibiotic antimycotic mixture of 10000 UI penicillin, 10000 g streptomycin, 25 g amphoterycin B and supplemented with 10% fetal calf serum [FCS]. Cells were grown in 96-well culture plates under a 5% CO₂/95% moist air atmosphere at 37°C

and were collected after confluence by trypsination with 0.05% trypsin in phosphate buffer saline [PBS]. The cells were harvested in 3 ml DMEM, centrifuged for 5 min. at 300g, and at 4°C the pellet was resuspended in 5 ml DMEM. To determine the cell viability we used trypan blue [0.4%] 1:1 as intravital colourant. The total cell number was determined in a 16 field chamber from Neubauer using an Olympus CK2 phase contrast microscope [Hamburg, Germany]. At least 100 to 150 cells were counted and the number of dead cells was calculated from the amount of trypan blue stained cells.

We used two procedures to incorporate carotenoids into RPE cells:

Procedure I

Volumes of 3 ml cell suspension containing 2.5×10^6 cells /ml DMEM were transferred to conical bottom flasks which each contains of 0.2 mg of β -carotene, canthaxanthin, lutein and zeaxanthin previously mixed with 10% FCS [as vehicle for carotenoids] in water-bath sonicator for 10 min. The IC was set to be approximately 26 μg carotenoid/ 10^6 cells. The mixture was incubated in dimmed light at 37°C for 48 h. Control cells were maintained at the same conditions of incubation and received an equivalent concentration of FCS. In all cases, mixtures were supplemented with 0.025% BHT to prevent further oxidation.

Procedure II

Volumes of 3 ml cell suspension containing 2.5×10^6 cells /ml DMEM were transferred to conical bottom flasks which contain each of 0.2 mg of β -carotene or canthaxanthin or lutein or zeaxanthin previously dissolved in tetrahydrofuran [THF] as solvent vehicle for carotenoids. THF was not found to be toxic to cells at concentrations that were $\leq 0.5 \%$ [Cooney *et al.*, 1993]. The IC was set to be approximately 26 μg carotenoid/ 10^6 cells. The mixture was incubated in dimmed light at 37°C at selected intervals [15 min, 0.5 h, 1h and 24 h]. Control cells were maintained at the same conditions of incubation and received an equivalent

concentration of THF. In all cases, mixtures were supplemented with 0.025% BHT to prevent further oxidation.

To evaluate the carotenoid uptake, at intervals of 15 min, 0.5 h, 1h, 24 h and 48h from the starting time, aliquots of the incubated suspension were taken into Eppendorf micro test tubes to quantify the carotenoid incorporation. The suspension was centrifuged at 14000 r.p.m for 30 min. The supernatant was harvested and the pellet was suspended in 1.5 ml Tris buffer [0.1M, pH 7.4]. Aliquots of 300 µl from a control cell suspension and from a cell suspension doped with carotenoids were solubilized using the detergent Triton X-100 to dissociate the membrane into its components and determine the actual content of carotenoids in the cells .

For the evaluation of the effective concentration of carotenoids in the retinal pigment epithelium cells, immediately after solubilization, aliquots of 50 µl of cell suspension loaded with carotenoid were extracted to 950 µl EtOH and on a Shimadzu UV-2102 PC Scanning Spectrophotometer the absorption spectra were displayed. From the UV-Vis spectra [300-550 nm], the EC was determined using the formula [Britton *et al.*,1995]:

$$\text{Carotenoid [mg]} = \frac{A * V * 1000}{\epsilon * 100}$$

where A is the optical density recorded , [its value should be between 0.2-0.8]. V is the volume of solution containing the carotenoid. ϵ is the specific extinction coefficient of each carotenoid in EtOH. The IY was calculated as the ratio between EC and IC.

3.9. Fluorescence studies on D407 RPE cells

Volumes of 5 µl Laurdan from a 1mM stock solution in DMF were added to each 100 µl cell suspension [control or doped with carotenoids] in 5 ml Tris buffer

[0.1M, pH 7.4]. Samples were incubated in dark at room temperature for 1 hour under magnetic mild stirring in order to allow the fluorescent probe to incorporate into the membrane. The emission spectra were recorded between 375 and 600 nm at λ_{exc} = 353 nm, and the excitation spectra between 300 and 420 nm at λ_{em} = 440 nm. All measurements were performed in triplicate at 25°C. From the spectroscopic data, Laurdan emission “generalized polarization” GP_{em} was calculated from the emission spectra as follows:

$$GP_{em} = [I_{440} - I_{490}] / [I_{440} + I_{490}]$$

I_{440} and I_{490} are the intensities at the emission maxima of 440 and 490 nm, respectively.

Fluorescence measurements were performed on a computer controlled Perkin Elmer LS-50 Luminescence Spectrometer equipped with a thermostatic cuvette [Julabo Labortechnik, Seelbach, Germany]. The scan speed was 100 nm/min and the monochromator slits were fixed at 8 nm.

4. Results and Discussion

4.1. Carotenoids incorporated into liposomes

4.1.1. Effective concentrations, incorporation yields and stability of carotenoids in liposomes

Based on the UV-Vis absorption spectra made from BC, CTX, LUT and ZEA after their incorporation into liposomes, we obtained their effective, final concentration in liposomes [EC] which was compared with the incubated concentrations [IC]. The incorporation yield [IY= IC/EC], was calculated for each carotenoid and also the stability of carotenoids in the liposomes at different times of storage [2 months and 6 months].

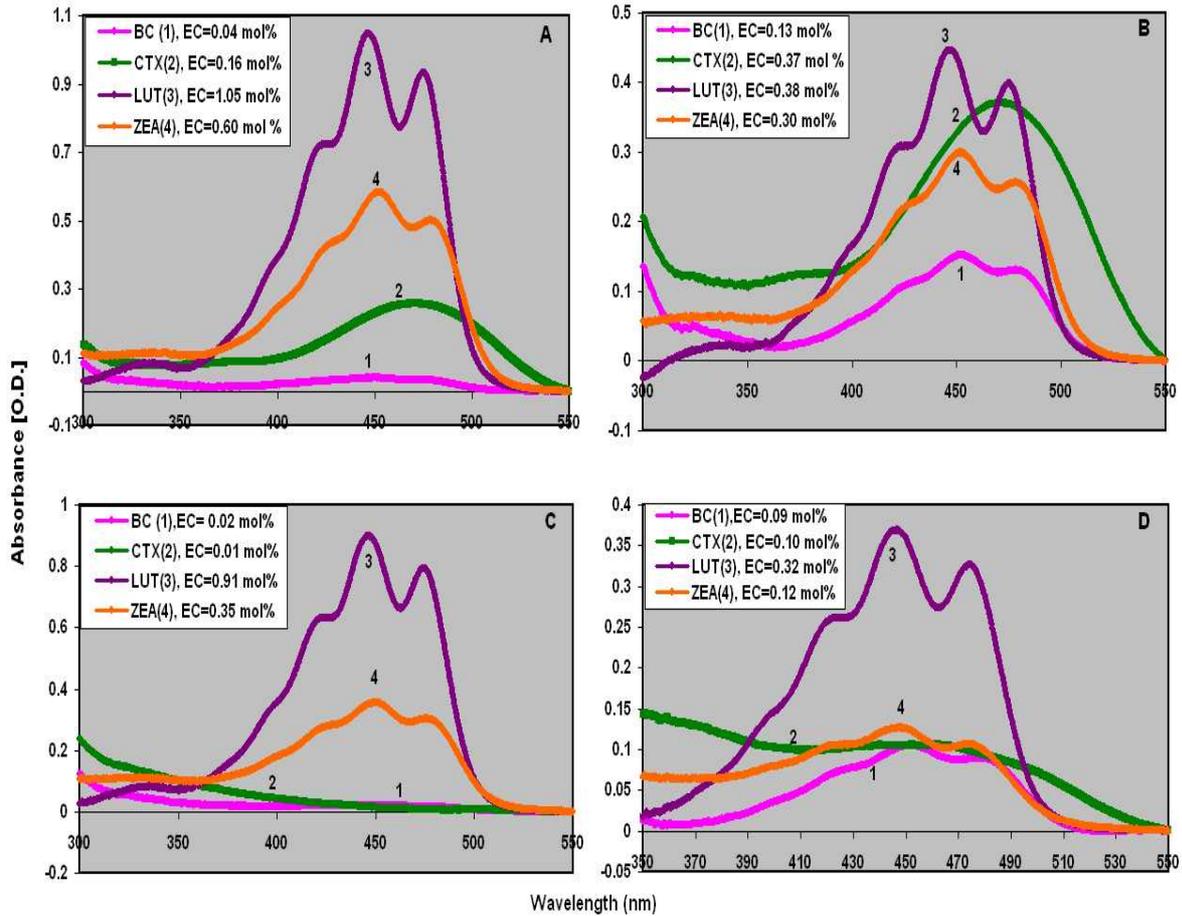
As it was mentioned in the section of Experimental we used two ways to incorporate carotenoids:

- *Preparation I*: an IC of 1.5 mol% was used for each carotenoid and then the EC and IY values were monitored.
- *Preparation II*: those IC [3 mol% for BC, 2 mol% for CTX and 0.5 mol% for LUT and ZEA] were used which achieved about the same EC in the liposomes.

By *Preparation I*, using same IC values, we intended to find informations about the specific capacity of each carotenoid to be inserted into liposomes. By *Preparation II*, based on the experience obtained from *Preparation I*, we tried to regulate/modify the IC for each carotenoid in such a way that the EC values were about the same for each carotenoid.

Fig.4-1 shows the different UV-Vis spectra of liposomes doped with carotenoids, after their separation and dissolution in ethanol. Fig.4-1A and C show the UV-Vis absorption spectra of these liposomes just after their preparation by

Preparation I and after 2 months of storage. Fig.4-1B and D show the UV-Vis absorption spectra of liposomes just after their preparation by *Preparation II* or after 2 months of storage. The EC values are mentioned in each case in the legend of these figures.



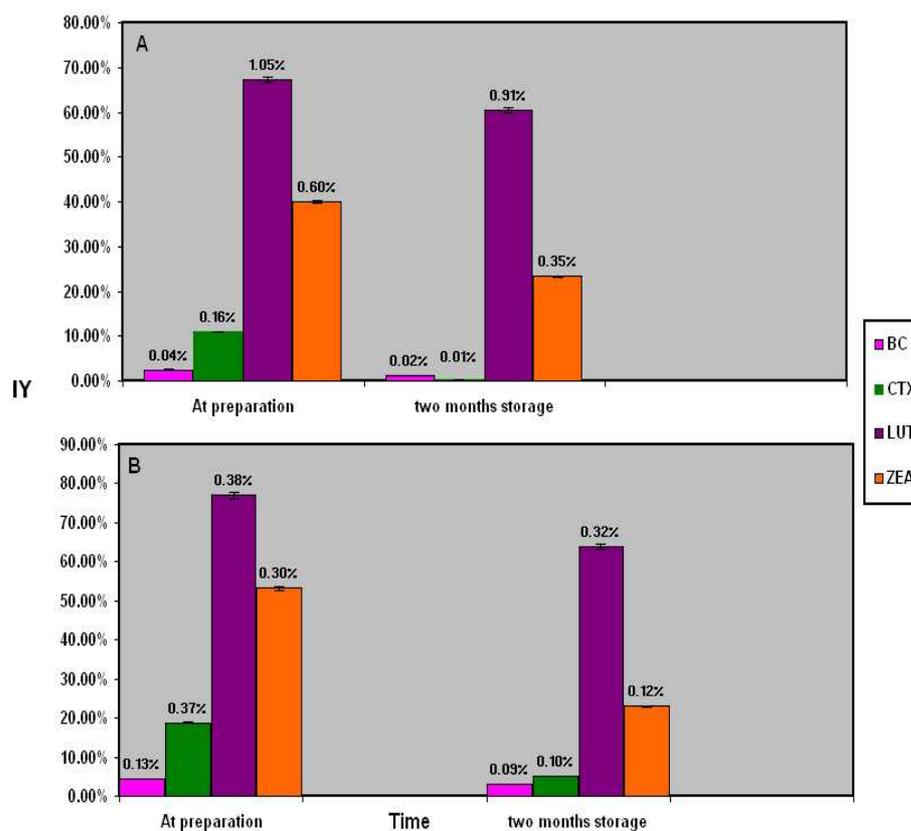
Fig[4-1]: UV-Vis spectra registered from liposomes with incorporated carotenoids by *Preparation I* just after their preparation [A] and after two months storage [C] respectively, and by *Preparation II* just after their preparation [B] and after two months storage [D] respectively. EC values are presented in the legends.

Table 4-1 presents the IC, EC and IY for all carotenoid-doped SUV liposomes, obtained according to *Preparations I and II*. Fig.4-2 presents the incorporation yields [IY] of all carotenoids in liposomes using *Preparations I or II* [Fig 4-2A or B], just after their preparation or after two months of storage. One can notice that incorporated concentrations of carotenoids [EC values] were higher for LUT and ZEA, using both preparations and at all moments [0, 2 or 6 months of

storage]. The EC values for the carotenoids do not depend on the preparation procedure neither just after their preparation nor after two months of storage. The highest IY is exerted just after preparation by LUT [about 72%], followed by ZEA [about 46%], CTX [about 15 %] and BC [about 3.5%]. After two months of storage a considerable amount of carotenoids get lost. It is only about 13 % for LUT but about 50% for ZEA and even about 80% for the CTX. The loss of BC is also high but it has not been determined exactly because its IY is anyway very low. So, LUT maintained stable to an higher extent than ZEA and CTX. The incorporation yield [IY] of phospholipids into control liposomes was about 94% in all cases.

Carotenoid	Preparation	IC [mol%] At direct preparation	EC [mol%]		IY [%]	
			At day of preparation	two months after preparation	At day of preparation	two months after preparation
BC	I	1.5	0.04	0.02	2.5	1.2
	II	3.0	0.13	0.09	4.5	3.0
CTX	I	1.5	0.16	0.01	11.0	0.4
	II	2.0	0.37	0.10	18.7	5.0
LUT	II	0.5	0.38	0.32	77.0	63.8
	I	1.5	1.05	0.91	67.2	60.5
ZEA	II	0.5	0.30	0.12	53.2	23.0
	I	1.5	0.60	0.35	40.0	23.3

Table 4-1: Incorporation yields [IY] of different initial [IC] and effective [EC] concentrations of carotenoids in liposomes, directly after preparation and after two months storage. Both preparations [I and II] were considered.



Fig[4-2]: Incorporation yield [IY%] for carotenoids at IC= 1.5 mol% [*Preparation I*, A], and at IC= 3mol% for BC, 2 mol% for CTX, 0.5 mol% for both LUT and ZEA [*Preparation II*, B] doped in liposomes. In both Preparations, the stability at preparation and after two months storage is also presented. EC values [mol%] are given on top of each column.

After 6 months storage, the UV-Vis spectra [data not shown] indicated that obviously BC and CTX suffered complete decays, while ZEA underwent a partial decay. LUT was the most stable one. A distribution of LUT into two differently oriented pools within the membrane [Fig.2-23] probably is related to the higher stability of this pigment to bleaching. Two orthogonal orientations of dipole transition moments of LUT molecules will provide the 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 absorbed 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 [Gruszecki *et al.*, 1999]. In that sense, LUT seems to be suited better than ZEA in acting like a screen against the excess of radiation. So, a main function of LUT in the membranes of retina is to decrease the level of blue light penetrating the membrane.[Sujak *et al.*, 1999]. LUT was found that it can exist also in an aggregated state in the membrane with the absorption band shifted towards shorter wavelengths [Gruszecki, 1999]. Such a hypsochromic shift makes it possible to filter out the high energy near UV radiation harmful to biological molecules. In addition, LUT has one less conjugated double bond [10 double bonds] than does the other carotenoids [11 double bonds], which may account for its somewhat slower reactivity to oxidation.

Major carotenoids of human plasma and tissues were exposed to radical-initiated autoxidation conditions. The consumption of lutein and zeaxanthin, the only carotenoids in the retina, and lycopene and β -carotene, the most effective quenchers of singlet oxygen in plasma, were compared. Under all conditions of free radical-initiated autoxidation of carotenoids which were investigated, the breakdown of lycopene and β -carotene was much faster than that of lutein and zeaxanthin. Under the influence of UV light in presence of Rose Bengal, by far the highest breakdown rate was found for β -carotene, followed by lycopene. Bleaching of carotenoid mixtures mediated by hypochloric acid [NaOCl], addition of azo-bis-isobutyronitril [AIBN], and the photo-irradiation of carotenoid mixtures by natural sunlight lead to the following sequence of breakdown rates: lycopene > β -carotene > zeaxanthin > lutein. The slow degradation of the xanthophylls zeaxanthin and lutein may explain the prevalence of zeaxanthin and lutein in the retina of man and other species. In correspondence to that, the rapid degradation of β -carotene and lycopene under the influence of natural sunlight and UV light is postulated to be

the reason for the nearly complete lack of those two carotenoids in the human retina [*Siems et al., 1999*].

Considering previous data obtained from different experiments in our laboratory or elsewhere we can assume that our results for EC and IY of LUT, ZEA, CTX and BC are comparable with previous data obtained for SUV liposomes [*Socaciu et al., 1999; Socaciu et al., 2000a; Socaciu et al., 2002*].

The efficacy of carotenoids to filter out blue light was investigated in unilamellar liposomes [*Junghans et al., 2001*]. In this model, LUT and ZEA showed a stronger filtering efficacy than β -carotene or lycopene. It was suggested that the more higher efficacy of LUT and ZEA is related to the orientation of the incorporated molecules in the liposomal membrane. Such microstructural differences may be a reason why LUT and ZEA can be incorporated into membranes in higher amounts than other carotenoids like β -carotene or lycopene and why LUT and ZEA but not other carotenoids are used for the protection of macula.

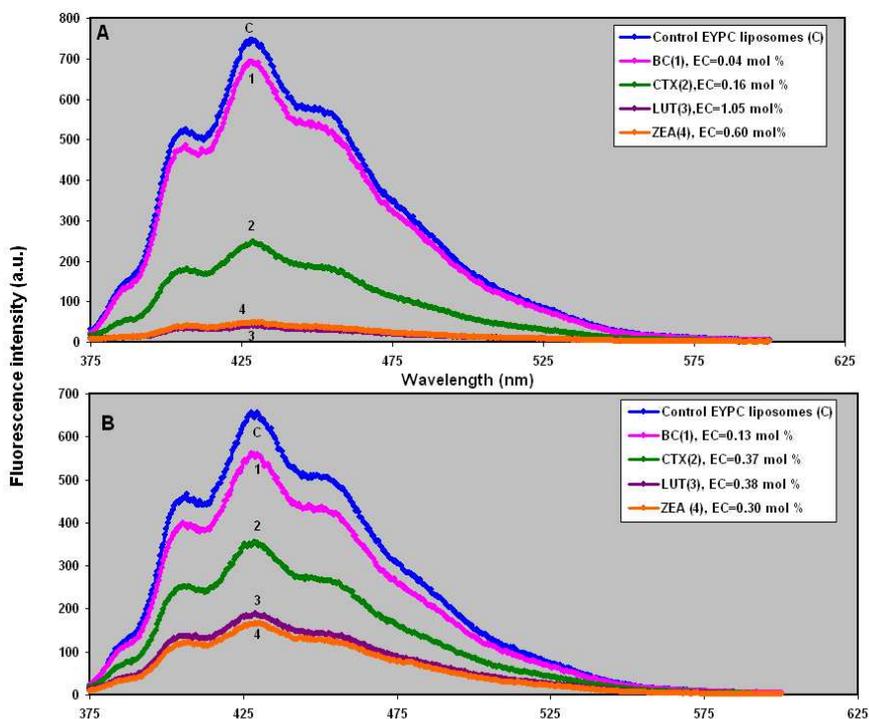
Conclusively, LUT and ZEA are best incorporated into liposomes, they cause the highest carotenoid/lipid ratio in the liposomes whereas BC causes the lowest one. These data suggest a preferential incorporation of polar carotenoids into SUV liposomes, LUT being favoured for incorporation and more stable in time. The threshold of incorporation [limit of saturation] seems to be around 0.3 mol% for LUT, ZEA and CTX and 0.1 mol% for BC. Lastly, in liposomes we could not succeed to have as high EC values for BC as for xanthophylls.

4.1.2. Effects of carotenoids on membrane properties, evaluated by fluorescence spectrometry of Laurdan - and DPH - labeled liposomes

Fig.4-3A and B show the emission spectra [375-600 nm, using $\lambda_{exc}=358$ nm] of DPH-labeled liposomes, previously doped with carotenoids by *Preparation I* [A]

and *Preparation II* [B], respectively. The polar carotenoids [LUT and ZEA] cause a strong quenching of the DPH emission peak. The maximal quenching activity of both LUT and ZEA was 94%, less for CTX [67%] and low for BC [7%].

Fig.4-3B shows the corresponding results using *Preparation II*. LUT and ZEA quench more efficiently the DPH emission, by 72% and 75% respectively, than CTX [46%] which is incorporated with about the same EC as LUT and ZEA. The lowest quenching efficiency was observed for BC [15%]. Here we must consider that BC we can incorporate only with an EC of about one third of the others and the quenching effect of BC is also one third that of CTX, but only one fifth that of LUT and ZEA. These differences can be explained by the assumption that BC and CTX locate at about the same space, the core of the membrane where they, although better accessible to DPH, are subjected to a less lipophilic environment, because they simultaneously cause a more fluid membrane which allows a higher degree of water intrusion.



Fig[4-3]: Emission spectra of DPH [0.5 mM in DMF] at $\lambda_{exc} = 358$ nm incorporated into liposomes doped with carotenoids by *Preparation I* [A], and liposomes doped with carotenoids by *Preparation II* [B]. The spectra were recorded at preparation time of carotenoid doped liposomes and EC values are presented in the legends.

Table 4-2 presents the quenching efficiencies of all carotenoids in DPH-labeled liposomes.

Carotenoid	Preparation	IC [mol%]	EC [mol%]	DPH Quenching % EM	Laurdan Quenching %	
					EXC	EM
BC	I	1.5	0.04	7.3	1.1	9.0
	II	3.0	0.13	15.0	42.2	44.5
CTX	I	1.5	0.16	67.3	58.1	71.0
	II	2.0	0.37	46.2	51.5	67.3
LUT	II	0.5	0.38	71.7	68.1	77.2
	I	1.5	1.05	94.5	82.1	89.3
ZEA	II	0.5	0.30	74.9	67.0	76.3
	I	1.5	0.60	93.8	76.8	87.1

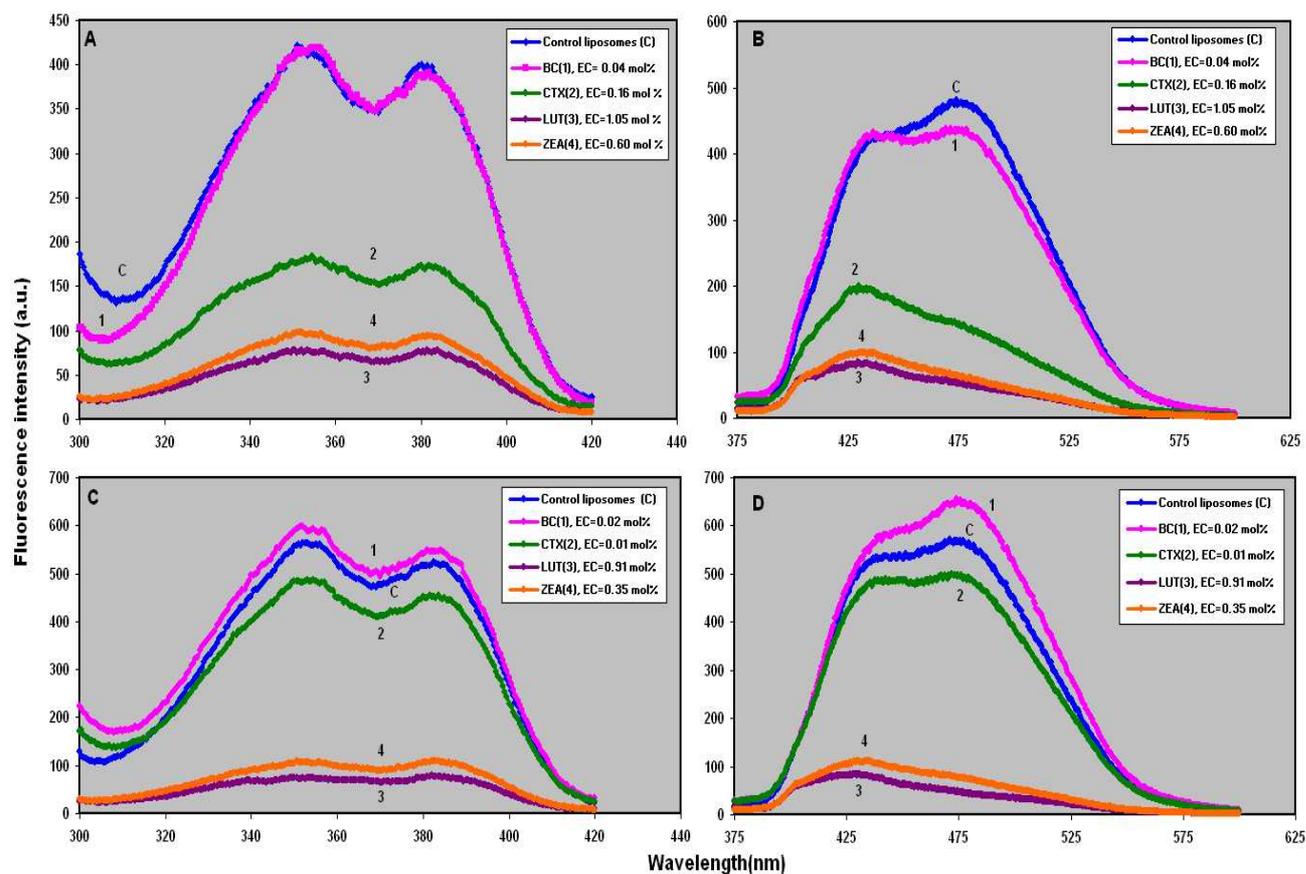
Table 4-2: Correlation between IC and EC values of carotenoids incorporated into liposomes and labeled with DPH or Laurdan. The fluorescence quenching efficiencies [%] of the individual carotenoids are also included. Both preparations [I and II] were considered.

Fig.4-4 A-D presents the excitation spectra [$\lambda_{em}= 440$ nm] [A and C] and emission spectra [$\lambda_{exc}= 353$ nm] [B and D] of Laurdan which was incorporated in the liposomes previously doped with carotenoids according to *Preparation I*. The spectra were registered either immediately after their preparation [A and B, respectively] or after 2 months of storage [C and D].

The incorporation of carotenoids exerts a strong quenching of Laurdan excitation peaks [A and C] especially in liposomes containing LUT and ZEA, less in

liposomes contain CTX and slightly in liposomes which contain BC. A significant quenching of fluorescence intensity was noticed for LUT and ZEA [82% and 77% respectively]. CTX was less efficient in quenching [58%] and BC had minimum quenching efficiency [1%]. The efficiency of quenching showed to be strongly correlated with the EC value and less related to the carotenoid structure.

The incorporation of carotenoids exerts also a strong quenching of Laurdan emission peaks [B and D] which was more efficient in liposomes containing LUT and ZEA, less efficient in liposomes containing CTX and low in liposomes containing BC. Carotenoids, at most LUT, ZEA and CTX, quenched very efficiently [89, 87 and 71%, respectively] the emission peak at 478 nm, while BC quenched only by 9%.

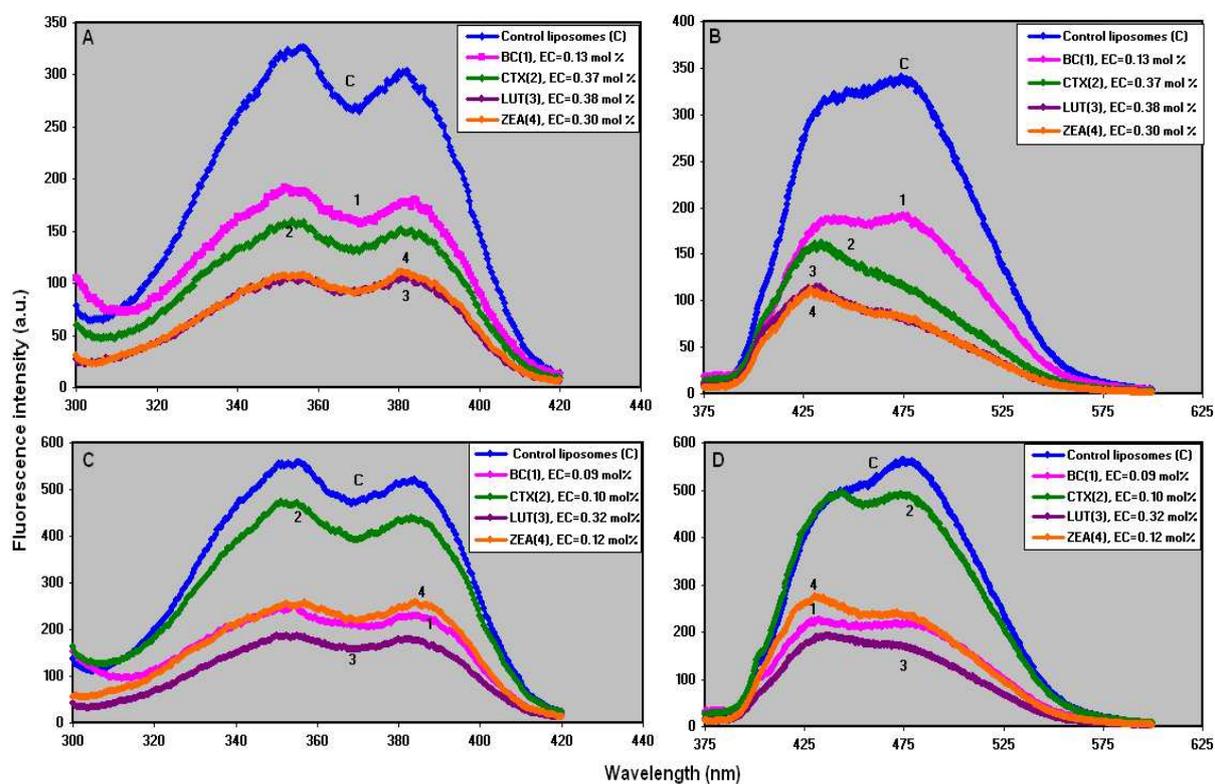


Fig[4-4]:Fluorescence spectra of Laurdan [excitation-A and C], [emission-B and D] incorporated into liposomes doped with carotenoids by Preparation 1 after direct preparation [A and B] and after 2 months storage [C and D].

Fig.4-5 A-D presents the excitation [$\lambda_{em}= 440$ nm] [A and C] and emission [$\lambda_{exc}= 353$ nm] [B and D] spectra of Laurdan which was incorporated in the liposomes previously doped with carotenoids according to *Preparation II*. The spectra were registered immediately after their preparation [A and B, respectively] or after 2 months of storage [C and D].

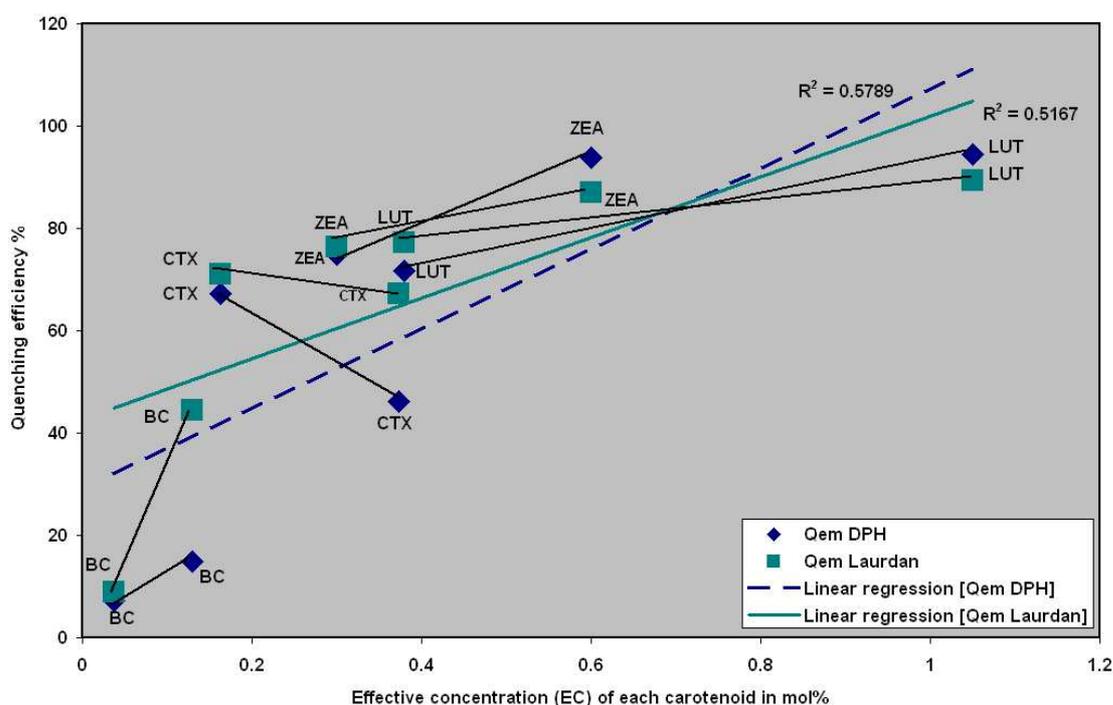
Fig.4-5A and C show the efficiency of LUT and ZEA to quench the fluorescence intensity of Laurdan excitation up to 68% against control. Other carotenoids such as CTX and BC were less active as quenchers [51% and 42%, respectively].

Fig.4-5B and D show the efficiency of LUT and ZEA to quench the fluorescence intensity of Laurdan emission up to 77% against control. Other carotenoids such as CTX and BC were less active as quenchers [65% and 44% respectively].



Fig[4-5]:Fluorescence spectra of Laurdan [excitation-A and C], [emission-B and D] incorporated into liposomes doped with carotenoids by *Preparation II* at preparation [A and B] and after 2 months storage [C and D].

Fig.4-6 presents the correlation between the quenching efficiency and the EC of the carotenoids. The correlation factors [R^2] are given for DPH and Laurdan. These values indicate statistically significant correlations [$R^2 = 0.5789$ for DPH and 0.5167 for Laurdan]. They show a generally positive correlation between the quenching efficiency and the EC values. LUT and ZEA are more close to the correlation line than BC which quenching efficiencies are below the correlation line, indicating a weak interaction with Laurdan.. The same holds partly for CTX.



Fig[4-6]: Correlation between the emission quenching efficiency [%] of carotenoids, as obtained from DPH and Laurdan spectra, depending on the EC values [mol%] for each carotenoid in liposomes.

Our results are in accordance with those published recently [Socaciu *et al.*, 2002], where LUT and ZEA were significantly more effective quenchers of Laurdan than BC. This can be explained by their intense interaction with the lipid bilayer, containing a non-polar dye like DPH or an amphiphilic dye like Laurdan [located in the hydrophobic-hydrophilic interface of membrane].

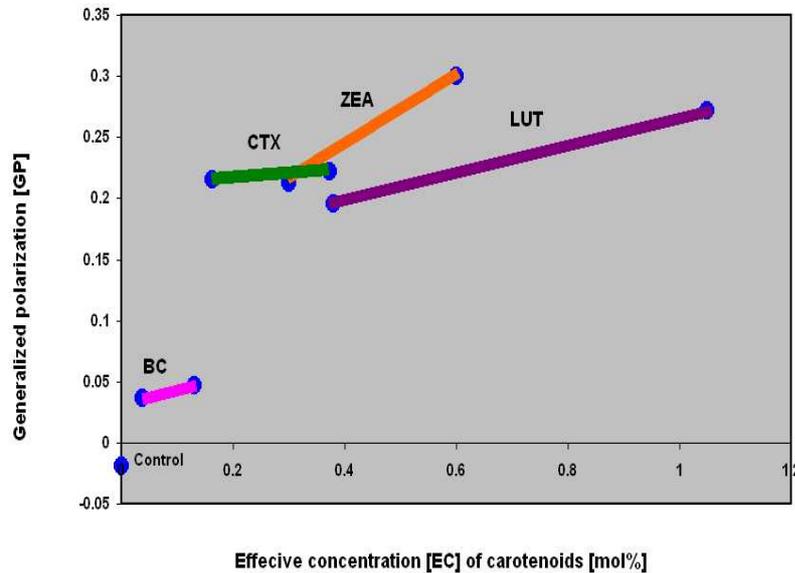
The quenching data registered for Laurdan [Fig.4-4 and 4-5, Table 4-2] show the efficiency of liposomes containing LUT and ZEA to display a strong

fluorescence quenching of Laurdan emission mainly in the 478 nm emission range. LUT and ZEA were also the most powerful quenchers of Laurdan excitation peaks [by 80%].

To investigate the influence of carotenoids on membrane rigidity, we calculated the generalized polarization values. Fig.4-7 presents the relationships between the GP_{em} values calculated for liposomes containing carotenoids and Laurdan, considering their EC values registered in both preparations I and II.

LUT, ZEA and CTX revealed high GP_{em} values while BC was nearly as low as the control value. It should be noticed that LUT and ZEA exerted the same strong quenching efficiency despite ZEA has only approximately the half EC value than LUT [Table 4-1]. The quenching capability of carotenoids depends on the number of conjugated double bonds. LUT has 10 conjugated double bonds, while ZEA contains 11 and it is indeed a better quencher than LUT [Conn *et al.*, 1991]. From this point of view, the preponderance of ZEA over LUT in the macular center where the incident light is most intense and the formation of reactive species is greatest, seems suitable.

An increase of the GP_{em} values with its membrane concentration is observed for LUT and ZEA [Fig.4-7]. It is known that the more is positive the GP_{em} , the more the membrane is rigid and hydrophobic. So, in the higher GP_{em} values for LUT and ZEA suggest a rigidifying of membrane. The low GP_{em} value of BC indicates a very small membrane rigidisation. This may be explained by its low EC value and by its preference to locate randomized in the non-polar core region of the bilayer. CTX shows an as intensive interaction with Laurdan as do LUT and ZEA, whereas it was similar to BC considering the interaction with DPH. CTX may form an intermolecular hydrogen bond with Laurdan which is located nearer to the membrane head-group than DPH and thus bring CTX in a location which is more similar to that of LUT and ZEA which rigidify the membrane.



Fig[4-7]: Correlation between the general polarization [calculated from emission spectra of Laurdan] and the EC values of carotenoids [mol%] incorporated into liposomes labelled with Laurdan [data collected from both *Preparations I and II* just after preparation].

A general rigidifying effect was observed for xanthophylls, correlated with their capacity to affect membrane dynamics, acting as reactive oxygen species quenchers, decreasing permeability, increasing hydrophobicity in the membrane interior and decreasing the permeability to water and oxygen, in the polar head-group region [Wisniewska and Subczynski, 1998]. The carotenoids found in cells and tissues reflect essentially the food composition but they seem to be selectively absorbed by membranes, depending on the structural carotenoid features [size, shape and polarity], as well as on membrane characteristics [composition, fluidity, cholesterol, protein content, ..etc] [Gruszecki, 1999 and references therein]. These properties determine the incorporation yield and the carotenoids's ability to fit into the membrane bilayer.

Membrane rigidity seems to be strongly related to LUT and ZEA since these carotenoids have higher limits of insertion in the bilayer than BC and CTX, for which the limits of incorporation are significantly lower. These data affect strongly the selectivity of membranes against different carotenoids or cholesterol or other lipids absorbed from food, as it was published in many articles [Gruszecki, 1999 and references therein; Sujak et al., 1999; Sujak and Gruszecki, 2000; Sujak et al., 2000 and Sujak et al., 2002].

It has been reported that, LUT broadens the spectral NMR lines representing the resonance of ^1H in CH_2 and of terminal CH_3 groups of the lipid acyl chains which is a direct demonstration of restriction of the lipid molecular motion brought about by interactions of the membrane embedded pigments [Chaturvedi and Kurup, 1986]. Gabrielska and Gruszecki [1996] have shown by proton NMR that incorporation of zeaxanthin into the lipid bilayer is associated with increased rigidity of the membrane which is not observed in liposomes prepared with β -carotene; they suggest that zeaxanthin molecules adopt a regular orientation in the membrane, with the polar ends contacting the aqueous milieu and the hydrophobic chain in the membrane's hydrophobic core, and that β -carotene, which has no polar moiety, by contrast becomes randomly organized in the membrane [Fig.4-8].

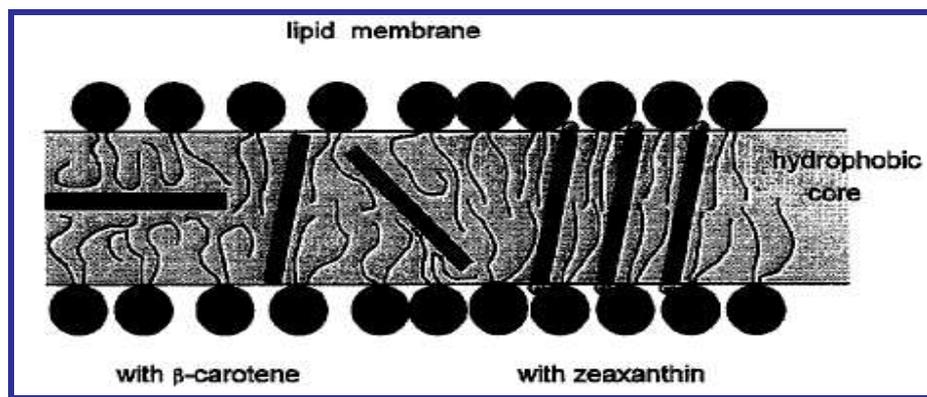


Fig.4-8. Schematic drawing of the EYPC membrane containing β -carotene and zeaxanthin. Please note the differences in the orientation of carotenoids, organization of alkyl chains and the distance between lipid head groups [adapted from Gabrielska and Gruszecki, 1996].

4.2. Carotenoids incorporated into microsomes

4.2.1. Effective concentrations, incorporation yields and stability of carotenoids in microsomes

The UV-Vis absorption spectra of BC, CTX, LUT and ZEA incorporated into microsomes, show the different abilities of the carotenoids to be incorporated and to be stable in the microsomal membrane. Their effective, final concentrations in microsomes [EC], the initial concentrations [IC] and the incorporation yields [IY] are presented in Table 4-3. To remember, we used two ways to incorporate carotenoids into microsomes:

- *Preparation I*: a fixed IC of 1.5 mol% carotenoid/lipid was used for each carotenoid and then the EC and IY values were monitored.
- *Preparation II*: those IC of the carotenoids [2.5 mol% for BC, 2 mol% for CTX and 1 mol% for LUT and ZEA] were used which achieved about the same EC in the microsomes

By *Preparation I*, using same IC values, we intended to find informations about the specific capacity of each carotenoid to be inserted into microsomes. By *Preparation II*, based on the experience obtained from *Preparation I*, we tried to regulate/modify the IC for each carotenoid in such a way that the EC values were about the same for each carotenoid.

When all IC were 1.5 mol% [carotenoid to microsomal lipid molecular ratio], very similar ECs and IYs were obtained for LUT and ZEA. The second preparation had the aim to obtain about the same EC for each carotenoid. This is achieved here in the case of microsomes while into liposomes this could not be achieved for BC. We see that BC can be incorporated better into microsomes than into liposomes [Table 4-3]. The very low EC and IY of BC in the case of the low IC does not fit to the other results. It must be mentioned that BC during the incubation process got lost to

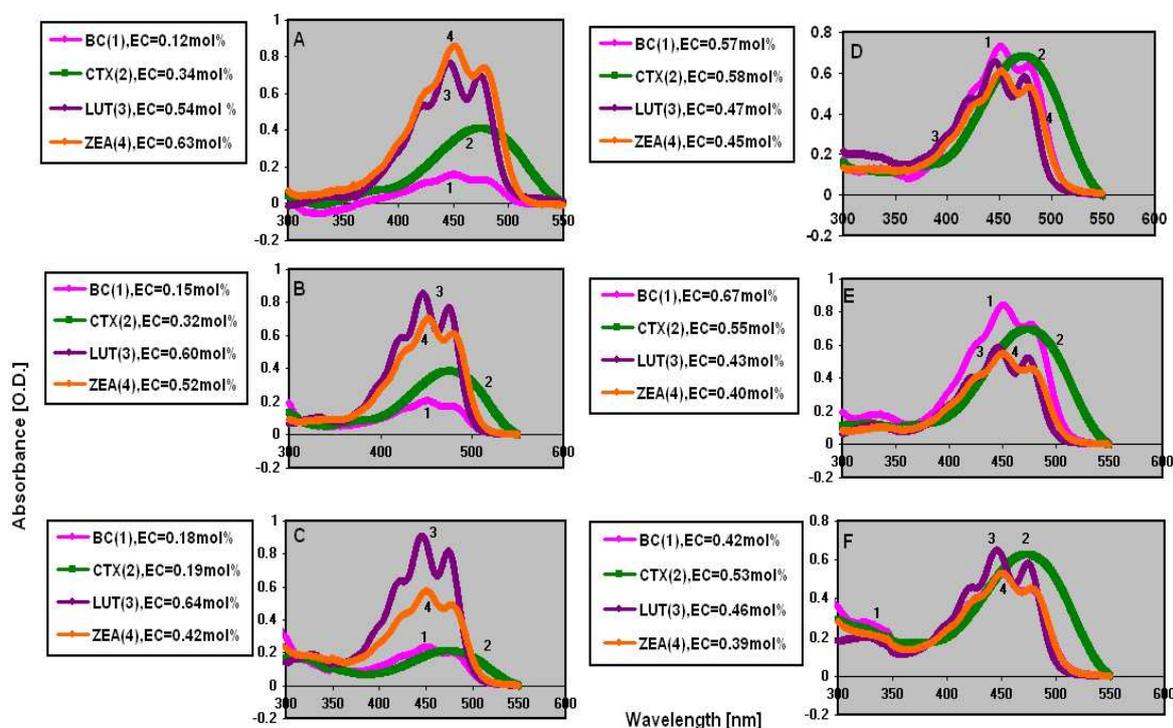
a considerable content whereas the other carotenoids did not. This holds also for the liposomes. In the discussion of results we consider this. The IY of LUT and ZEA are around 42%, CTX at around 26%. BC incorporates with the lowest IY. The stability of the carotenoids in microsomes is much more stable than in liposomes even after 7 months [-20°C].

Carotenoid	Preparation	IC [mol%] At direct preparation	EC [mol%]			IY [%]		
			At day of preparation	After one month of preparation	After seven months of preparation	At day of preparation	After one month of preparation	After seven months of preparation
BC	I	1.5	0.12	0.15	0.18	8.6	10.0	11.7
	II	2.5	0.57	0.67	0.42	23.0	26.7	17.0
CTX	I	1.5	0.34	0.32	0.19	23.0	21.8	13.0
	II	2.0	0.58	0.55	0.53	29.1	28.0	26.5
LUT	II	1.0	0.47	0.43	0.46	47.0	43.1	46.0
	I	1.5	0.54	0.60	0.64	36.5	39.9	42.7
ZEA	II	1.0	0.45	0.40	0.39	45.0	39.9	39.2
	I	1.5	0.63	0.52	0.42	42.0	34.2	27.8

Table 4-3: Incorporation yields [IY] of carotenoids at different initial concentrations [IC] and effective final concentrations [EC] in microsomes. The data were registered at day of preparation or after one or seven months of storage. Both preparations [I and II] were considered.

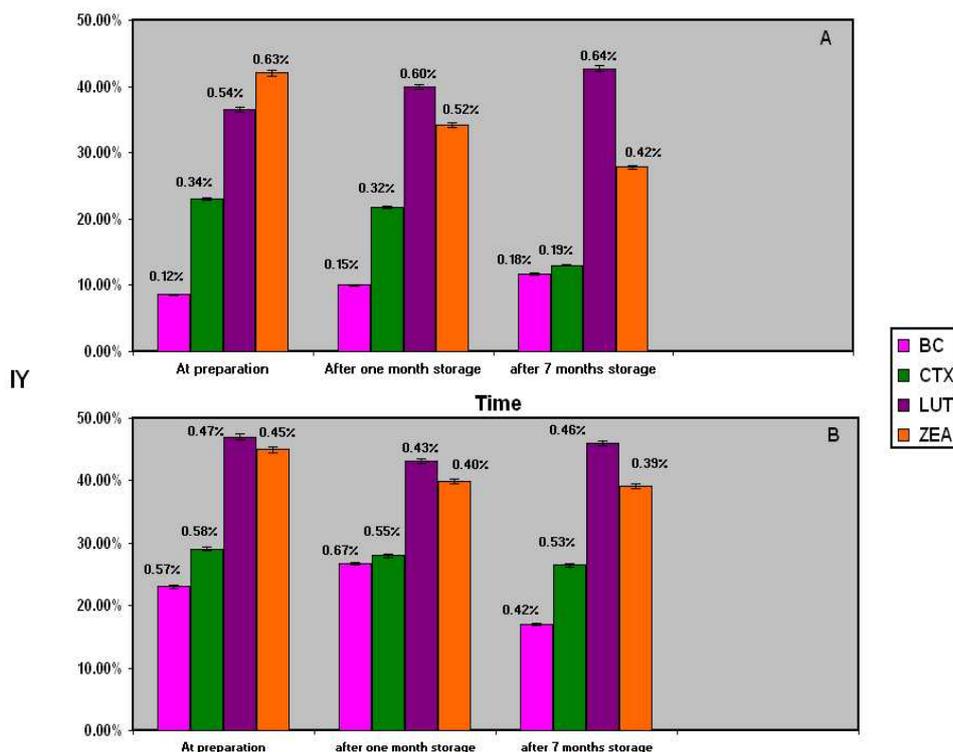
Fig.4-9A-F show the UV-Vis spectra of microsomes doped with carotenoids after their separation and dissolution in ethanol. The spectral characteristics of carotenoids [fine structure of the absorption peaks] are well kept which demonstrates their stability also after storage. Fig.4-9A, B and C show the UV-Vis absorption spectra from carotenoid doped microsomes just after *Preparation I* and after one and seven months of storage, respectively. Fig.4-9D, E and F show the

UV-Vis absorption spectra of microsomes just after *Preparation II* and after one and seven months of storage, respectively. The corresponding EC values are given.



Fig[4-9]:UV-Vis spectra registered from microsomes with incorporated carotenoids by *Preparation I* [A, B and C], and by *Preparation II* [D, E and F]. The spectra were registered at preparation, after one and seven months storage, respectively. EC values are presented in the legends

Fig.4-10 summarizes the IY values from both preparations I and II [Fig.4-10A or B] just after the preparation and after one and seven months of storage. The decay over the time is generally much weaker than in liposomes. In microsomes as well as in liposomes LUT appears to be the most stable and the ranking of IY is LUT > ZEA > CTX > BC. LUT proved to be better incorporated into liposomes than into microsomes [LUT around 70% in liposomes and 42% in microsomes], while ZEA was equally incorporated in both membrane types [around 46% in liposomes and 42% in microsomes]. In both systems LUT was highest incorporated and most stable in both membranes. BC and CTX were more favoured to enter and to keep stable in microsomes, and LUT seems to be incorporated better into liposomes than into microsomes.



Fig[4-10]:Incorporation yield [IY%] for carotenoids at IC= 1.5 mol% [Preparation I, A], and at IC= 2.5 mol% for BC, 2 mol% for CTX, 1mol% for both LUT and ZEA [Preparation II, B] doped in microsomes. In both preparations, the stability at preparation and one or seven months storage is also presented. EC values [mol%] are given on top of each column.

Our results for EC and IY of LUT, ZEA, CTX and BC are in good agreement with previous data obtained for microsomes [Socaciu *et al.*, 2000c].

With regard to the high stability of LUT, it has been found that songbirds selectively incorporate LUT into and exclude ZEA from yellow feathers [McGraw *et al.*, 2003]. The authors suggest that LUT may out-compete ZEA for binding sites in feather follicles. At the functional level, it is especially perplexing why a more conjugated [and thus more colourful] molecule like ZEA is not shunted to brightly coloured feathers that probably are used as sexual or social signals to attract mates or repel competitors. One intriguing possibility is that ZEA containing more conjugated bounds is a superior antioxidant than LUT, such that ZEA may be saved in the living tissue for intracellular free radical-scavenging rather than made inaccessible by allocating it to non-living feathers. Another idea is that extrinsic selection pressures such as predation and light environment favour yellow LUT-

based plumage colouration over the more orange hue that would be generated by the incorporation of ZEA into feathers.

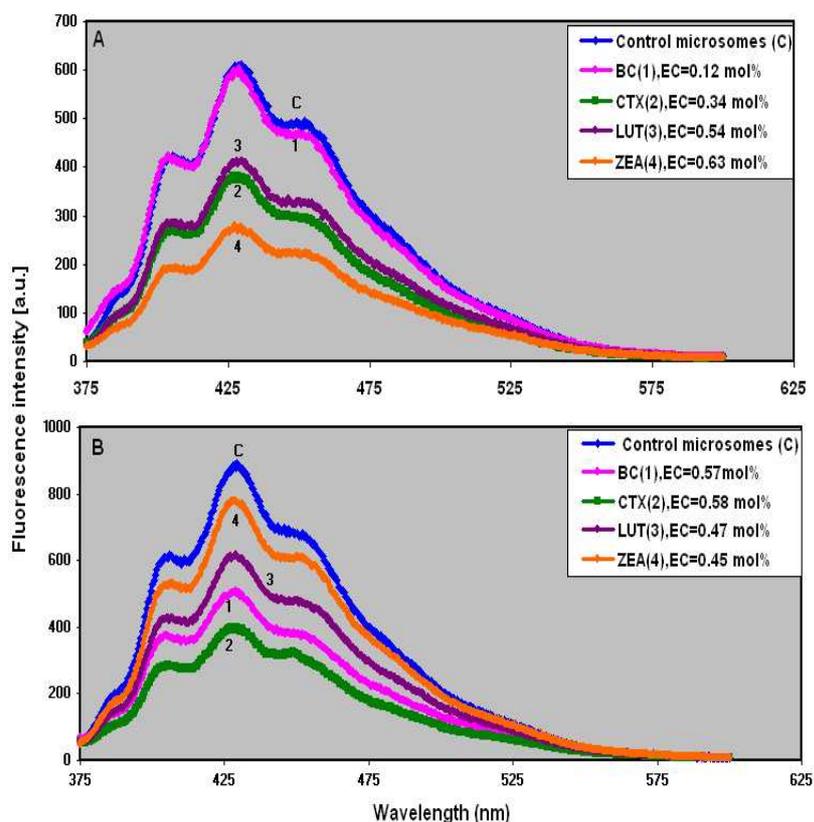
A demonstration that LUT is more stable than ZEA was also given by *Khachick et al. [1995]*. They purified LUT from marigold flowers and ZEA from *Lycium chinense*, a berry used in Chinese traditional medicine, and administered suspensions in olive oil to three volunteers. Daily doses of 10 mg were given for 18 [LUT] or 21 [ZEA] days. HPLC showed that the serum levels of both carotenoids peaked after one week: LUT at 0.9 $\mu\text{mol/L}$, and ZEA considerably lower at 0.1 $\mu\text{mol/L}$.

Considering the general ratio of IYs for BC: CTX: LUT: ZEA, this was 1: 2: 3: 3 in microsomes comparing with 1: 5: 20: 15 in liposomes. Overall, one can deduce that microsomes favour BC and CTX more and LUT less than liposomes do. One of the possible explanations for the discrimination of LUT and ZEA is, that ZEA is oriented in the lipid bilayer as close to the normal as possible. It induces minute disturbances of membrane structure or even exerts an ordering effect. It is likely, that this specific property of ZEA is due to the geometry and rigidity of its structure. The only stable position of the ZEA molecule in the lipid bilayer is such that its two hydroxyls interact with two opposite polar regions. In other words, the ZEA molecule must span the hydrophobic core of bilayer. Relatively free rotation of one ring in the LUT molecule make possible that molecules of LUT can be parallel to the plane of membrane with their hydrophobic portions in the core of bilayer and polar groups interacting with polar head region at the same side of the bilayer [Fig.2-23]. Such a carotenoid orientation introduces disorder to the membrane [*Sielewiesiuk et al., 1997*].

We conclude that microsomes are a membrane system which can be useful for further studies of carotenoid effects on natural membranes.

4.2.2. Effects of carotenoids on membrane properties, evaluated by fluorescence spectrometry of Laurdan - and DPH - labeled microsomes

Fig.4-11A and B shows the emission spectra [375-600 nm, using $\lambda_{exc}=358$ nm] of DPH-labeled microsomes, previously doped with carotenoids by *Preparation I* [A] and *Preparation II* [B], respectively.



Fig[4-11]: Emission spectra of DPH [0.5 mM in DMF] at $\lambda_{exc}=358$ nm incorporated into microsomes doped with carotenoids by *Preparation I* [A], and microsomes doped with carotenoids by *Preparation II* [B]. The spectra were recorded at preparation time of carotenoid doped microsomes. EC values are presented in the legends.

Table 4-4 shows the absolute values of the quenching efficiencies of all carotenoids in DPH-labeled microsomes. The maximal quenching efficiency for LUT was 32% and for CTX 45%. BC and ZEA showed very different quenching efficiencies: only 2.7 % [*Preparation I*] and 43.8 % [*Preparation II*] for BC and 13.2% and 55% for ZEA respectively. By *Preparation II* [which realized almost equal ECs for all carotenoids] one can say that BC and CTX quenched more

efficiently the DPH fluorescence than LUT. ZEA has a very low quenching efficiency [Fig.4-11B].

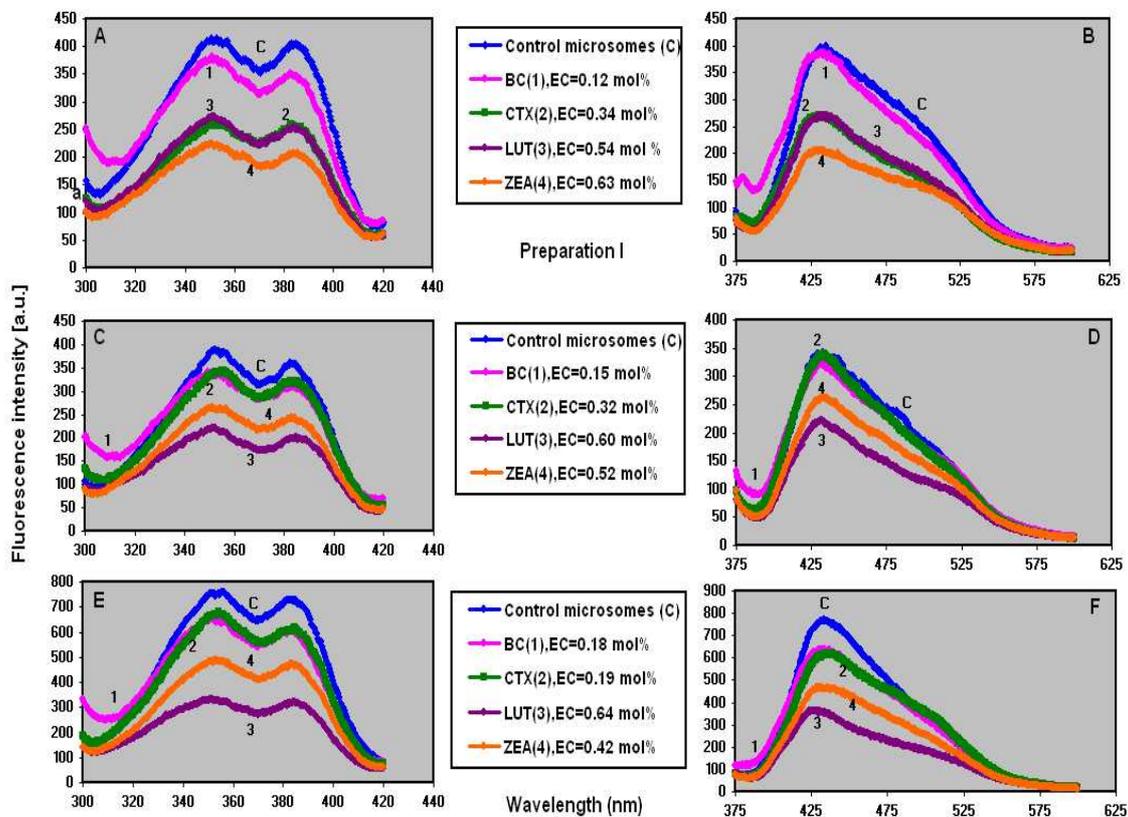
Carotenoid	Preparation	IC [mol%]	EC [mol%]	DPH Quenching [%] EM	Laurdan Quenching [%]	
					EXC	EM
BC	I	1.5	0.12	2.7	8.8	4.0
	II	2.5	0.57	43.8	59.0	57.4
CTX	I	1.5	0.34	38.8	37.9	32.5
	II	2.0	0.58	55.0	65.0	64.5
LUT	II	1.0	0.47	31.4	40.6	40.0
	I	1.5	0.54	32.4	35.0	33.0
ZEA	II	1.0	0.45	13.2	22.3	23.7
	I	1.5	0.63	54.5	46.6	49.5

Table 4-4: Correlation between IC and EC values of carotenoids incorporated into microsomes and labeled with DPH or Laurdan. The fluorescence quenching efficiencies [%] of individual carotenoids are also included. Both preparations [I and II] were considered.

These data suggest that in the non-polar region of the membrane, where DPH is located, BC and CTX are interacting more with DPH, while ZEA insertion does not interfere considerably with this region. It seems that the correlation between quenching efficiency and EC values is more important in microsomes than in liposomes. The correlation curve [Fig.4-14] suggests this.

Fig.4-12 A-F presents the excitation spectra [$\lambda_{em}= 440$ nm] [A, C and E] and emission spectra [$\lambda_{exc}= 353$ nm] [B, D and F] of Laurdan which was incorporated

into the microsomes doped with carotenoids according to *Preparation I*. The spectra were registered either immediately after their preparation [A and B, respectively] or after 1 month of storage [C and D] and after 7 months of storage [E and F]. To remember, *Preparation I* showed a preference of incorporation for ZEA and LUT compared to CTX and BC. In parallel to this preference, ZEA and LUT had higher quenching efficiencies, showing that they interact stronger with Laurdan in the membrane than BC does.

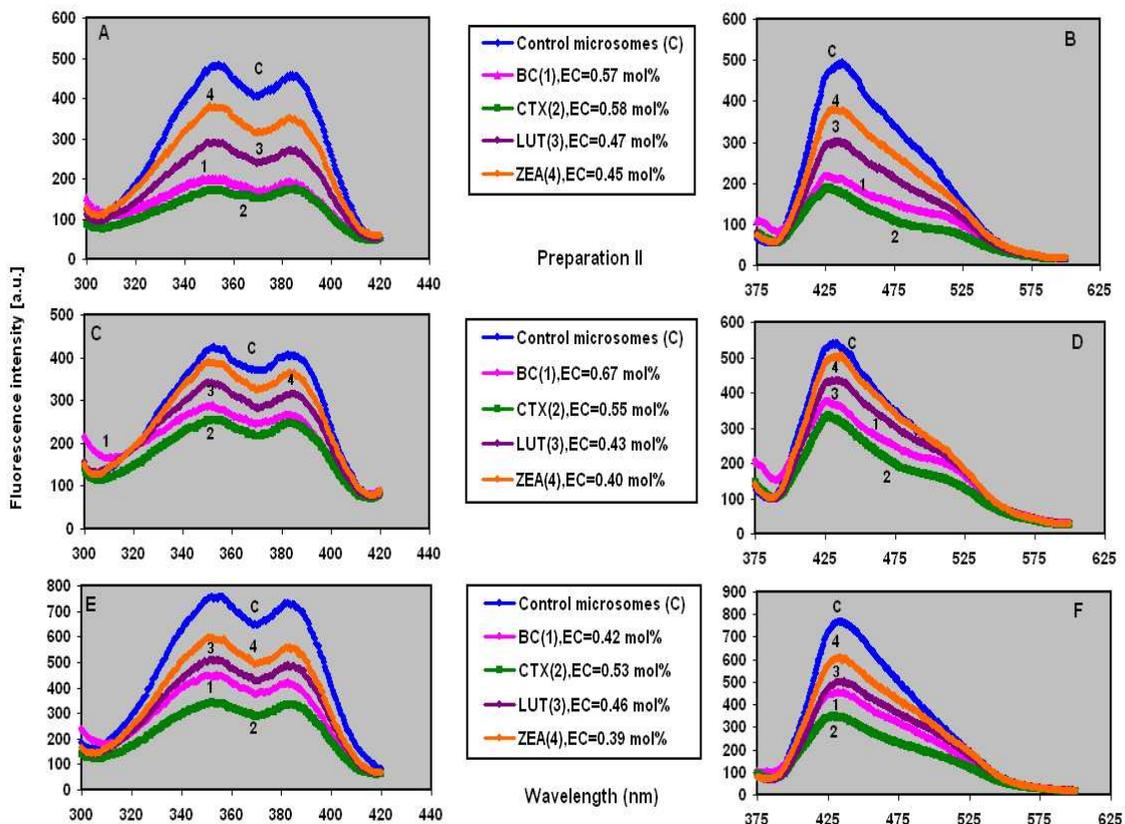


Fig[4-12]: Fluorescence spectra of Laurdan [excitation-A, C and E], [emission-B, D and F] incorporated into microsomes doped with carotenoids by *Preparation I*, at preparation [A and B], after one month storage [C and D] and after seven months storage [E and F] respectively. EC values are presented in the legends.

Storage did not change the quenching effect with the exception of ZEA which decayed in time and decreased its quenching ability compared to LUT [Fig.4-12C and D]. The order of quenching is the same in the emission and excitation spectra of Laurdan. Under storage, there is not much change. All carotenoids seem to increase the rigidity. The GP_{em} values quantify this [Fig.4-15].

The efficiency of quenching is strongly correlated with the EC value for both dyes, Laurdan and DPH [Fig.4-14].

Fig.4-13 A-F presents the excitation [$\lambda_{em}= 440$ nm] [A, C and E] and emission [$\lambda_{exc}= 353$ nm] [B, D and F] spectra of Laurdan which was incorporated in the microsomes previously doped with carotenoids according to *Preparation II*. The spectra were registered immediately after their preparation [A and B, respectively] or after one month of storage [C and D] or after 7 months [E and F].



Fig[4-13]: Fluorescence spectra of Laurdan [excitation-A, C and E], emission [B, D and F] incorporated into microsomes doped with carotenoids by *Preparation II*, at preparation (A and B), after one month storage [C and D] and after seven months storage [E and F] respectively. EC values are presented in the legends.

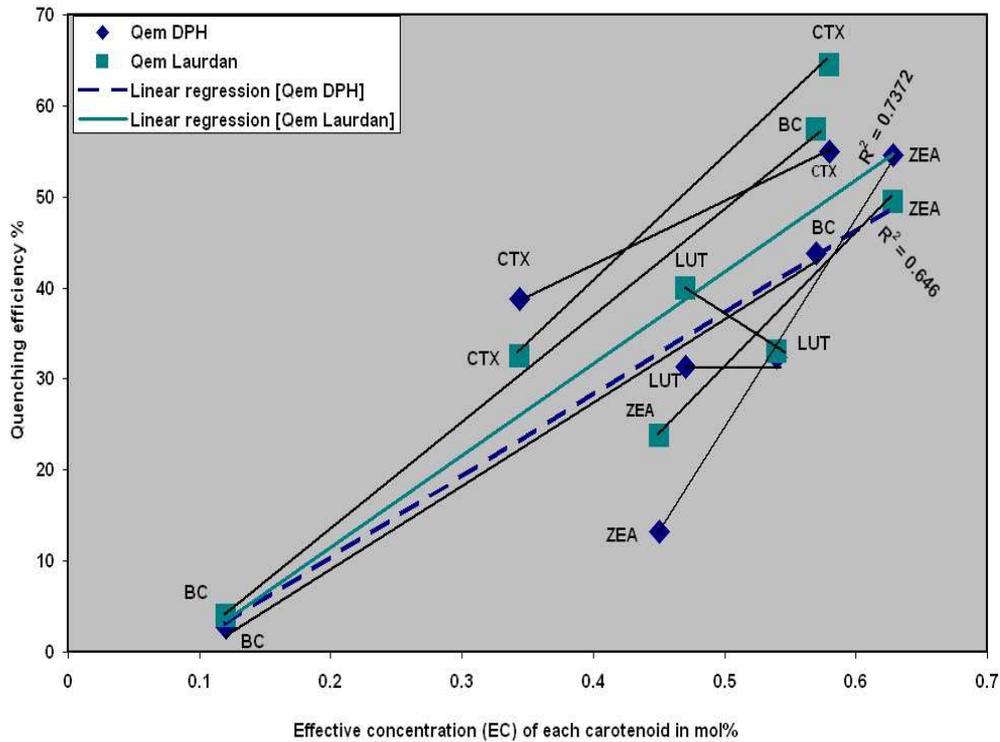
To remember, by *Preparation II* we realized almost equal concentrations of carotenoids in microsomes. Under these conditions, we observed for BC and CTX the highest quenching efficiency and not for LUT and ZEA. This interaction keeps constant during storage for 1 and 7 months and can be seen in both, excitation and emission, spectra. The high quenching efficiency of BC may be attributed to its

different location in microsomes and liposomes, BC in microsomes may not completely located in the core of the membrane as it does in liposomes, but partly will span the membrane and orient perpendicular to the membrane plane because of a stronger interaction of the aromatic rings of BC and cholesterol moieties [Socaciu *et al.*, 2000c]. It may be that CTX plays a similar role in our system.

The ranking of the quenching efficiency is similar with Laurdan and DPH. But the quenching of Laurdan is stronger than that of DPH. This suggests that all the carotenoids have close interaction with Laurdan. For BC and CTX this happens because of their partly orientation to the head-group area. It is remarkable that despite equal EC, BC and CTX quench Laurdan fluorescence more effective than LUT and ZEA, just opposite as it is the case in liposomes. Obviously the incorporation of ZEA and LUT into the microsomal membrane is restricted by the presence of cholesterol. From Fig.4-13B, D and F one can see the tendency of carotenoids to rigidify the membrane, comparing with control [quantitatively demonstrated by the GP_{em} values, see Fig.4-15]. Table 4-4 summarizes all data concerning the quenching efficiencies of carotenoids using DPH and Laurdan. Considering this percentage of quenching observed from the spectra of both fluorescent dyes [DPH and Laurdan] which labeled carotenoid-doped microsomes [presented in Fig.4-11, 4-12 and 4-13], we tried to establish the relationships and correlations between the EC values of carotenoids, their structure and the efficiency of quenching.

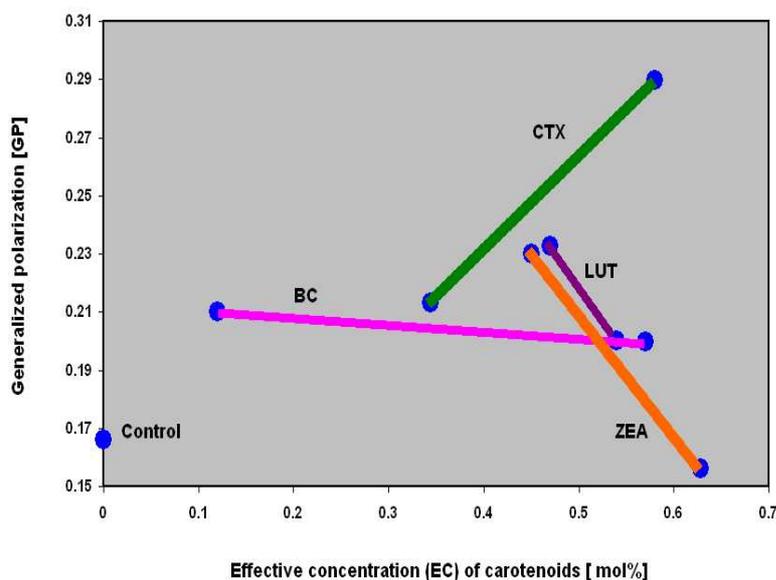
Fig.4-14 presents the correlation curves of the quenching efficiencies with the EC values, and the correlation factors [R^2]. Positive correlations are obtained between the quenching efficiencies and the EC values of the carotenoids, the values of R^2 being statistically significant [0.737 for Laurdan and 0.646 for DPH]. The correlation coefficients are higher in microsomes than in liposomes, especially for Laurdan. This gives us the possibility to evaluate the carotenoid concentrations

from intact microsomes by a fluorospectrometry instead of dissolving the microsomes, extracting the carotenoids and then determine the carotenoid spectrophotometrically.



Fig[4-14]: Correlation between the emission quenching efficiency [%] of carotenoids, from DPH or Laurdan spectra, and the EC values [mol%] for each carotenoid in microsomes.

To investigate the influence of carotenoids on membrane rigidity, we calculated the generalized polarization values from the emission spectra of Laurdan. Fig.4-15 presents the relation between the GP_{em} values and the EC values of the incorporated carotenoids.



Fig[4-15]: Correlation between the general polarization [calculated from emission spectra of Laurdan] and the EC values of carotenoids [mol%] incorporated into microsomes labelled with Laurdan [data collected from both Preparations I and II just after preparation].

The membrane rigidity which is obtained by the low incorporation concentrations of ZEA and LUT turns to a more fluid state at high incorporation concentrations. This may be explained by a kind of incorporation saturation, where the membrane well fitting locations for ZEA and LUT are reduced by the presence of cholesterol which leads to a more unordered way of incorporation in microsomes, but not in liposomes. CTX revealed a rigidifying effect, proportional to its concentration in the membrane. The higher GP_{em} values for CTX may account possibly to develop crystal deposition as has been known for CTX in monkey retina [Goralczyk *et al.*, 2000]. A similar behavior but a less rigidifying effect and less well correlated with the EC value was seen for BC. Our conclusion is that xanthophylls [ZEA and LUT] are just modulators of membrane rigidity and not real rigidifying molecules. Socaciu *et al.*, 2000b, concluded from their investigations that the incorporation of carotenoids competes with the incorporation of cholesterol [as a main component in

microsomes] with respect to their modulating effect on membrane mechanics. Also *Grolier et al., 1992*, showed a high competition of cholesterol and carotenoids with respect to their incorporation into liposomes. Other investigations using multilamellar liposomes prepared from DMPC or EYPC with various concentrations of LUT, ZEA and cholesterol, revealed that the membrane ordering effect induced by 10 mol% of xanthophylls was comparable to an effect induced by 20 mol% of cholesterol [*Subczynski et al., 1994*]. **Cholesterol causes a fluidizing effect** in the membrane core and an increased oxygen transport across the membrane, **while carotenoids have opposite effects in this region**. While dihydroxy carotenoids [LUT and ZEA] behave like ‘rivets’ spanning the entire membrane bilayer and bracing together the two halves of the bilayer, cholesterol acts as ‘nails’ inserted into one half of the lipid bilayer [Fig.4-16]. In another peripheral but potentially important aspect of carotenoid biochemistry, *Jialal et al 1991*, suggested that **carotenoids may play a role in preventing cholesterol formation and reducing the amount of plaque deposits that are formed inside arteries in patients suffering from atherosclerosis** [hardening of the arteries].

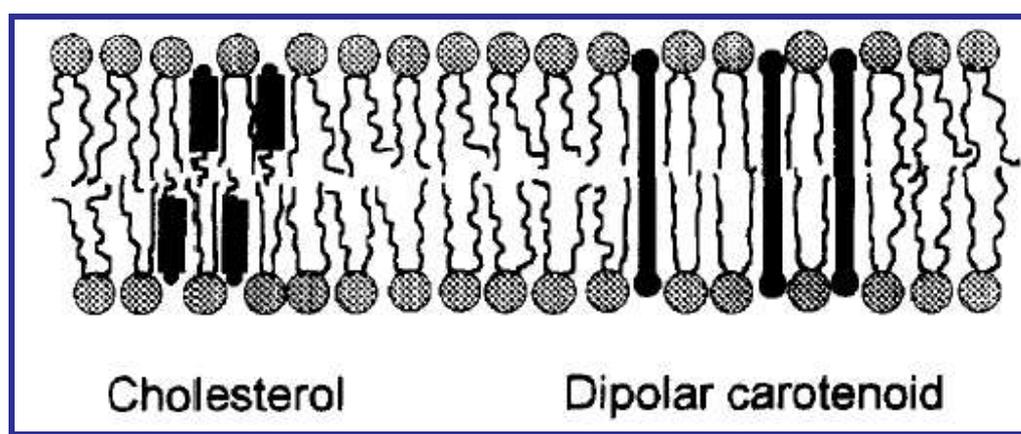


Fig.4-16. Schematic representation of an artificial lipid membrane containing cholesterol and dipolar carotenoids [adapted from *Havaux, 1998*].

Membrane rigidity seems to be very important since some carotenoids which have higher limits of insertion in the bilayer induce an increase of hydrophobicity.

These properties affect strongly the selectivity of membranes against different carotenoids absorbed from food, as it was published in many articles [*Gruszecki, 1999 and references therein*]. In our experiments with microsomes, it seems that the bioavailability for unpolar and polar carotenoids is similar. It was concluded that carotenoids can be incorporated into microsomes with good efficiency, but limited to a concentration which does not increase essentially normal membrane anisotropy [*Socaciu et al., 2000c*]. It is known that proteins and other naturally present compounds like tocopherols may stabilize carotenoids in biological environments [*Britton, 1995*]. Proteins may thus protect carotenoids from destruction and pro-oxidant effect on lipid oxidation. Particularly important in relation to the functioning of carotenoids is the role of proteins in maintaining the correct position of the carotenoid with respect to other molecules. Also, it has been shown that polar and unpolar carotenoids are both active in inhibiting microsomal phospholipid peroxidation [*Palozza and Krinsky, 1992; Nagakawa et al., 1997*].

4.3. Carotenoids incorporated into D407 RPE cells

4.3.1. Effective concentrations and incorporation yields of carotenoids in cells

From UV-Vis absorption spectra of the carotenoids BC, CTX, LUT and ZEA, incorporated into cells, the abilities of the carotenoids to be incorporated and to be stable in the cellular membrane were investigated. Their effective, final concentrations in cells [EC] initial concentrations [IC] and the incorporation yields [IY] are presented in Table 4-5.

A: Procedure I [Carotenoids delivered to cells in Fetal Calf Serum]

Carotenoid	IC [μg carotenoid/ 10^6 cells]	Incubation Time	EC [μg carotenoid/ 10^6 cells]	IY%
BC	26	48 h	0.02	0.1
CTX	26	48 h	0.08	0.3
LUT	26	48 h	0.90	3.0
ZEA	26	48 h	1.10	4.1

B: Procedure II [Carotenoids delivered to cells in THF]

Carotenoid	IC [μg carotenoid/ 10^6 cells]	Incubation Time	EC [μg carotenoid/ 10^6 cells]	IY%
BC	26	0.25 h	0.20	0.7
		0.5 h	0.23	0.9
		1 h	0.19	0.7
		24 h	0.20	0.8
CTX	26	0.25 h	1.40	5.3
		0.5 h	1.24	4.7
		1 h	1.45	5.4
		24 h	0.60	2.3
LUT	26	0.25 h	1.98	7.4
		0.5 h	1.66	6.3
		1 h	1.60	6.0
		24 h	0.94	4.0
ZEA	26	0.25 h	2.50	9.2
		0.5 h	3.37	13.0
		1 h	4.00	15.0
		24 h	2.30	9.0

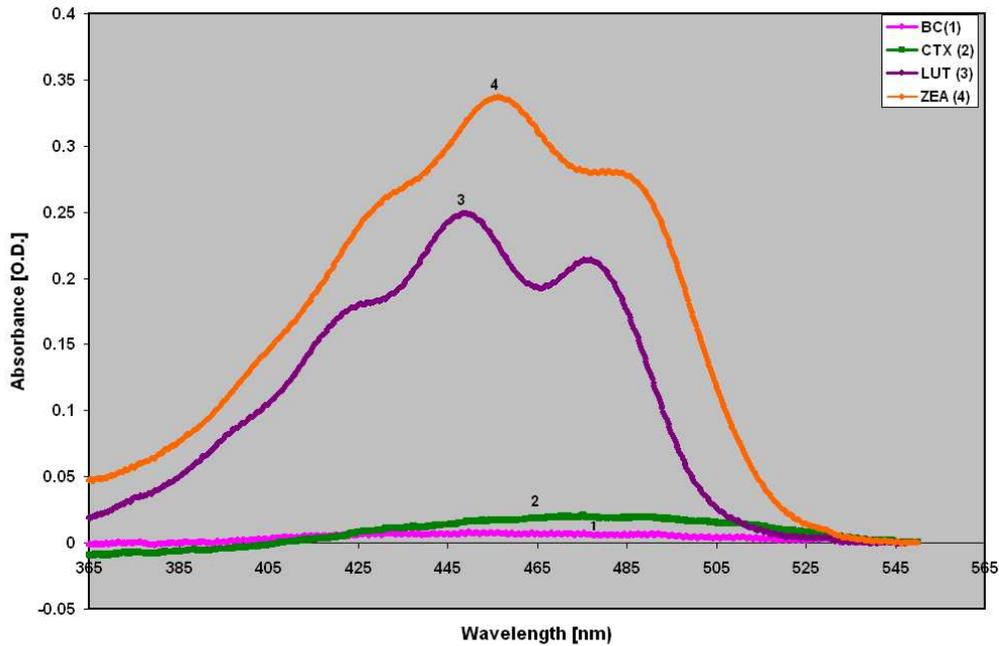
Table 4-5: Incorporation yields [IY%] of carotenoids co-cultivated with RPE cells by procedure I [A] and procedure II [B] at IC = 26 μg carotenoid/ 10^6 cells. EC values of each carotenoid are also presented.

To remember, we used two different ways to incorporate carotenoids into cells:

- *Procedure I:* carotenoids were delivered to cells in fetal calf serum as vehicle at IC = 26 μg carotenoid/ 10^6 cells, and then the EC and IY values were monitored after 48 h of incubation.
- *Procedure II:* carotenoids were delivered to cells in THF as vehicle at IC = 26 μg carotenoid/ 10^6 cells, and then the EC and IY values were monitored after various times of incubation.

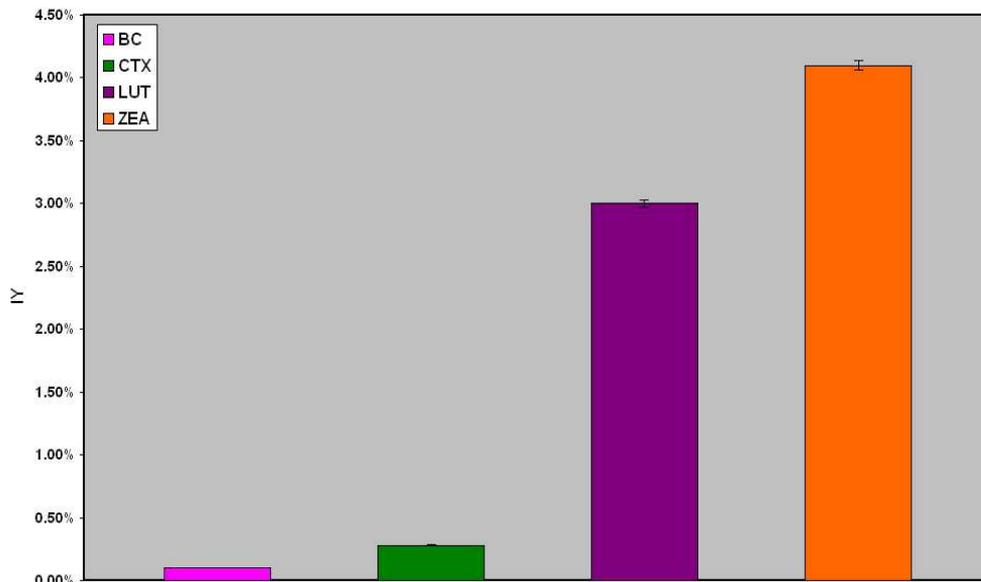
Different procedures to incorporate carotenoids into RPE cells have been used because from the literature no clear argumentation for a certain method was available. Obviously THF is the better solvent for the carotenoids without doing harm to the cells [Table 4-5]. From the Tables 4-5, 4-3 and 4-1 we see that the incorporation yields of all the carotenoids are smaller in RPE cells than in microsomes and liposomes. But this observation may result from the fact that very high ICs were used in order to get sufficient ECs, and a comparison of absolute values between liposomal vesicles, microsomal vesicles and cells is not possible. But the ranking of IYs is to be discussed for comparable ICs. In cells and in microsomes we find ZEA > LUT > CTX > BC, whereas for liposomes the ranking distinctly is: LUT > ZEA > CTX > BC. Therefore microsomes are good model in this respect. The data indicate that the xanthophylls [ZEA and LUT] appear to accumulate much better in RPE cells than the less polar carotenoid CTX and, least, the non-polar carotenoid BC

Fig.4-17 shows the UV-Vis spectra of RPE cells doped with carotenoids by procedure I, after cell solubilization by Triton X-100 and the release of carotenoid into an ethanol solution. One can identify the incorporated carotenoids and verify highest incorporation for ZEA and LUT.



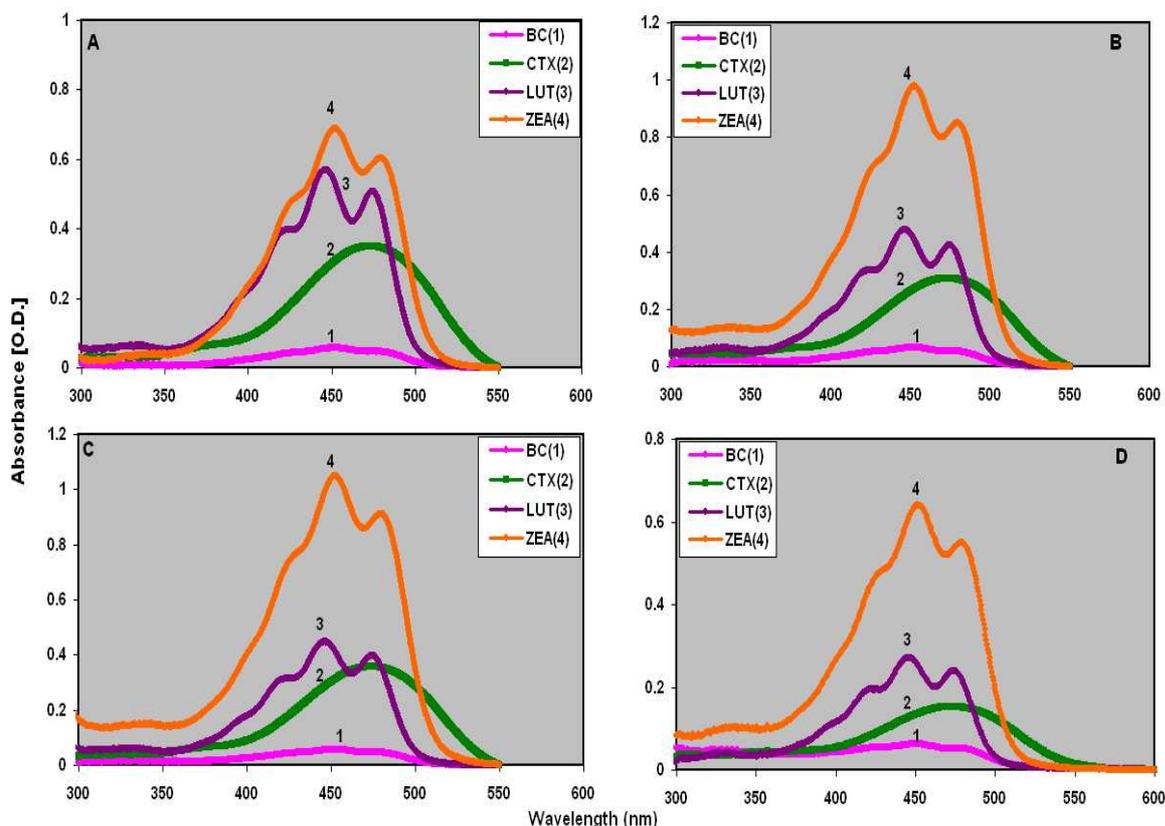
Fig[4-17]: UV-Vis spectra registered after 48 hours carotenoid incubation with RPE cells by procedure I at IC= 26 μg carotenoid / 10^6 cells. The spectra were registered after cell solubilization by Triton X-100, then carotenoids were extracted into ethanol. For EC values see Table 4-5.

Fig.4-18 summarizes the IY values of all carotenoids incorporated into RPE cells using procedure I. It shows the clear preference of ZEA and LUT.



Fig[4-18]: Incorporation yield [IY%] for carotenoids co-cultivated with RPE cells by procedure I at IC= 26 μg carotenoid/ 10^6 cells. The incubation time of carotenoids with RPE cells was 48 hours.

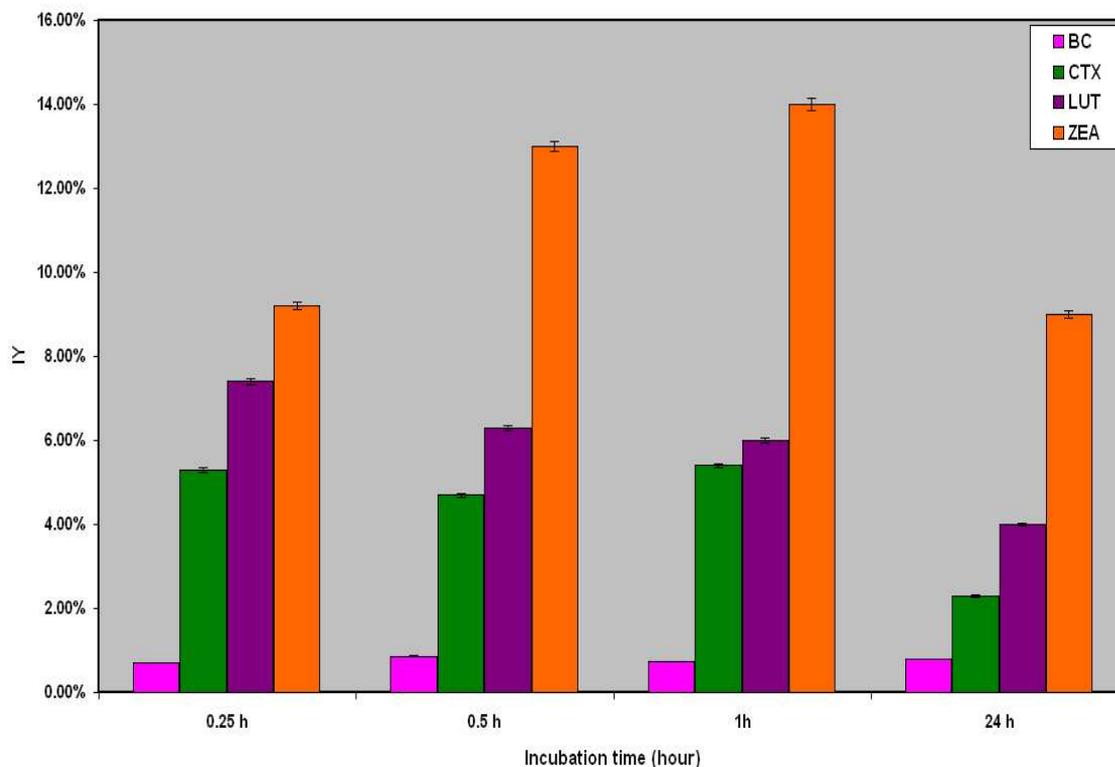
Fig.4-19 presents the UV-Vis spectra of RPE cells doped with carotenoids by procedure II during one day. It is obvious that ZEA has certain traits that render it better than LUT at cellular and retinal deposition. Again we see that RPE cells favour ZEA more than LUT and the other carotenoids which points to the actual function of ZEA to absorb damaging light and to neutralize toxic free radicals. *Scheidegger et al., 1998*, hypothesized that ZEA plays a major role in the protection of macular tissue from oxidative damage. And ZEA appeared to be a better photo-protector than LUT during a prolonged UV exposure [*Sujak et al., 1999*]. Working with quail as an animal model, it was demonstrated that light damage is strongly influenced by the amount of zeaxanthin in the retina, and that a significantly greater retinal protection is provided at dietary levels higher than those normally occurring in the diet. *Thomson et al., 2002*, suggest that ZEA is well suited to its role in maintaining retinal health, and it may be the backbone for a strategy to prevent or intervene in macular degeneration. It preferentially accumulates in the macula where it absorbs harmful blue light, and it accumulates in the RPE and the most vulnerable portions of the photoreceptors where its potent anti-oxidant capacity can prevent oxidative damage, a problem that increases with aging.



Fig[4-19]: UV-Vis spectra of RPE cells which incorporated carotenoids by procedure II at IC=26 μg carotenoid/ 10^6 cells. The spectra were registered at different incubation times : 0.25 h [A], 0.5h [B], 1h [C], and 24h [D] and after cell solubilization by Triton X-100 and carotenoid solubilization in ethanol. For EC values see Table 4-5.

Fig.4-20 presents the IY values of all carotenoids in RPE cells doped by procedure II during one day of incubation. ZEA achieved the highest IY [about 12%], followed by LUT [6%], and BC only 0.8%. ZEA reaches its IY maximum after one hour of incubation and then it decays from 15% to 9% after 24 h of incubation. LUT reaches its saturation earlier and decays faster than ZEA. In microsomes there is not a big difference in the incorporation yields and decay rates of ZEA and LUT. But in liposomes LUT incorporates with the highest yield and remains most stable. So, there must be a mechanism which prefers ZEA for its prevalence in RPE cells. Two RPE-special explanations shall be discussed. First, the higher IY and slower decay of ZEA compared to LUT [Fig.4-20] prefers its diffusion process from the blood plasma to the retinal cell and farther to the macula as it is indicated in Figure 2-11. Secondly, in the retina may exist an enzymatic

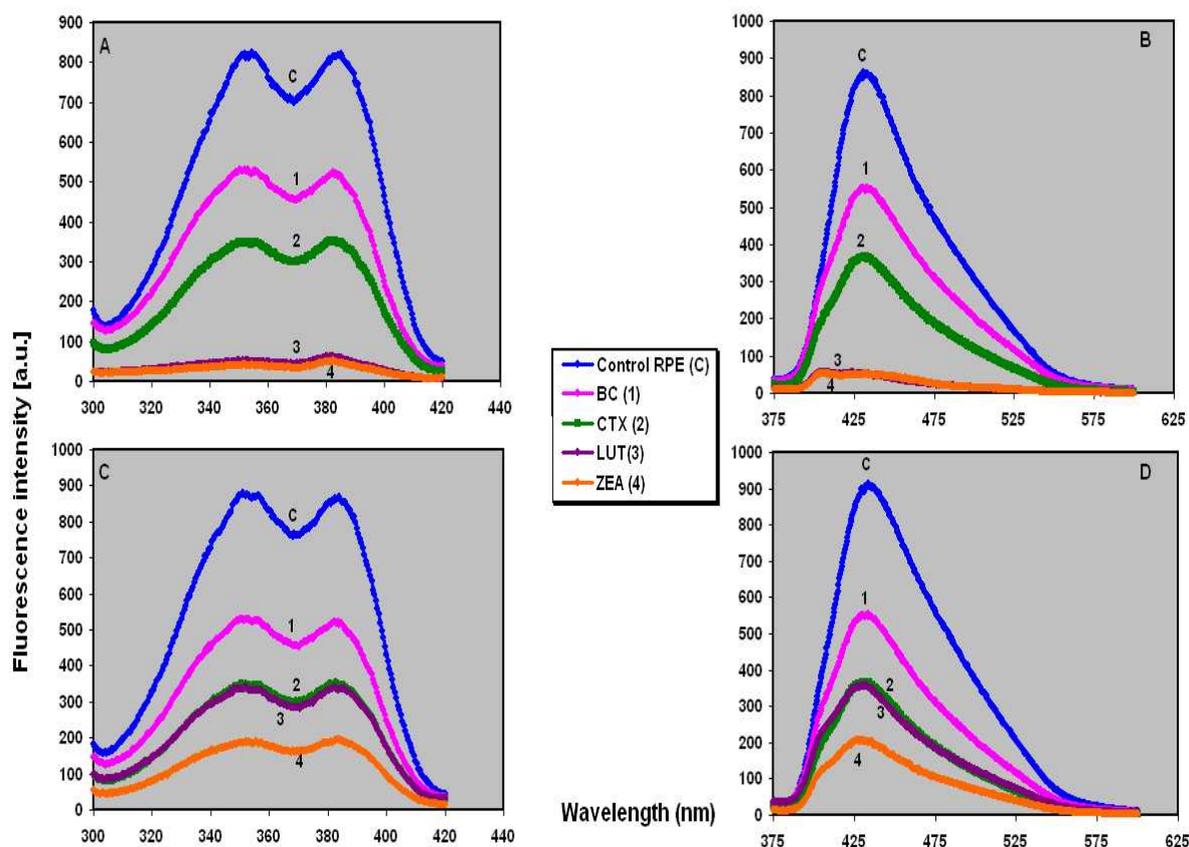
metabolisation of LUT to ZEA as we discussed already in chapter 2.2.4 based on a finding of *Bone et al., 1993*.



Fig[4-20]: Incorporation yields [IY%] of carotenoids incubated with RPE cells for different periods by procedure II, at IC= 26 μg carotenoid/ 10^6 cells.

4.3.2. Effects of carotenoids on membrane properties, evaluated by fluorescence spectrometry of Laurdan-labeled cells

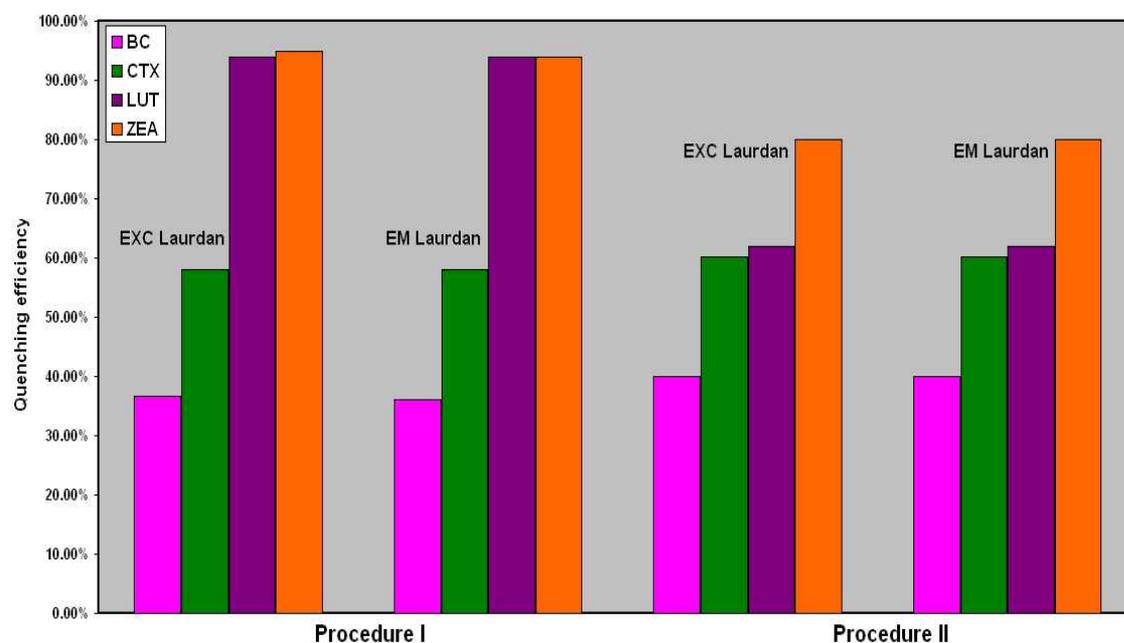
Fig.4-21 presents the excitation spectra [$\lambda_{em}= 440$ nm] and emission spectra [$\lambda_{exc}= 353$ nm] of Laurdan which was incorporated in the RPE cells previously doped with carotenoids. The incorporation of carotenoids exerts a strong quenching of Laurdan fluorescence. The ranking of the quenching efficiency is ZEA = LUT > CTX > BC for procedure I and ZEA > LUT = CTX > BC for procedure II.



Fig[4-21]:Fluorescence spectra of Laurdan [excitation and emission-A and B] incorporated into RPE cells co-cultivated with carotenoids by procedure I, and by procedure II [excitation and emission-C and D]. The spectra were registered after 48 h of carotenoid incubation with cells by procedure I. Similar fluorescence spectra were obtained when carotenoids were incubated with cells by procedure II.

Fig.4-22 summarizes the results concerning the quenching efficiencies of the carotenoids to Laurdan. In order to get an experimental confirmation both, the excitation and the emission spectra, have been plotted for both procedures. The agreement from different measurements is excellent. By procedure II the quenching was less efficient for LUT and ZEA than by procedure I. One of the possible explanations of this finding is that THF [a five-member ring containing an oxygen] penetrates into the head-group region of the membrane and dislocates LUT and ZEA from a more perpendicular-to-membrane location to a more in-membrane-plane location where their interaction with Laurdan is weaker. This argument is supported by the fact that the quenching efficiency of CTX and BC which are

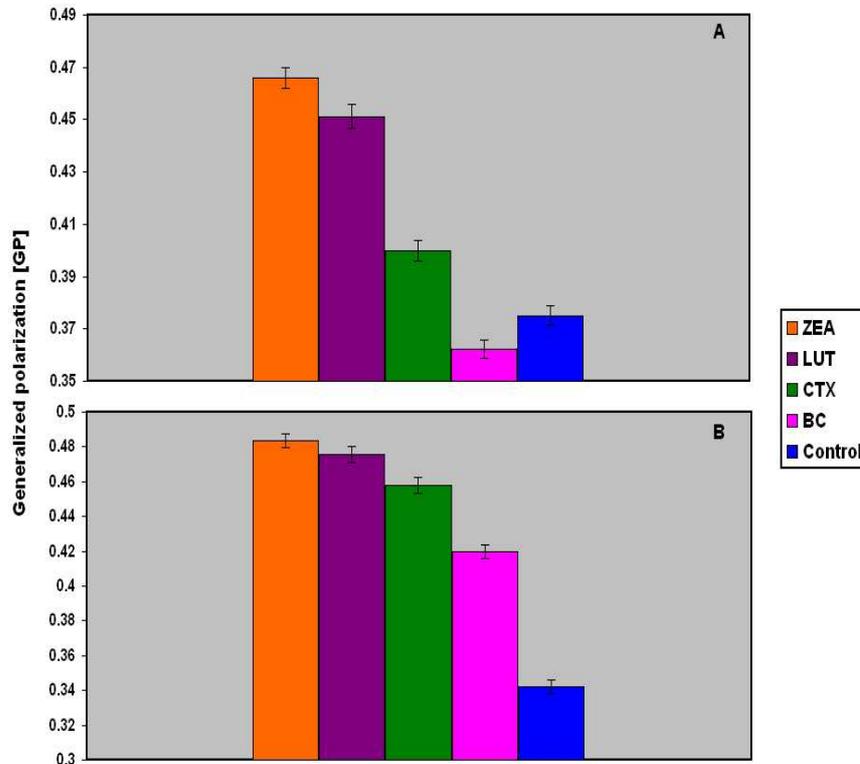
anyway more located in the core region of the membrane do not depend on the method of their incorporation.



Fig[4-22]: Laurdan quenching efficiency [%] of carotenoids co-cultivated with RPE cells by both procedures, I and II, respectively. [data collected from Fig.4-21]

To investigate the influence of carotenoids on membrane rigidity, we calculated the generalized polarization values. Fig.4-23 presents the GP_{em} values for RPE cells containing carotenoids and Laurdan. With the exception of the low BC concentration [here the GP_{em} value is not significantly different from the control], we find a membrane rigidisation by the carotenoid incorporations. The ranking is: ZEA > LUT > CTX > BC. The GP_{em} values of ZEA and LUT do not depend on the method of their incorporation. This is not in agreement with our former argumentation that THF dislocates partly ZEA and LUT, otherwise their GP_{em} values should be lower in the case of the THF vehicle. Therefore we have to consider also the possibility that THF excludes Laurdan from cell membrane to

some extent. The quenching efficiency can also be explained by this. The high GP_{em} values of LUT and ZEA suggest a rigidifying of the retinal cell membrane.



Fig[4-23]: General polarization values calculated from emission spectra of Laurdan incorporated into RPE cells co-cultivated with carotenoids by procedure I [A] and procedure II [B] respectively. Data represent means of 3 experiments \pm SD.

The incorporation of ZEA into phospholipid membrane systems has at least three known effects, which are not restricted to antioxidant activity:

1] ZEA increases the ordering of the hydrocarbon chains by its ability to integrate into the membrane, effectively modifying the structure of the membrane [Gruszecki and Sielewiesiuk, 1990], 2] ZEA decreases the oxygen diffusion rate which controls the rate of chemical reactions with oxygen and should help to protect the fatty acids from oxidation [Gruszecki and Sielewiesiuk, 1990], and 3] ZEA [but not β -carotene] mechanically stabilizes model membranes and decreases the water flow across them [Subczynski et al., 1991].

In plants ZEA has a well accepted photo-protective role. When plants are exposed to excess light, ZEA is synthesized and it helps to dissipate energy that can not be used for photosynthesis so that the plant is protected from light damage. When plants return to moderate light levels, ZEA must be removed or the energy continues to be dissipated and the normal efficiency of photosynthesis is diminished. In fact, ZEA concentrations go through a daily cycle, low at night and high at midday, whereas β -carotene concentrations fluctuate less and are lower when the light is more intense. These and other observations have led to the conclusion that ZEA is the carotenoid that protects the photosynthetic apparatus when photon flux density is high [*Schubert et al., 1994*]. The accumulation of ZEA to protect the primate fovea appears to be exploiting an ancient and ubiquitous evolutionary adaptation from the plant kingdom.

The polarity of the carotenoids may therefore be crucial for transport into the RPE cells. β -carotene was not even detected in postmortem retina from a subject who had been taking very high doses of a combination of β -carotene and canthaxanthin [*Daicker et al., 1987*]. The less polar canthaxanthin, on other hand, was present in substantial amounts but still much less than zeaxanthin and lutein.

5. Conclusion

Carotenoid pigments are ubiquitous in plant and animal organisms and they are known to have several important physiological functions, such as: light harvesting in photosynthesis [Paulsen, 1999], protection of neighbouring biomolecules against oxidative damage which is realized via the quenching of triplet states of photosensitizers and deactivation of singlet oxygen and free radicals [Krinsky, 1998], modification of dynamic and structural properties of lipid membranes [Gruszecki, 1999] and proteins [Moskalenko and Karapetyan, 1996] and other biological roles [Britton, 1995].

The biological importance of the two carotenoid pigments lutein and zeaxanthin found in the retinal membranes of the eye, [Snodderly, 1984] is still not fully understood and is one of the subjects of interest motivating recent research [Landrum and Bone, 2001; Sommerburg et al., 1999; Gruszecki et al., 1999; Sujak et al., 1999]. Lutein and zeaxanthin are present in the membranes of retina and in particular in the *macula lutea*, the yellow spot located in the retina, close to the optical axis of an eye [Snodderly, 1984]. The role of these two pigments in the *macula* is most probably directly related to the protection against the free radical attack on the membrane components and also to the attenuation of the short-wavelength radiation penetrating the retina and being potentially harmful by photosensitization [Sommerburg et al., 1999; Gruszecki et al., 1999; Sujak et al., 1999]. Both the radical-scavenger [Woodall et al., 1997; Stahl et al., 1998] and the filtering efficacy [Junghans et al., 2001; Britton et al., 1995] of zeaxanthin and lutein in the lipid membranes are potentially dependent on the organization of xanthophyll-lipid membranes.

In particular, the localization of a carotenoid molecule in the lipid membrane, its orientation and optical properties have to be taken into account while considering the action of carotenoid pigments in membranes [*Gruszecki, 1999*].

The effect of carotenoid pigments on structural and dynamic properties of biological membranes and model lipid membranes has been extensively studied in recent years by means of several experimental techniques: electron paramagnetic resonance [EPR], light scattering in liposome suspension, differential scanning calorimetry, X-ray diffractometry and nuclear magnetic resonance applied to phosphorous nuclei [^{31}P -NMR] and carbon nuclei [^{13}C -NMR] of phospholipid molecules [see *Gruszecki, 1999* for a review]. The effect of carotenoid pigments on structural and dynamic properties of lipid membranes observed in those biophysical studies may be summarized as follows:

[1] The chromophore of carotenoid pigment molecules is located in the hydrophobic core of a membrane. Xanthophyll pigments [LUT and ZEA] with their polar group located at the opposite ends of a rigid, rod-like molecule are oriented to place their polar groups in the two opposite polar zones of the bilayer.

[2] The effect of carotenoid pigments on molecular dynamics of a lipid membrane is based on hydrophobic, Van der Waals interaction between the rigid pigment molecules and alkyl lipid chains undergoing continuous gauche-trans isomerization and hydrogen bonding formation between polar groups of xanthophylls and lipid heads. These interactions result in a fluidization of the well-ordered structure of a lipid membrane in the “crystal state” and a rigidifying effect of a membrane in its fluid state. The effect of the increase of motional freedom of lipid molecules in a membrane, referred to as the fluidization, is reported to be more distinctly pronounced in the case of β -carotene while the opposite - the rigidifying effect - is reported in the case of polar xanthophylls (LUT and ZEA). The dihydroxy carotenoids [ZEA and LUT] are more effective in affecting

membrane properties than β -carotene. They have been shown to increase the rigidity [as evaluated by using a generalized polarization parameter from Laurdan spectra] of the membrane and act as “molecular rivets” bracing together the two leaflets of the bilayers to reinforce them [see Figures 4-7 and 4-23]. Incorporation of polar carotenoids into a lipid bilayer usually results in a decrease in membrane fluidity and an increase in membrane stability. The inclusion of a non-polar carotene into the membrane increases the motional freedom of the polar head groups, presumably via generation of a free space in that portion of the bilayer. β -carotene is not effective in reinforcement of the membrane structure but makes the membrane less compact in its polar region. It may be that canthaxanthin plays a similar role as β -carotene that is to perturb the acyl chain packing and to increase bilayer permeability.

The UV-Visible study showed that the membrane structure was affected very differently by incorporating polar carotenoids or β -carotene. The incorporation of β -carotene at different concentrations resulted in a very small miscibility of β -carotene with phospholipid bilayer, although β -carotene may primarily localize within the hydrophobic core of the membrane without well defined orientation [Gabrielska and Gruszecki, 1996] [see Figures 4-1, 4-9A-C, 4-17, and 4-19; tables 4-1, 4-3 and 4-5]. The molecular assembly of LUT and ZEA in the membrane is more stable than that of β -carotene. LUT and ZEA can easily fit into the phospholipid lamellar structure [an ordering effect] and they should be oriented parallel to the hydrocarbon chains in the phospholipid bilayer. The two polar ends of them are in contact with an opposite polar zone of the bilayer, achieving a very high miscibility with the phospholipid bilayer. The results presented in this work clearly demonstrate that the interactions between carotenoids and lipids in the membrane strongly depend on the structure of the carotenoid, the polarity and the rigidity of the molecule being the most important factors. These findings give

reason to suppose an evolutionary selection of certain carotenoids to optimise their function in certain membranes.

To approach the *in vivo* environment pig liver microsomes and RPE cells were chosen and we found that ZEA, whose distribution in the membrane is more homogenous, has certain traits that render it better than LUT at cellular deposition [see Figures 4-9A, 4-17, 4-18, 4-19 and 4-20]. ZEA is oriented in the lipid bilayer as close to the normal as possible. It induces minute disturbances of the membrane structure and even exerts an ordering effect while the relatively free rotation of one ring in the LUT molecule allows that molecules of LUT can be located parallel to the plane of membrane with their hydrophobic portions in the core of bilayer and polar groups interacting with polar head region at the same side of the bilayer [Fig.2-23]. Such a carotenoid orientation introduces disorder to the membrane [Sielewiesiuk *et al.*, 1997].

LUT was found to be the most stable carotenoid against bleaching during storage while ZEA underwent a partial decay [see Figures 4-1, 4-2, 4-9B, C and F, 4-10]. This finding may provide the speculation that LUT be converted into ZEA by the retina [Bone *et al.*, 1997]. Because LUT is more widely available in food than ZEA, such a conversion would have the advantage of giving the retina a stronger source for its ZEA. The existence of two orthogonally oriented pools of LUT in lipid bilayers increases considerably the chances of light to be captured by a carotenoid molecule and to cover all the possible orientations of the electric vector of the excitation beam, which may interact with pigment and assure effective light absorption. In that sense, LUT seems to be suited better than ZEA in acting like a screen against the excess of radiation [Sujak *et al.*, 1999]. In addition LUT has one conjugated double bond less [10 double bonds] than the other carotenoids [11 double bonds] have, which may account for its somewhat slower reactivity to oxidation.

Resonance Raman spectroscopic studies indicate that β -carotene is restricted to the hydrophobic core of the bilayer [Van de Ven *et al.*, 1984]. β -Carotene, and presumably lycopene, would thus be susceptible to be attacked by peroxy radicals generated in this area, but less accessible to radicals generated in the aqueous phase. Zeaxanthin, a dihydroxy carotenoid, spans across the DMPC bilayer [Gruszecki and Siewiesiuk, 1990] and adopts a similar orientation in EYPC bilayers with the polar hydroxyl groups located close to the hydrophobic-hydrophilic interface of the lipid bilayer. Zeaxanthin is thus held in close proximity and approximately parallel to the lipid acyl chains. This is the optimum position to provide membrane protection against peroxy radicals at all depths in the hydrophobic phase. Zeaxanthin shall also be able to intercept peroxy radicals entering the lipid layer from the aqueous phase. A direct presence of xanthophylls in close proximity of lipid molecules potentially increases their chances to be protected against oxidative damage. Such a statement follows directly from the ability of xanthophyll pigments to:

- [1] conserve the membrane integrity via reinforcement of the lipid bilayer structure,
- [2] increase the hydrophobic barrier of the membrane,
- [3] limit oxygen penetration within the membrane,
- [4] quench photo-chemically reactive species responsible for initiation and prolongation of the lipid peroxidation in the membrane [Edge *et al.*, 1997].

Fluorescence techniques are specifically sensitive and broadly applied. Fluorophores are incorporated into the membranes and the resulting fluorescence properties (quenching, spectral shifts, depolarisation, fluorescence life time) are used for the evaluation of membrane properties.

Lipophilic fluorophores like DPH and Laurdan are molecules which easily share membrane locations with carotenoids. They come into such a tight vicinity

that they interact with each other and it is not always easy to discriminate if a specific fluorescence signal is determined by the membrane which has been modulated by the pigment, or if the signal is directly determined by an interaction of the fluorophore with the pigment. In any case, informations about the location of the carotenoid are obtained if the location of the fluorophore is known as it is the case for Laurdan. Its fluorescence in membranes is strongly quenched by carotenoids. This property is proportional to the carotenoid content and can even be used to check the pigment concentrations. It has been demonstrated that the xanthophylls [LUT and ZEA] are significantly more efficient quenchers of DPH and Laurdan in membrane than BC [see Figures 4-3, 4-4, 4-5, 4-11A, 4-12, 4-21, 4-22], indicating that the physical interaction of the carotenoid with the bilayer is an important determinant of quenching activity. LUT and ZEA are densely located between incoming light and photoreceptors such that they act efficiently as a blue light filter, shielding the most sensitive part of the retina from radiation. LUT and ZEA are also efficient singlet oxygen quenchers and are capable to scavenge the product of photooxidative reactions initiated by blue light.

Literature Cited

- Alberts B**, Bray D, Lewis J, Raff M, Roberts K and Watson J .1994. *Molecular Biology of the Cell*. Garland Publishing, Inc. New York London. p477.
- Andely UP**. 1987. *Photodamage to the eye*. Photochem Photobiol 46: 1057-1066.
- Ascherio A**, Stampfer MJ, Colditz GA, et al. 1992. *Correlation of vitamin A and E intakes with the plasma concentrations of carotenoids and tocopherols among American men and women*. J Nutr 122:1792-1801.
- Bone RA**, Landrum JT and Tarsis SL. 1985. *Preliminary identification of the human macular pigment*. Vision Res 25:1531–1535.
- Bone RA**, Landrum JT, Fernandez L and Tarsis SL. 1988. *Analysis of the macular pigment by HPLC: retinal distribution and age study*. Invest Ophthalmol Vis Sci 29:843-849.
- Bone RA**, Landrum JT, Friedes LM, Gomez CM, Kilburn MD, Menendez E, Vidal I, and Wang W. 1997. *Distribution of lutein and zeaxanthin stereoisomers in the human retina*. Exp Eye Res 64: 211–218.
- Bone RA**, Landrum JT, Hime GW, Cains A and Zamor J. 1993. *Stereochemistry of the human macular carotenoids*. Invest Ophthalmol Vis Sci 34:2033–2040.
- Bressler NM**, Bressler SB and Fine SL. 1988. *Age-related macular degeneration - a major review*. Survey Ophthalmol 32: 375- 412.
- Britton G**, Liaan-Jensen S, and Pfander H. 1995. *UV/visible spectroscopy*. In *Carotenoids* (Britton G, Liaan-Jensen S, and Pfander H., Eds.), Vol. 1B, pp. 13–62, Birkhauser Verlag, Berlin.
- Britton G**.1995. *Structure and properties of carotenoids in relation to function*. FASEB J. 9:1551–1558.
- Cantrell A**, McGarvey DJ, Truscott TG, Rancan F and Böhm F. 2003. *Singlet oxygen quenching by dietary carotenoids in a model membrane environment*. Arch Biochem Biophys 412:47-54.
- Chan C**, Leung I, Lam KW and Tso MO. 1998. *The occurrence of retinol and carotenoids in human subretinal fluid*. Curr Eye Res 17:890-895.

- Chaturvedi VK and Ramakrishna Kurup CK.** 1986. *Interaction of lutein with phosphatidylcholine bilayers.* Biochim Biophys Acta 860:286-292.
- Chen G and Djuric Z.** 2001. *Carotenoids are degraded by free radicals but do not affect lipid peroxidation in unilamellar liposomes under different oxygen tensions.* FEBS Lett 505: 151-154.
- Cognis,** May 2003. *Carotenoids and eye health.*
www.cognis.com/veris/FactSheets/EYE_FS_May_03_Final.pdf
- Conn PF, Schalch W and Truscott GT.** 1991. *The singlet oxygen carotenoid interaction.* J Photochem Photobiol 11:41-47.
- Cooney RV, Kappock TJ, Pung A and Bertram JS.** 1993. *Solubilisation, cellular uptake, and activity of β -carotene and other carotenoids as inhibitors of neoplastic transformations in cultured cells.* Methods Enzymol 214:55-68.
- Daicker B, Schiedt K, Adnet JJ and Bermond P.** 1987. *Canthaxanthin retinopathy-an investigation by light and electron microscopy and physicochemical analysis.* Graefe's Arch Clin Exp Ophthalmol 225:189-197.
- David L Atkins.** 1998. *Structure of the plasma membrane: a tutorial in basic neurobiology.* Department of biological sciences, George Washington University.
http://sky.bsd.uchicago.edu/lcy_ref/synap/plasmalemma.html#phospholipid
- Davis AA, Bernstein PS, Bok D, Turner J, Nachtigal M and Hunt RC.** 1995. *A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture.* Invest Ophthalmol Vis Sci 36:955-964.
- Dayhaw-Barker P.** 1986. *Ocular photosensitisation.* Photochem Photobiol 46: 1051-1055.
- Deamer DW and Uster PS.** 1983. *Preparation of liposomes.* In: Ostro M.J.ed. Liposomes. Marcel Dekker, New York. P.1.
- Edge R, McGarvey DJ and Truscott TG.** 1997. *The carotenoids as anti-oxidants - a review.* J Photochem Photobiol Biology 41:189-200.

- Erdman J.** 1988. *The physiologic chemistry of carotenes in man.* Clin Nutr 7:101–106.
- Fine SL.** 1999. *Photodynamic therapy with verteporfin is effective for selected patients with neovascular age-related macular degeneration.* Arch Ophthalmol 117:1400-1402.
- Frank RN.** 1989. *Macular degeneration.* JAMA 261:767-768.
- Gabrielska J and Gruszecki WI.** 1996. *Zeaxanthin (dihydroxyl- β -carotene) but not β -carotene rigidifies lipid membranes: a $^1\text{H-NMR}$ study of carotenoid-egg phosphatidylcholine liposomes.* Biochim Biophys Acta 1285:167–174.
- Goralczyk R, Barker FM, Buser S, Liechti H and Bausch J.** 2000. *Dose dependency of canthaxanthin crystals in monkey retina and spatial distribution of its metabolites.* Invest Ophthalmol Vis Sci 41:1513-1522.
- Gottsch JD, Pou S, Bynoe LA and Rosen GM.** 1990. *Hematogenous photosensitization. A mechanism for the development of age-related macular degeneration.* Invest Ophthalmol Vis Sci 31:1674-1682.
- Grolier P, Arais-Braesco V, Zelmire L and Fessi H.** 1992. *Incorporation of carotenoids in aqueous systems: uptake by cultured rat hepatocytes.* Biochim Biophys Acta 1111:135–138.
- Gross J.** 1987. *Pigments in fruits.* New York: Academic Press:1-303.
- Gross J.** 1991. *Pigments in vegetables.* New York: Van Nostrand Reinhold:1-351.
- Gruszecki WI and Siewiewsiuk J.** 1990. *Orientation of xanthophylls in phosphatidylcholine multibilayers.* Biochim Biophys Acta 1023:405- 412.
- Gruszecki WI, Sujak A, Strzalka K, Radunz A and Schmid GH.** 1999. *Organisation of xanthophyll-lipid membranes studied by means of specific pigment antisera, spectrophotometry and monomolecular layer technique lutein versus zeaxanthin.* Z. Naturforsch 54c: 517-525.
- Gruszecki WI.** 1999. *Carotenoids in membranes,* in: Frank HA, Young AJ, Britton G, and Cogdell R (Eds.), *The Photochemistry of Carotenoids,* Kluwer Acad Publ, Dordrecht/Boston/London, 363-379.

- Ham WT and Müller WA.** 1989. *The photopathology and nature of the blue-light and near-UV retinal lesion produced by lasers and other optical sources.* In: Wolbarsht ML, editor. *Laser applications in medicine and biology.* New York; Plenum Press: 191-246.
- Handelman GJ, Dratz EA, Reay CC and van Kuijk FJGM.** 1988. *Carotenoids in the human macula and whole retina.* *Invest Ophthalmol Vis Sci* 29:850-855.
- Hart DJ and Scott KJ.** 1995. *Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK.* *Food Chem* 54:101-111.
- Havaux M.** 1998. *Carotenoids as membrane stabilizers in chloroplasts.* *Trends Plant Sci* 3(4): 147-151.
- Jezowska I, Wolak A, Gruszecki WI and Strzalka K.** 1994. *Effect of β -carotene on the structural and dynamic properties of model phosphatidylcholine membranes. II. A ^{31}P -NMR and ^{13}C -NMR study.* *Biochim Biophys Acta* 1194:143–148.
- Jialal I, Norkus EP, Cristol L and Grundy SM.** 1991. *β -Carotene inhibits the oxidative modification of low-density lipoprotein.* *Biochim Biophys Acta* 1086: 134-138.
- Junghans A, Sies H and Stahl W.** 2001. *Macular pigments lutein and zeaxanthin as blue light filters studied in liposomes.* *Arch Biochem Biophys* 391(2):160–164.
- Khachick F, Beecher G and Smith JC.** 1995. *Lutein, lycopene and their oxidative metabolites in chemoprevention of cancer.* *J Cell Biochem* 22 (suppl):236-246.
- Khachik F, Spangler CJ and Smith JC.** 1997. *Identification quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum.* *Anal Chem* 69: 1873-1881.
- Kirschfeld K.** 1982. *Carotenoid pigments: their possible role in protecting against photooxidation in eyes and photoreceptor cells.* *Proc. R. Soc. Lond. B* 216:71–85.

- Krinsky NI, Russett MD, Handelman GJ and Snodderly DM.** 1990. *Structural and geometrical isomers of carotenoids in human plasma.* J. Nutr 120:1654-1661.
- Krinsky NI.** 1968. *The protective function of carotenoid pigments.* Photophysiology 3: 123-195.
- Krinsky NI.** 1979. *Carotenoid protection against oxidation.* Pure & Appl Chem 51:649-660.
- Krinsky NI.** 1998. *The antioxidant and biological properties of the carotenoids.* Ann N.Y. Acad Sci 854: 443-447.
- Lam K-W and But P.** 1999. *The content of zeaxanthin in Gou Qi Zi, a potential health benefit to improve visual acuity.* Food Chemistry 67:173-176.
- Landrum JT and Bone RA.** 2001. *Lutein, zeaxanthin, and the macular pigment.* Arch Biochem Biophys 385:28-40.
- Liebler DC, Stratton SP and Kaysen KL.** 1997. *Antioxidant actions of β -carotene in liposomal and microsomal membranes: role of carotenoid-membrane incorporation and alpha-tocopherol.* Arch Biochem Biophys 338: 244-250.
- Machlin LJ and Bendich A.** 1987. *Free radical tissue damage: Protective role of antioxidant nutrients.* FASEB J 1:441-445.
- Mateo CR, Acuna AU and Brochon J-C.** 1995. *Liquid-crystalline phases of cholesterol/lipid bilayers as revealed by the fluorescence of trans-parinaric acid.* Biophys J 68: 978-987.
- McCarty C and Taylor HR.** 1999. *Light and risk for age-related eye diseases.* In: Taylor A, editor. Nutritional and environmental influences on the eye. Boca Raton; CRC Press: 215-250.
- McGraw KJ, Beebee MD, Hill GE and Parker RS.** 2003. *Lutein-based plumage coloration in songbirds is a consequence of selective pigment incorporation into feathers.* Comp Biochem Physiol B135:689-696.
- Micozzi MS, Beecher GR, Taylor PR and Khachik F.** 1990. *Carotenoid analyses of selected raw and cooked foods associated with a lower risk for cancer.* JNCI 82:282-285.

- Milon A**, Wolff G, Ourisson G and Nakatani Y. 1986a. *Organization of carotenoid-phospholipid bilayer systems. Incorporation of zeaxanthin, astaxanthin, and their C₅₀ homologues into dimyristoyl phosphatidylcholine vesicles.* Helv Chim Acta 69:12–24.
- Moskalenko AA and Karapetyan NV.** 1996. *Structural role of carotenoids in photosynthetic membranes.* Z. Naturforsch 51c:763–771.
- Müller H.** 1996. *Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection.* Z Lebensm Unters Forsch A 204:88-94.
- Nakagawa K**, Kang SD, Park DK, Handelman GJ and Miyazawa T. 1997. *Inhibition of β -carotene and astaxanthin of NADPH-dependent microsomal phospholipid peroxidation.* J. Nutr. Sci. Vitaminol. (Tokyo) 43:345-355. *of peroxynitrite with zeaxanthin.* Nitric Oxide 2:8–16.
- Olmedilla B**, Granada F, Gil-Martinez E, et al. 1997. *Reference values for retinol, tocopherol, and main carotenoids in serum of control and insulin-dependent diabetic Spanish subjects.* Clin Chem 43:1066-1071.
- Palozza P and Krinsky NI.** 1992. *Astaxanthin and canthaxanthin are potent antioxidants in a membrane model.* Arch Biochem Biophys 297:291-295.
- Palozza P**, Calviello G and Bartoli GM. 1995. *Prooxidant activity of β -carotene under 100% oxygen pressure in rat liver microsomes.* Free Rad Biol Med 19:887-892.
- Palozza P**, Moualla S and Krinsky NI. 1992. *Effects of β -carotene and α -tocopherol on radical-initiated peroxidation of microsomes.* Free Rad Biol Med 13:127-136.
- Parasassi T**, De Stasio G, Ravagnan G, Rusch RM and Gratton E. 1991. *Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence.* Biophys J 60:179–189.
- Parasassi T**, Di Stefano M, Loiero M, Ravagnan G and Gratton E. 1994. *Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence.* Biophys J 66:120–132.

- Park JS**, Chew BP and Wong TS. 1998. *Dietary lutein absorption from marigold extract is rapid in BALB/c mice*. J Nutr 128: 1802-1806.
- Paulsen H**. 1999. *Carotenoids and the assembly of light-harvesting complexes*, in: H.A. Frank, A.J. Young, G. Britton, R. Cogdell (Eds.), *The Photochemistry of Carotenoids*, Vol. 8, Kluwer Academic Publications, Dordrecht, Boston, London, pp. 123–135.
- Peterson GL**. 1977. *A simplification of the protein assay method of Lowry et al., which is more generally applicable*. Anal Biochem 83:346-356.
- Rapp LM**, Maple SS and Choi JH. 2000. *Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina*. Invest Ophthalmol Vis Sci 41:1200–1209.
- Rengel D**, Diez-Navajas A, Serna-Rico A, Veiga P, Muga A and Milicua JCG. 2000. *Exogenously incorporated ketocarotenoids in large unilamellar vesicles. Protective activity against peroxidation*. Biochim Biophys Acta 1463:179-187.
- Schalch W**. 1992. *Carotenoids in the retina — a review of their possible role in preventing or limiting damage caused by light and oxygen*. In: Emerit I, Chance B, editors. Free radicals and aging. Basel; Birkhäuser Verlag: 280-298.
- Schalch W**. 2000. *Possible contribution of lutein and zeaxanthin, carotenoids of the macula lutea, to reducing the risk for age macular degeneration: a review*. HKJ Ophthalmol 4(1):31-42.
- Scheidegger R**, Pande AK, Bounds PL and Koppenol WH. 1998. *The reaction*
- Schubert H**, Kroon BMA and Matthijs HCP. 1994. *In vivo manipulation of the xanthophyll cycle and the role of zeaxanthin in the protection against photodamage in the green alga Chlorella pyrenoidosa*. J Biol Chem 269: 7267-7272.
- Shibata A**, Kiba Y, Akati N, Fukuzawa K and Terada H. 2001. *Molecular characteristics of astaxanthin and β -carotene in the phospholipid monolayer and their distributions in the phospholipid bilayer*. Chem Phys Lipids 113:11-22.

- Sielewiesiuk J, Matula M and Gruszecki WI.** 1997. *Photo-oxidation of chlorophyll a in digalactosyldiacylglycerol liposomes containing xanthophyll pigments: indication of a special photoprotective ability of zeaxanthin.* Cell Mol Biol Lett 2: 59-68.
- Siems WG, Sommerburg O and van Kuijk F JGM.** 1999. *Lycopene and β -carotene decompose more rapidly than lutein and zeaxanthin upon exposure to various pro-oxidants in vitro.* Biofactors 10:105-113.
- Singer SJ and Nicolson GL.** 1972. *The fluid mosaic model of the structure of cell membranes.* Science 175: 720-731.
- Snodderly DM, Brown PK, Delori FC and Auran JD.** 1984. *The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas.* Invest Ophthalmol Vis Sci 25:660-673.
- Snodderly DM.** 1995. *Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins.* Am J Clin Nutr 62 (Suppl.):1448-1461.
- Socaciu C, Bojarski P, Aberle L and Diehl HA.** 2002. *Different ways to insert carotenoids into liposomes affect structure and dynamics of the bilayer differently.* Biophys Chem 99:1-15.
- Socaciu C, Jessel R and Diehl HA.** 2000b. *Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy.* Chem Phys Lipids 106:79-88.
- Socaciu C, Jessel R and Diehl HA.** 2000c. *Carotenoid incorporation into microsomes: yields, stability and membrane dynamics.* Spectrochimica Acta Part A 56 :2799-2809.
- Socaciu C, Jessel R, Haertel S and Diehl HA.** 2000a. *Carotenoids in 1,2-dipalmitoyl-sn-glycero-3-phosphoryl-choline liposomes: incorporation and effects on phase transition and vesicle size.* J Med Biochem 4:71-82.
- Socaciu C, Lausch C and Diehl HA.** 1999. *Carotenoids in DPPC vesicles: membrane dynamics.* Spectrochimica Acta Part A 55:2289-2297.

- Sommerburg OG**, Siems WG, Hurst JS, Lewis JW, Kliger DS and van Kuijk FJ. 1999. *Lutein and zeaxanthin are associated with photoreceptors in the human retina*. *Curr Eye Res* 19:491–95.
- Stahl W**, Junghans A, de Boer B, Driomina ES, Briviba K and Sies H. 1998. *Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein*. *FEBS Lett* 427: 305-308.
- Stewart JCM**. 1980. *Colorimetric determination of phospholipids with ammonium ferrothiocyanate*. *Anal Biochem* 104:10-14.
- Strzalka K and Gruszecki WI**. 1994. *Effect of β -carotene on the structural and dynamic properties of model phosphatidylcholine membranes. I. An EPR spin label study*. *Biochim Biophys Acta* 1194:138–142.
- Subczynski WK**, Markowska E and Sielewiesiuk J. 1991. *Effect of polar carotenoids on the oxygen diffusion-concentration product in lipid bilayers. An EPR spin label study*. *Biochim Biophys Acta* 1068:68-72.
- Subczynski WK**, Wisniewska A, Yin JJ, Hyde JS and Kusumi A. 1994. *Hydrophobic barriers of lipid membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol*. *Biochemistry* 33:7670-7681.
- Sujak A and Gruszecki WI**. 2000. *Organization of mixed monomolecular layers formed with the xanthophyll pigments lutein or zeaxanthin and dipalmitoylphosphatidylcholine at the argon-water interface*. *J Photochem Photobiol Biology* 59:42- 47.
- Sujak A**, Gabrielska J, Grudzecki W, Borc R, Mazurek P and Gruszecki WI. 1999. *Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: the structural aspects*. *Arch Biochem Biophys* 371(2):301-307.
- Sujak A**, Mazurek P and Gruszecki WI. 2002. *Xanthophyll pigments lutein and zeaxanthin in lipid multibilayers formed with dimyristoylphosphatidylcholine*. *J Photochem Photobiol Biology* 68(1):39-44.
- Sujak A**, Okulski W and Gruszecki WI. 2000. *Organisation of xanthophyll pigments lutein and zeaxanthin in lipid membranes formed with dipalmitoylphosphatidylcholine*. *Biochim Biophys Acta* 1509 (1-2):255-263.

- Taylor HR**, West S, Munoz B, et al. 1992. *The long-term effects of visible light on the eye*. Arch Ophthalmol 110:99-104.
- Ten Grotenhuis ER**, Demel A, Ponec M, Boer DR, van Miltenburg JC and Bouwstra JA. 1996. *Phase behavior of stratum corneum lipids in mixed Langmuir–Blodgett monolayers*. Biophys J 71: 1389–1399.
- Thomson LR**, Toyoda Y, Langner A, Delori FC, Garnett KM, Craft N, Nichols CR, Cheng KM and Dorey CK. 2002. *Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail*. Invest Ophthalmol Vis Sci 43(11):3538-3549.
- Van de Ven M**, Kattenberg M, Van Ginkel G and Levine YK. 1984. *Study of the orientational ordering of carotenoids in lipid bilayers by resonance-Raman spectroscopy*. Biophys J 45: 1203-1210.
- Weber G and Farris FJ**. 1979. *Synthesis and spectral properties of a hydrophobic fluorescent probe:6-propionyl-2-(dimethylamino)naphthalene*. Biochemistry 18:3075–3078.
- Webvision**, October 2003. *The organization of the retina and visual system*. www.webvision.med.utah.edu
- Weiser H and Kormann AW**. 1993. *Provitamin A activities and physiological functions of carotenoids in animals*. Ann NY Acad Sci 691:213-215.
- Wisniewska A and Subczynski WK**. 1998. *Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers*. Biochim Biophys Acta 1368(2):235-246.
- Wolfe SL**.1993. *Molecular and Cellular Biology*. Wadsworth Publishing Company. Belmont, California.
- Woodal AA**, Britton G and Jackson MJ. 1997. *Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxy radicals: relationship between carotenoid structure and protective ability*. Biochim Biophys Acta 1336: 575-586.
- Wrona M**, Rozanowska M and Sarna T. 2004. *Zeaxanthin in combination with ascorbic acid or α -tocopherol protects ARPE-19 cells against photosensitized peroxidation of lipids*. Free Rad Biol Med 36(9):1094-1101.