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The solubilisation pattern of lutein, zeaxanthin, canthaxanthin and β-carotene differ characteristically in liposomes, liver microsomes and retinal epithelial cells

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Abstract

The incorporation efficiencies of lutein, zeaxanthin, canthaxanthin and β-carotene into Retinal Pigment Epithelial (RPE) cells (the human RPE cell line D 407), liver microsomes and EYPC liposomes are investigated. In RPE cells the efficiency ratio of lutein and zeaxanthin compared to canthaxanthin and β-carotene is higher than in the other membranes. The preferential interactions of lutein and zeaxanthin with RPE cells are discussed considering special protein binding properties. Incorporation yields were obtained from the UV–Vis spectra of the carotenoids. Membrane modulating effects of the carotenoids were obtained from the fluorescence spectra of co-incorporated Laurdan (6-dodecanoyl-2-dimethylaminonaphtalene).

The Laurdan fluorescence quenching efficiencies of the membrane bound carotenoids offer an access to direct determinations of membrane carotenoid concentrations.

Fetal calf serum as carrier for carotenoid incorporation appears superior to tetrahydrofuran.

Keywords: RPE cells; Liver microsomes; EYPC liposomes; Lutein-; Zeaxanthin-; Canthaxanthin-; β-carotene-incorporation

1. Introduction

Among the more than 600 carotenoids existing in nature [1,2], three are of basic importance for the function of the retinal pigment epithelial (RPE) cells in the mammalian eye: β-carotene, lutein and zeaxanthin. β-carotene itself is not abundant but its derivative 11-*cis*-retinal is part of the visual pigment rhodopsin [3], whereas lutein and zeaxanthin are the only carotenoids of high abundance in RPE cell membranes [4]. Previously we studied the incorporation of various carotenoids into liposomes [5,6] and pig liver microsomes [7]. Now we extend the investigations to RPE cells. We investigate the incorporation yields of lutein and zeaxanthin, isomeric di-hydroxy compounds of different stereometry, of the di-keto compound canthaxanthin and of the non-polar β-carotene. The structures of these four carotenoids are presented (Fig. 1). The differential comparison of the incorporation yields of these four carotenoids into each of the three membrane types shall allow us to evaluate membrane specific carotenoid incorporation properties.

A main physiological function of the xanthophylls lutein and zeaxanthin consists in their protection of the eye from potentially harmful short-wavelength radiation [8]. The overlapping of the blue light hazard spectrum and the absorption spectrum of the macular pigments has been demonstrated [9]. Antioxidant and

Abbreviations: BC, β-carotene; BHT, Di-*t*-butyl-*p*-toluene; CTX, Canthaxanthin; DMEM, Dulbecco Modified Eagle Medium; DMF, Dimethyl formamide; EC, Effective concentration; EM, Emission; EtOH, Ethanol; EXC, Excitation; EYPC, Egg Yolk phosphatidylcholine; FCS, Fetal calf serum; GP, Generalized Polarization; GSTP1, Pi Isoform of Glutathione S-Transferase; HPLC, High Performance Liquid Chromatography; IC, Incubation concentration; IY, Incorporation yield; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; LUT, Lutein; MLVs, Multilamellar vesicles; PBS, Phosphate buffer saline; RPE, Retinal pigment epithelium; SUVs, Small unilamellar vesicles; THF, Tetrahydrofuran; Tris, Tris-hydroxymethyl-aminomethane; XBP, Xanthophyll binding proteins; ZEA, Zeaxanthin.

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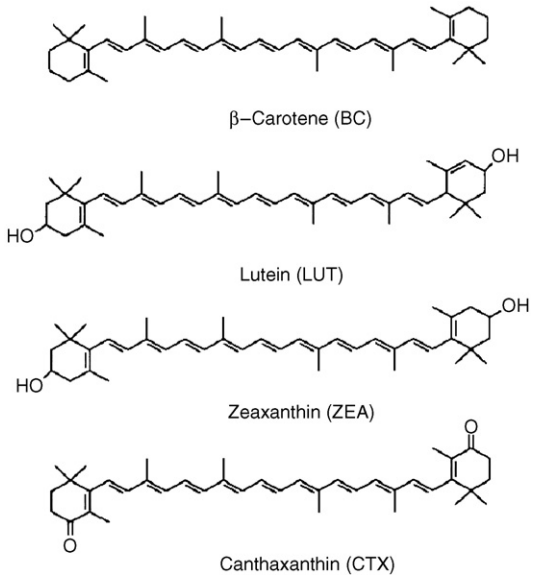

Fig. 1. The chemical structures of the carotenoids involved in the study: β-carotene, lutein, zeaxanthin, canthaxanthin.

radical scavenging effects have as well been reported [8,10,11]. A poor absorption of xanthophylls from food matrix or disturbances in their supply to the RPE is supposed to be a risk factor for the age dependent macular degeneration [12,13].

A basic biophysical question is why only the two xanthophylls, lutein and zeaxanthin out of more than 20 carotenoids present in human plasma are incorporated into the macula [14,15]. In this paper we look for incorporation features which may favour the exclusive predominance of the macular xanthophylls in RPE cells. We investigate the incorporation rates of the carotenoids into liposomes, microsomes and the RPE cells and its consequences on the membrane fluidity using β-carotene and canthaxanthin as reference pigments. The aim is not to compare quantitatively these three membrane types with regard to their capability to be supplemented with carotenoids, but to compare the carotenoids lutein, zeaxanthin, canthaxanthin and β-carotene in their quantitative relation to each other in which they incorporate differentially into each of these membranes.

The method we apply for the quantification of the carotenoids is UV–Vis absorption spectrometry using the characteristic absorption bands of the carotenoids. To detect the membrane modulating effect of the carotenoids we co-incubate the fluorophor Laurdan and evaluate its fluorescence spectra.

2. Experimental

2.1. Chemicals

β-carotene, lutein, and zeaxanthin were bought from 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). High Purity Egg Yolk phosphatidylcholine (EYPC), MW 750, was purchased from Lipoid KG (Ludwigshafen, Germany). The lipid purity of the preparation was higher than 99% and used without further purification.

The fluorescent probe used was Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene), MW 353.55. It was purchased from Molecular Probes (Eugene, OR, USA). 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 were bought from Nunc (Wiesbaden, Germany) and Trypan blue 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*-butyl-*p*-toluene (BHT), ethanol, ammonium ferrothiocyanate, were from Sigma (Deisenhofen, Germany). Dimethyl formamide (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.

2.2. Preparation of liposomes

Aliquots of 20 mg of EYPC were dissolved in 10 ml EtOH, and evaporated in a Rotavapor (Vacuum System). The film was dried under vacuum (Vacutherm, Heraeus Instr., Hannover, Germany) overnight at 42 °C. The liposomes were prepared following classical methods [20] as described below.

2.3. Incorporation of carotenoids into liposomes

Two alternative incorporation strategies were performed: (i) to start from identical carotenoid incubation concentrations, IC, or (ii) to achieve finally similar effective concentrations, EC, of carotenoids in the liposomes.

2.4. Liposome preparation I

The incubation concentration, [IC], of carotenoids in lipids according to alternative (i) was set to be approximately 1.5 mol%. 20 mg of EYPC were mixed with 0.21 mg of β-carotene or 0.2273 mg of lutein or 0.2273 mg of zeaxanthin or 0.225 mg of canthaxanthin in round bottom flasks. 10 ml of EtOH were added to each mixture. The mixture was evaporated and dried as before.

2.5. Liposome preparation II

According to our previous experience [5] to realize alternative (ii), we use appropriate incubation concentrations, IC, for each carotenoid in order to obtain identical final effective concentrations, EC, in the liposomal membrane. To achieve this, 20 mg of EYPC were mixed with 0.429 mg of β-carotene or 0.3 mg of canthaxanthin or 0.07575 mg of either lutein or zeaxanthin in round bottom flasks. This corresponds to 3 mol% β-carotene, 2 mol% canthaxanthin and 0.5 mol% of either lutein or zeaxanthin. To facilitate solution, 10 ml of EtOH was added to each mixture. The mixtures were evaporated under the same conditions and the film was dried as before.

The dried film was vortexed for 10 min at 42 °C with 10 ml Tris buffer [0.1 M, pH 7.4], giving a heterogeneous 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 vesicle suspensions (SUVs) were centrifuged at 6000 r.p.m. for 5 min and the supernatant was harvested. The pellet was suspended in Tris buffer, centrifuged again for 5 min at 6000 r.p.m., the supernatant was collected and mixed with the former supernatant. The supernatant fractions represent a heterogeneous population of unilamellar liposomes while the pellet contains aggregates of non-incorporated molecules.

The unilamellar liposomes were extruded using the thermostabilized membrane extruder (Thermostat Membrane Extrusion Equipment, Lipex Biomembranes Inc., Vancouver, Canada) through polycarbonate filters of 0.4 and 0.2 μm. To characterize the carotenoid concentration and stability before and after incorporation, UV–Vis spectra of the liposomal suspensions and of the carotenoids released from the liposomes in an ethanol solution were taken on a Shimadsu UV-2102 PC Scanning Spectrophotometer. From UV–Vis spectra the incorporation yield, IY, was calculated as the ratio between EC and IC. The phospholipid concentration of the liposomes was determined by the Steward method [21].

2.6. Preparation of microsomes

Pig livers were provided from a public slaughter house. Microsomal fractions were prepared by differential centrifugation at 105,000 ×*g* [22]. Microsomes were suspended in a 0.01 M potassium phosphate buffer, pH 7.7, with 20% glycerol and stored at −80 °C. Microsomal protein was determined according to Peterson [23]. A mirosomal fraction of 24 mg protein/ml was used.

2.7. Incorporation of carotenoids into microsomes

As for liposomes, two alternative incorporation strategies were performed: (i) to start from nearly identical carotenoid incubation concentrations, IC, and (ii) to achieve finally nearly the same final effective concentrations, EC, in the microsomes by starting from an appropiate IC.

2.8. Microsome preparation I

For alternative (i) the ICs of carotenoids were set to be approximately 20 nmol carotenoid/mg protein. With respect to the microsomal lipid content the ICs were about 1.5 mol%. Aliquots of 2 ml of microsomal suspensions were homogenized with 0.5 mg β-carotene, 0.54 mg canthaxanthin or 0.55 mg of either lutein or zeaxanthin for 15 min on ice using a Potter–Elvehjem tube at 400 r.p.m. 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.

2.9. Microsome preparation II

To realize alternative (ii), i.e. in order to achieve about the same EC of about 0.5 mol% 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 either lutein or zeaxanthin in a Potter–Elvehjem tube at 400 r.p.m. to incubate the thin film of carotenoids spread at the tube's wall. The ICs correspondingly used were 31.1 nmol β-carotene/mg protein, 26.6 nmol canthaxanthin/mg protein or 13.3 nmol either lutein or zeaxanthin/mg protein.

The microsomes supplemented with carotenoids were ultracentrifuged at 105,000 ×*g* for 1 h at 4 °C. The supernatant was collected and the pellet containing the coloured microsomes was resuspended in 2 ml Tris buffer (0.1 M pH 7.4) and used for all determinations. For the evaluation of the final effective concentrations, ECs, of the carotenoids in microsomes, aliquots of 30 μl of microsomal suspension were added to 970 μl EtOH, and on a Shimadzu UV-2102 PC Scanning Spectrophotometer the absorption spectra were displayed. From UV–Vis spectra (300–550 nm), the incorporation yield, IY, was calculated as the ratio between EC and IC.

2.10. Cultivation of the RPE cell line type D407

The human retinal pigment epithelial cell line D407 was gratefully obtained from Dr. Richard Hunt (University of South Carolina, USA). It retains many characteristics of the original cells like the ability to phagocytize [24]. D407 cells were cultivated in high glucose DMEM containing 4.5 mg/l glucose, 4 mM glutamine, 3.7 g/l sodium bicarbonate, 1 mM sodium pyruvate, 1% antibiotic antimycotic mixture of 10,000 UI penicillin, 10,000 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% CO_2/95% moist air atmosphere at 37 °C and were collected after confluence by trypsination with 0.05% trypsin in PBS. The cells were harvested in 3 ml DMEM, centrifuged for 5 min at 300 g, and at 4 °C the pellet was resuspended in 5 ml DMEM. To determine the cell viability we used 0.4% trypan blue 1:1 as intra-vital colorant. 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 carriers to incorporate carotenoids into RPE cells: fetal calf serum (FCS) or tetrahydrofuran (THF).

2.11. Carrier FCS

Volumes of 3 ml cell suspension containing 2.5×10^6 cells/ml DMEM were transferred to conical bottom flasks which each contains 0.2 mg of either β-carotene or canthaxanthin or lutein or zeaxanthin previously mixed with 10% FCS in a water-bath sonicator for 10 min. The IC was set to be approximately 45 nmol carotenoid/10^6 cells. The mixture was incubated in dimmed light at 37 °C for 48 h. Control cells

received an equivalent concentration of FCS. The mixtures were supplemented with 0.025% BHT to prevent further oxidation.

2.12. Carrier THF

Volumes of 3 ml cell suspension containing 2.5×10^6 cells/ml DMEM were transferred to conical bottom flasks which each contains 0.2 mg of either β-carotene or canthaxanthin or lutein or zeaxanthin previously dissolved in 15 μl tetrahydrofuran [THF]. We checked that the procedure was toxic to less than 10% of the cells. THF was found not to be toxic to cells at concentrations below 0.5% [25]. The IC was set to be approximately 45 nmol carotenoid/10^6 cells. The mixture was incubated in dimmed light at 37 °C for 15 min, 0.5 h, 1 h and 24 h. Control cells received an equivalent concentration of THF. The mixtures were supplemented with 0.025% BHT to prevent further oxidation.

To evaluate the carotenoid uptake at intervals of 15 min, 0.5 h, 1 h, 24 h and 48 h, aliquots of the incubated suspension were taken into Eppendorf micro test tubes. The suspension was centrifuged at 14,000 rpm for 30 min. The supernatant was harvested and the pellet was suspended in 1.5 ml Tris buffer (0.1 M, pH 7.4). Aliquots of 300 μl from a control cell suspension and from a cell suspension supplemented with carotenoids were solubilized using the detergent Triton X-100 to dissociate the membrane into its components and to determine the actual content of carotenoids in the cells. Immediately after solubilization, aliquots of 50 μl of the cell suspension supplemented with carotenoid were extracted to 950 μl EtOH, and the absorption spectra were displayed on a Shimadzu UV-2102 PC Scanning Spectrophotometer. From the UV–Vis spectra the incorporation yield, IY, was calculated as the ratio between EC and IC.

2.13. Fluorescent assays for liposomes, microsomes and D407 RPE cells

A stock solution of 1 mM Laurdan in DMF was prepared and 4 μl of this solution were added either to 100 μl liposomal suspension or to 50 μl microsomal suspension and suspended into 5 ml Tris buffer (0.1 M, pH 7.4). The ratio of dye to lipid in liposomes was 1.5 mol% and about the same in microsomes. In the studies performed on RPE cells, volumes of 5 μl Laurdan were added to 100 μl cell suspension in 5 ml Tris buffer (0.1 M, pH 7.4). All samples were incubated in dark at room temperature for 1 h under mild magnetic 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. The spectra were recorded on a Perkin Elmer LS-50 Luminescence Spectrometer equipped with a temperature-stabilized cuvette (Julabo Labortechnik, Seelbach, Germany). The scan speed was 100 nm/min and the monochromator slits were fixed at 5 nm.

All measurements were performed in triplicate. From the spectroscopic data, the Laurdan emission "generalized polarization" GP_{em} [26] was calculated from the emission spectra as follows:

$$GP_{em} = [I_{440} - I_{490}]/[I_{440} + I_{490}] \quad (1)$$

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

3. Results

3.1. Liposomes

Two sets of incorporation experiments were performed. One was intended to measure the incorporation rates of the carotenoids: the incubation concentration was 1.5 mol% related to liposomal lipids for each carotenoid.

The other set of experiments intended to evaluate the effect of the incorporated carotenoids onto the membranes. To obtain carotenoid specific, and not concentration dependent results, we incubated the liposomes with such carotenoid concentrations (IC) that the carotenoid concentration obtained in the membrane (EC) was about the same for the different carotenoids. For lutein, zeaxanthin and canthaxanthin this could be achieved for an EC of (0.34 +/− 0.04) mol%. For this, the IC had to be chosen to be 0.5 mol% for each, lutein and for zeaxanthin, and 2 mol% for canthaxanthin. β-carotene could not be incorporated up to 0.34 mol% but only to 0.13 mol%.

Fig. 2 shows the absorption spectra which have been recorded to determine the concentrations of the carotenoids incorporated into the liposomal membrane.

To evaluate changes of membrane properties caused by the incorporation of carotenoids, we labelled the liposomal membranes with the fluorophor Laurdan. Fig. 3 presents its fluorescence spectra from the preparations as they have been used in Fig. 2B. Lutein, zeaxanthin and canthaxanthin which are present in the liposomal membrane with about the same concentration of (0.34 +/− 0.4) mol%, quench the Laurdan fluorescence to round about the same extent, only a bit less effective is canthaxanthin. β-carotene the concentration of which is only about one third of the others quenches Laurdan nearly as strong as canthaxanthin. So, all the carotenoids quench efficiently the Laurdan fluorescence. Here it was not the aim to establish an efficiency ranking. However, the fluorescence spectra indicate characteristic differences between the differently supplemented liposomes. The long-wavelength emission peak around 478 nm is quenched stronger with increasing EC of the carotenoids than the short-wavelength peak at 436 nm. We assume that the increasing emission ratio 436 nm/478 nm is caused by a decrease of membrane fluidity [6]. Furthermore, the stronger carotenoid quenching action on Laurdan fluorescence intensity is related to the increase of membrane rigidity.

3.2. Pig liver microsomes

Pig liver microsomes we use as a natural model for cellular membranes which with regard to complexity is intermediate between liposomes and RPE cell membranes. Our investigations

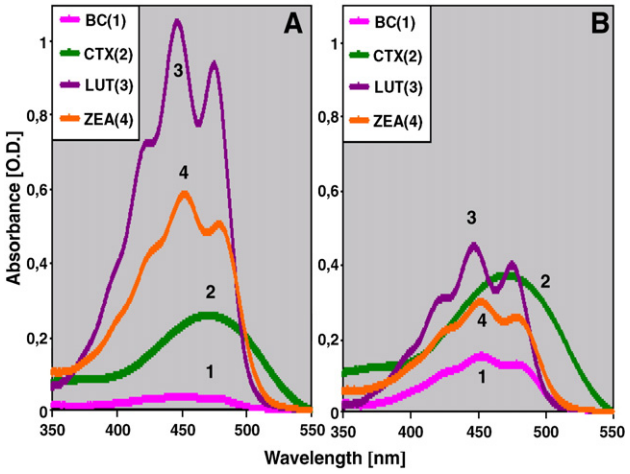

Fig. 2. Absorption spectra taken from liposomes after incorporation of carotenoids. A — incubation concentration 1.5 mol% (Preparation I), B — incubation concentration 3 mol% β-carotene, 2 mol% canthaxanthin, 0.5 mol% lutein and 0.5 mol% zeaxanthin (Preparation II).

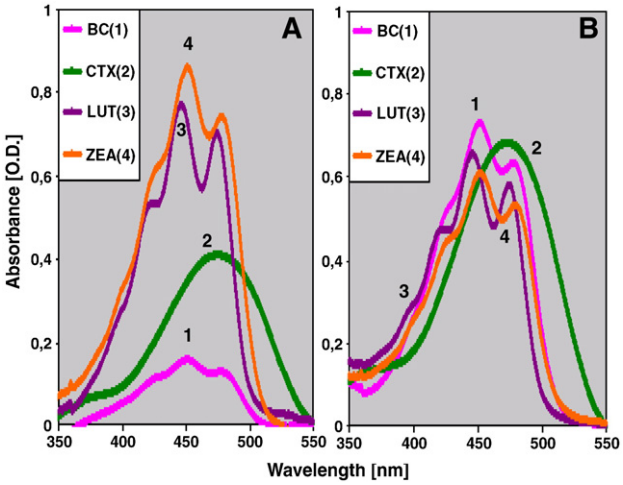

Fig. 4. Absorption spectra taken from microsomes after incorporation of carotenoids. A — incubation concentration 20 nmol carotenoid/mg protein (Preparation I), B — incubation concentration 31 nmol β-carotene/mg protein, 27 nmol canthaxanthin/mg protein, 13 nmol lutein/mg protein and 13 nmol zeaxanthin/mg protein (Preparation II).

on microsomes follow the same strategy as before. First, the incorporation yields, IY, are determined via carotenoid absorption measurements. Fig. 4A shows absorption measurements made to determine the incorporation yields. The incubation concentration was approximately 20 nmol carotenoid/mg protein. Table 1 summarizes the incorporation yields obtained from Fig. 2A for liposomes, from Fig. 4A for microsomes and from Fig. 6A for RPE cells. In liposomes the incorporation yields of β-carotene:canthaxanthin:lutein:zeaxanthin behave like 1:4:27:16, in microsomes like 1:3:4:5 and in RPE cells like 1:3:30:41. The carotenoid distribution pattern in RPE and liposomes are similar but different from the pattern in microsomes. The absorption spectra shown in Fig. 4B have been taken from microsomes which contain equimolar carotenoid concentrations. These samples are used to evaluate the Laurdan fluorescence excitation (Fig. 5A) and emission (Fig. 5B) spectra from microsomes which contain round about 0.5 mol% of a carotenoid. The quenching efficiency of the carotenoids on the Laurdan fluorescence increases in the order control < zeaxanthin < lutein < β-carotene < canthaxanthin, indicated from both, excitation and emission spectra. Contrarily to the corresponding results on liposomes (Fig. 3), the shape of the Laurdan emission spectra, related to the membrane fluidity of the microsomes, does not depend on the specific carotenoid.

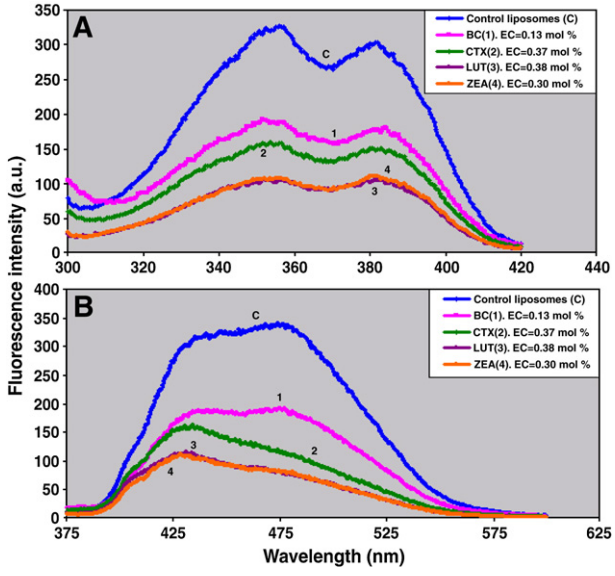

Fig. 3. Fluorescence spectra of Laurdan taken from liposomes which have been incorporated with carotenoids as in Fig. 2B. The effective carotenoid concentrations are given in the inserts. A — excitation spectra [λ_{em}=440 nm], B — emission spectra [λ_{exc}=353 nm].

3.3. Retinal pigment epithelial cell line

Fig. 6 shows the absorbances of the carotenoids released from the cells which had been incorporated via FCS (Fig. 6A) or via THF (Fig. 6B). Lutein is nearly as good as zeaxanthin incorporated from the carrier FCS (Fig. 6A). This does not hold for the carrier THF from which zeaxanthin seems to be incorporated much better (Fig. 6B). As we found in liposomes

Table 1
Incorporation yields [IY] of carotenoids into liposomes, microsomes and RPE cells

	IY [%]		
Carotenoid	Liposomes	Microsomes	RPE cells
β-carotene	2.5	8.6	0.1
Canthaxanthin	11	23	0.3
Lutein	67.2	36.5	3
Zeaxanthin	40	42	4.1

The incubation concentrations were 1.5 mol% carotenoid/lipid for liposomes, 20 nmol carotenoid/mg protein for microsomes, and 45 nmol carotenoid/10^6 RPE cells.

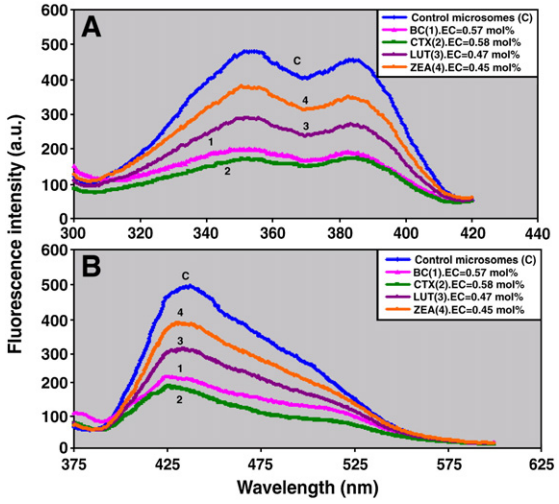

Fig. 5. Fluorescence spectra of Laurdan taken from microsomes which have been incorporated with carotenoids as in Fig. 4B. The effective carotenoid concentrations are given in the inserts. A — excitation spectra [λ_{em}=440 nm], B — emission spectra [λ_{exc}=353 nm].

and in microsomes, we find also here that Laurdan in the membranes is quenched by co-incorporation of the carotenoids (Fig. 7). The quenching pattern appears to be inverse to the absorption pattern whichever carrier is used FCS (Fig. 7A and B) or THF (Fig. 7C and D). Those carotenoids which incorporate strongest quench strongest. The same results are obtained from emission (Fig. 7 B and D) and excitation (Fig. 7A and C) spectra. Fig. 8 summarizes the results from Fig. 7 by plotting the quenching efficiencies at the peak wavelength in a block diagram. The Laurdan quenching effects from the cells loaded with carotenoids via FCS are stronger than from those loaded via THF, although the absorption measurements (Fig. 6) tell us the opposite. This leads us to the conclusion that the assays with the carrier THF contain carotenoids which are not incorporated into the membranes.

In order to find out if the modulation of the RPE cell membrane is more determined by the carotenoid concentration or by the carotenoid structure, we plotted the Generalized Polarizations (GP_{em}) of the Laurdan fluorescence against the carotenoid concentration. Fig. 9 shows that there is a correlation between carotenoid concentration and GP_{em}.

4. Discussion

The xanthophylls lutein and zeaxanthin are the only carotenoids which are located in retinal pigment epithelial (RPE) cell membranes, particularly in the macula lutea. We investigate their incorporation yields into the human RPE cell line D407. We included two reference carotenoids: (i) β-carotene and canthaxanthin as a completely unpolar and less polar one, respectively, and (ii) we compared the incorporation pattern in RPE cells with those in liposomal and in microsomal membranes as physical and biological models, respectively.

The incorporation yield, IY, is defined as the ratio of the carotenoid concentration which has been achieved in the membrane (EC) for a certain carotenoid incubation concentration (IC) in the experimental assay. In a first experimental set we used carotenoid incubation concentrations of 1.5 mol%. In the experiments with RPE cells we used a carotenoid incubation concentration of 45 nmol/10^6 cells. Figs. 2A, 4A and 6 belong to this set of experiments. From there the incorporation yields have been calculated and summarized in Table 1. As is already known, lutein and zeaxanthin are the two carotenoids with the highest available incorporation yields into membranes. But we emphasize that the absolute values given in Table 1 should not be compared between different membranes because the incubation concentrations have a common reference only within one membrane type but not between different membrane types. The carotenoid concentrations have been referred to the membrane lipid content in liposomes, to protein content in microsomes, and put to 45 nmol carotenoid/10^6 RPE cells. The incorporation yields of lutein and zeaxanthin into RPE cells are at least ten times higher than those of β-carotene and canthaxanthin (Table 1). In microsomes and liposomes this ratio is considerably smaller than ten. Lutein in liposomes achieves an exceptionally high incorporation yield which may be explained by the bigger distribution space available in the liposomal than in the microsomal membrane, as lutein has been reported to be incorporated into two different orientations, perpendicular and parallel to the membrane plane [27,28].

According to the pattern of incubation yields (Table 1) neither liposomes nor microsomes may be considered to be a model for the incorporation of carotenoids into RPE cells. Only the incorporation ratio between lutein and zeaxanthin resemble in RPE and liposomes.

In order to find out what modifications the carotenoids impose to the membrane properties we labeled the membranes with the fluorophor Laurdan which is known to sense the polarity of its environment [26]. Carotenoids modify membrane fluidities and thus also the Laurdan environment [6]. Laurdan

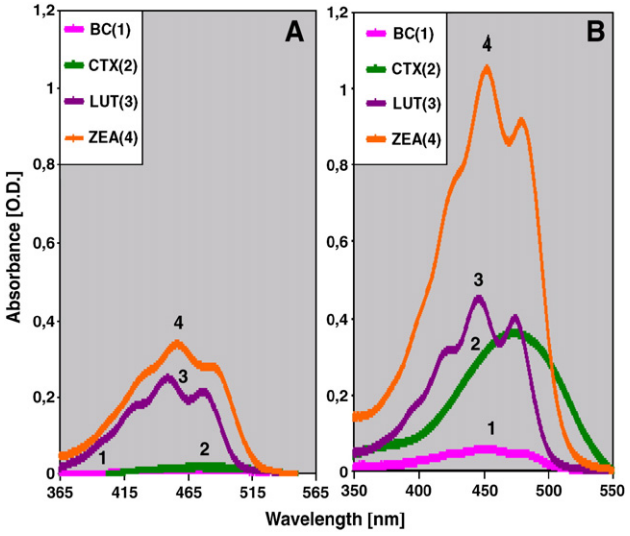

Fig. 6. Absorption spectra taken from RPE cells after incorporation of 45 nmol carotenoids/10^6 RPE cells. A — carotenoid carrier FCS, B — carotenoid carrier THF.

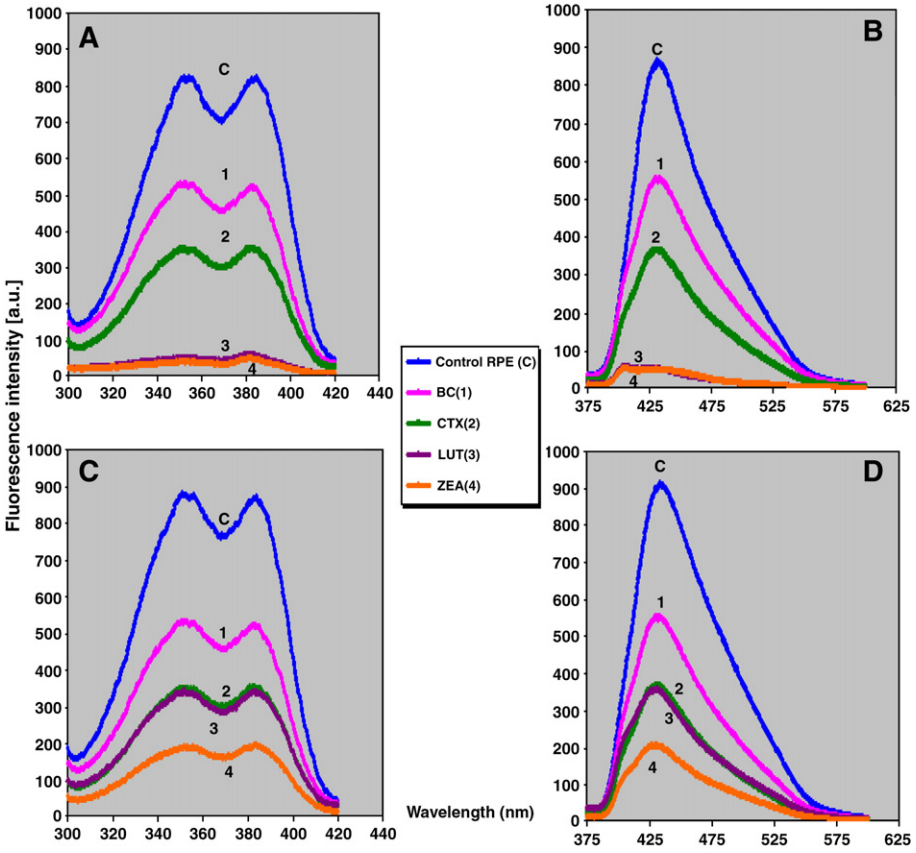

Fig. 7. Fluorescence spectra of Laurdan taken from RPE cells which have been incorporated with carotenoids as in Fig. 6. Incubation concentration was 45 nmol carotenoid/10^6 cells. A and C — excitation spectra [λ_{em}=440 nm], B and D — emission spectra [λ_{exc}=353 nm]. A and B — carotenoid carrier FCS, C and D — carotenoid carrier THF.

fluorescence does not only sense the polarity changes but it is also quenched by co-incorporated carotenoids [29].

To compare the ability of various carotenoids to modify membrane properties it is necessary to have them incorporated at equimolar concentrations. We achieved this for canthaxanthin, lutein and zeaxanthin. In liposomes and RPE cells β-carotene could only be incorporated to a much lower extent (Figs. 2B and 6). The modulating effects of the carotenoids on the fluorescence spectra of the co-incorporated Laurdan must be discussed on this background. Two effects have to be discriminated: (i) the quenching effects, best to be seen from the excitation spectra (Figs. 3A, 5A and 7A and C), and (ii) the membrane changing properties to be seen from the Laurdan emission spectra (from liposomes as shown in Fig. 3B; from microsomes as shown in Fig. 5B and for RPE cells as shown Fig. 7 B and D.

Fig. 8 summarizes the fluorescence quenching efficiencies of the carotenoids onto the co-incorporated Laurdan. To get an experimental confirmation, both, the results from excitation and emission spectra have been plotted. The quenching effects obtained with the carotenoid solvent THF are smaller for lutein and zeaxanthin, but not for canthaxanthin and β-carotene than those from the carrier FCS, although the corresponding Fig. 6B shows a higher carotenoid absorption rate via the carrier THF than via the carrier FCS (Fig. 6A). Obviously, using the carrier THF not all xanthophyll molecules are incorporated into the membrane, there is absorption from xanthophylls which have not been incorporated. Fluorescence quenching can only be performed by incorporated ones. This leads to the conclusion that FCS is the better carrier for xanthophylls and should be used instead of THF. We cannot be sure that we do not have free xanthophylls also when using the carrier FCS, but if there are

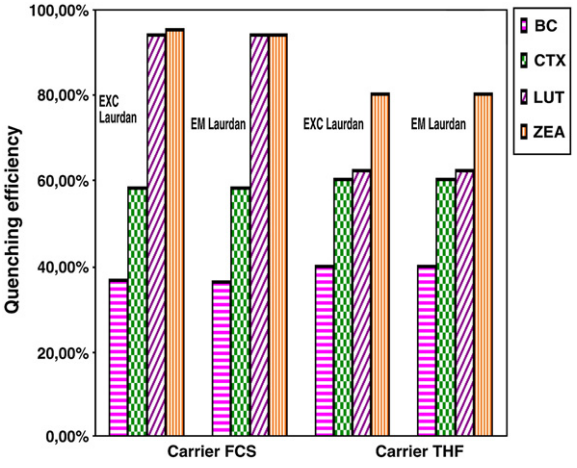

Fig. 8. Laurdan fluorescence quenching efficiencies from RPE cells which have been co-incubated with Laurdan and carotenoids (45 nmol carotenoids/10^6 RPE cells). Carriers FCS and THF have been used. The quenching efficiencies have been plotted from excitation and emission spectra.

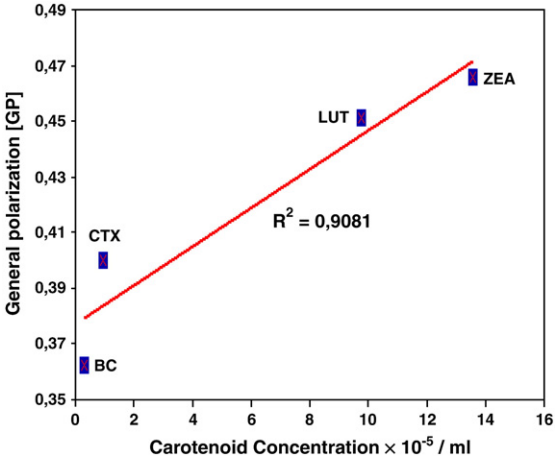

Fig. 9. Generalized polarization according to Eq. (1) is plotted against the effective carotenoid concentrations in RPE cells. Carotenoid carrier FCS. R=correlation coefficient.

free xanthophylls the more the conclusion in favour of the incorporation efficiency of this carrier is true. For β-carotene and canthaxanthin the same argumentation as for the xanthophylls may hold. But as their quenching efficiency does not depend on the carrier type (Figs. 7 and 8) although their absorption does (Fig. 6), an incorporation saturation also may be discussed for β-carotene and canthaxanthin considering their low incorporation efficiency.

The incorporation of xanthophylls into liposomes changes the Laurdan emission profile considerably (Fig. 3B) indicating a membrane rigidisation. Yet microsomes (Fig. 5B) and also RPE cells (Fig. 7B and D) appear in that rigidisation state even without carotenoid incorporations, which is not more changed significantly by carotenoid incorporations.

Comparing the Laurdan quenching efficiencies of carotenoids from liposomes (Fig. 3) and from microsomes (Fig. 5), we see that the xanthophylls and the carotenes behave oppositely: in liposomes the xanthophylls quench the Laurdan fluorescence stronger than the carotenes; in microsomes it is the reverse. Probably in microsomes the lipophilic molecules Laurdan and carotenes come into a closer contact because the microsomal intrinsic cholesterol limits their available space. And also, Laurdan because of the intrinsic cholesterol is better shielded from penetrating water and therefore less effectively quenched in microsomes than in liposomes.

In RPE cells like in liposomes, but not in microsomes, the xanthophylls most strongly quench the Laurdan fluorescence (Fig. 7). This result is not in line with the result from the incorporation experiments where we found the xanthophyll incorporation patterns for RPE cells (Fig. 6A) and microsomes Fig. 4A) to resemble. According to our reasoning that fluorescence quenching can only be performed by membrane incorporated carotenoids, we argue that the binding efficiency of xanthophylls in RPE cells is much stronger than in microsomes. A stronger binding of xanthophylls in RPE cells may be achieved by special binding proteins which are abundant in RPE cells but not in microsomes. Moreover, an enhanced protein binding of the xanthophylls in RPE cells would provide a more polar environment for Laurdan leading to its stronger fluorescence quenching in RPE cells than in microsomes. We have no direct proof for this hypothesis. But some arguments from literature are available. The existence of membrane-associated xanthophyll-binding proteins has been reported [16–18]. Retinal tubulin has been identified to bind xanthophylls [19]. Moreover, a Pi Isoform of Glutathione S-Transferase (GSTP1) has been reported to have a high specificity and affinity to bind zeaxanthin in contrast to apparently weaker interactions with lutein in the macula of the human eye [18].

The fluorescence emission spectra gave us indications for membrane fluidity changes caused by the incorporation of carotenoids. Are these membrane changes caused mainly by the specificity of the carotenoid as has been reported for liposomes [30], or are they, independent from the carotenoid specificity, correlated with carotenoid concentration. For RPE cells we find the latter hypothesis fulfilled as we conclude from the plot of the Generalized Polarisation against carotenoid concentration (Fig. 9). The xanthophylls achieve the highest incorporation yields and they also affect most the membrane fluidity reducing it.

From our results we propose the hypothesis that a reverse correlation exists between the effectively incorporated membrane carotenoid concentration and the Laurdan fluorescence quenching efficiency. This offers a method to determine the carotenoid contents intrinsically from membranes instead to dissolve the membranes and to determine the carotenoid content released. To validate this hypothesis with regard to a certain carotenoid and to a certain membrane type, a calibration between Laurdan fluorescence quenching and carotenoid concentration must be performed.

5. Conclusions

The incorporation preference for lutein and zeaxanthin compared to β-carotene and canthaxanthin into RPE cells is significantly higher than into liver microsomes and into liposomes.

As a carrier for carotenoid incorporation into RPE cells fetal calf serum is recommended instead of tetrahydrofuran.

Membrane bound Laurdan fluorescence quenching may reveal a base to determine directly carotenoid concentrations incorporated into membranes.

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