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Different ways to insert carotenoids into liposomes affect structure and dynamics of the bilayer differently

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Abstract

We apply and quantify two techniques to incorporate carotenoids into liposomes: (i) preparation of unilamellar liposomes from mixtures of phospholipids and a carotenoid or cholesterol; (ii) insertion of carotenoids into prepared liposomes. Homogeneous liposomal fractions with a vesicle size diameter of approximately 50 nm were obtained by an extrusion method. The resulting vesicles were subjected to a three-dimensional light scattering cross-correlation measurement in order to evaluate their size distribution. The fluorescent dyes Laurdan, DiI-C_{18}, C_6-NBD-PC were used to label the liposomes and to evaluate modulations 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.

Keywords: Carotenoids; Doped liposomes; Three-dimensional light scattering; Fluorescence labelling

Abbreviations: ASTA, Astaxanthin; BC, β-Carotene; BHT, Di-*t*-buthyl-*p*-cresol; C_6-NBD-PC, Hexanoyl (7-nitro-2,1,3-benzoxadiazol-4-yl) 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; CHOL, Cholesterol; CHOL1, Cholesterol 10 mol%; CHOL2, Cholesterol 40 mol%; CTX, Canthaxanthin; DCM, Dichloromethane; DiI-C_{18}, 1,1-Dioctadecyl-3,3,3,3′-tetramethylindocarbo-cyanine perchlorate; DMF, Dimethylformamide; DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DPPE, 1,2-Dipalmitoyl phosphatidyletanol-amine; DPPS, 1,2-Dipalmitoyl diphosphatidylserine; DSC, Differential scanning calorimetry; EC, Effective concentration; EtOH, Ethanol; EYPC, Egg yolk phosphatidylcholine; IC, Initial concentration; IY, Incorporation yield; Laurdan, 6-Dodecanoyl-2-dimethylaminonaphthalene; LSM, Light scattering microscopy; LUT, Lutein; LYC, Lycopene; MeOH, Methanol; MLV, Multilamellar vesicles; PL, Phospholipid; SUV, Small unilamellar vesicles; ZEA, Zeaxanthin

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1. Introduction

It is known how carotenoids act in plant membranes to promote photosynthesis and photoprotection, but few data are available about the location, distribution and actions of dietary carotenoids in animal and human cells and membranes [1]. 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.) [2]. These properties determine the incorporation yield and the carotenoids' ability to fit into the membrane bilayer [3]. The predominant carotenoids found in plasma are α-, β-carotene, lycopene, lutein, β-cryptoxanthin and zeaxanthin [4,5]. Some of these (β-carotene, lycopene, zeaxanthin, lutein) were extensively used in in vitro experiments to demonstrate their action. The effects are proved to be associated with their antioxidant capacity, which is usually considered to explain their beneficial effects on human health [6–8].

Controversial data are reported about location and distribution of carotenoids in the membrane bilayers, the data being cumulated from a diversity of experimental protocols, membrane types, carotenoid specificity and their incubation method.

Differential scanning calorimetry, ^{1}H-NMR and EPR were applied in multilamellar or unilamellar liposomes of known composition but uncontrolled dimensions, using non-polar hydrocarbons (β-carotene and lycopene) and the polar carotenoids (xanthophylls) zeaxanthin and lutein. Different hypotheses regarding specific interactions with membranes were proposed.

Hydrocarbons locate into the inner, hydrophobic part of the membrane having a randomised orientation, increasing the motional freedom of the bilayer headgroups and decreasing the membrane order in its crystalline state. This effect is stronger below the main phase transition temperature, but it depends on the phospholipid composition [9,10]. Based on the more pronounced effects in the crystalline state than in the fluid state of the membrane, these carotenoids may induce membrane fluidisation.

The polar dihydroxy xanthophylls (studies have been made with zeaxanthin, mainly) are spanning the membrane, acting as rivets which decrease the motional freedom in the polar region of the membrane, so they may increase the membrane rigidity especially in the fluid-phase [11]. These molecules increase the hydrophobicity in the membrane interior but decrease it, as well as the permeability to water and oxygen, in the polar head-group region [12]. In gel phase membranes, polar carotenoids increased the motional freedom of most of the EPR spin labels, showing a regulatory effect on membrane fluidity [13,14]. The orientations of lutein and zeaxanthin in the membrane were proposed to be different from each other. Other studies made by EPR, show that polar carotenoids can regulate the membrane fluidity in a way similar to cholesterol, although they locate differently within the lipid bilayer membrane [15–17].

The antioxidant capacity of the two classes of carotenoids proved to be dependent on their different location in the bilayer: while β-carotene and lycopene are able to quench radicals in the hydrophobic part of the membrane, zeaxanthin was effective as an antioxidant in the polar region, exposed to an aqueous environment [18]. Various membrane types loaded with carotenoids and other antioxidants were used to study the dynamic membrane properties [18–20].

The concentrations of carotenoids used in those membrane experiments have usually been very high, up to or even above the aggregation limits. Recent experiments on the organisation of lutein–DPPC and zeaxanthin–DPPC monolayers have been interpreted in terms of aggregation limits, which revealed a molar fraction of 5 mol% for zeaxanthin and a molar fraction of 20 mol% for lutein. Different orientations of both xanthophylls at the interface were found [21].

Until our recent studies, no quantitative data were obtained about the incorporation yields of various polar and non-polar carotenoids into membrane bilayers and their effects on membrane structure and dynamics. We had incorporated β-carotene, lutein, zeaxanthin, canthaxanthin and astaxanthin into multilamellar or unilamellar liposomes and evaluated incorporation yields, concen-

trations and membrane ordering effects [22–25]. The methods applied were DSC, UV-Vis spectrometry and fluorescence spectroscopy. Generally, polar carotenoids (lutein and zeaxanthin) were better incorporated than β-carotene and they caused similar effects as cholesterol (ordering and rigidifying effects and increase of membrane anisotropy) suggesting that they can affect the membrane structure and dynamics even at much lower effective concentrations than cholesterol. The incubation doses used did not depass 5 mol%, thus resembling physiological concentration ranges.

This paper reports on alternatives of carotenoid incorporation. (i) Preparation of liposomes from mixtures of phospholipids and carotenoids or (ii) by insertion of carotenoids into liposomes yet prepared. Homogeneous populations of liposomes of controlled vesicle size were obtained by an extrusion method. The fluorescent dyes Laurdan, DiI-C_{18} and C_6-NBD-PC have been used to label the liposomes. Special fluorescence characteristics obtained from liposomes which have been doped with β-carotene or lutein or zeaxanthin, were evaluated in order to interpret membrane mechanics involved.

2. Materials and methods

2.1. Chemicals

β-Carotene (BC) used in all experiments was kindly offered by the Department of Vitamins and Fine Chemicals, Hoffmann la Roche, Basel, Switzerland. Lutein (LUT) and zeaxanthin (ZEA) were purified from natural sources (flowers of *Tagetes erecta* and *Physalis alkekengy*) and checked for purity by HPLC.

High purity 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC), egg yolk phosphatidylcholine (EYPC), 1,2-dipalmitoyl phosphatidyletanolamine (DPPE), 1,2-dipalmitoyl diphosphatidylserine (DPPS) were purchased from Lipoid KG (Ludwigshafen, Germany). The lipid purity of the preparation was higher than 99% and we used them without further purification.

The fluorescent probes used in the experiments were: 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan); 1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbo-cyanine perchlorate (DiI-C_{18}); and hexanoyl (7-nitro-2,1,3-benzoxadiazol-4-yl) 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (C_6-NBD-PC). They were purchased from Molecular probes (Eugene, OR, USA).

Di-*t*-buthyl-*p*-cresol (BHT), cholesterol (CHOL), dichloromethane (DCM), methanol (MeOH), chloroform (CHL), ethanol (EtOH), ammonium ferrothiocyanate were from Sigma (Deisenhofen, Germany). Dimethylformamide (DMF) was obtained from ACROS (Geel, Belgium). All chemicals were of research grade. Solutions were prepared in deionized ultra pure water.

2.2. Preparation of liposomes from mixtures of phospholipids and carotenoids or cholesterol. Size measurements

A mixture of 100 mg phosholipids (75 mg EYPC, 17 mg DPPE and 8 mg DPPS, 17:5:3 molar ratio) were dissolved in 10 ml DCM/MeOH (2:1) and evaporated in the Rotavapor (vacuum system). The film was dried under vacuum (Vacutherm, Heraeus Instr., Hannover, Germany) overnight at 45 °C. This standard mixture of phospholipids used to build liposomes was used in all experiments and is abbreviated as PL.

A mixture of 100 mg phospholipids (PL) was dissolved in 10 ml DCM/MeOH (2:1) together with 5.9 mg of each carotenoid (BC, LUT or ZEA) previously dissolved in 10 ml DCM containing 0.002% BHT. The mixture was evaporated under the same conditions as before and the film was similarly dried. The initial concentration (IC) of carotenoids to PL was set to approximately 2.5 mol%.

In parallel, to 200 mg phospholipid mixture (PL), 10.5 or 41 mg cholesterol dissolved in DCM were added, the ratio to PL being 10 mol% (CHOL1) and 40 mol% (CHOL2), respectively. The procedure to obtain the film was the same as before.

In all cases, the dried film was vortexed for 10 min at 48 °C with 10 ml Tris buffer (0.1 mM, pH 7.4), giving a heterogeneous suspension of multilamellar vesicles (MLVs). Then the 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, for 15 min.

After sonication the MLV suspensions were centrifuged at 12 000 rev./min and the supernatant was harvested. The pellet was suspended in Tris buffer (same molarity and pH), centrifuged again for 5 min at 12 000 rev./min, the supernatant was collected and mixed with the former supernatant.

In all cases the supernatant fractions represented a heterogeneous population of unilamellar liposomes (empty ones or doped with carotenoids or cholesterol) while the pellet contained aggregates of non-incorporated molecules (carotenoids, phospholipid, cholesterol). The unilamellar liposomes were extruded using the thermostabled membrane extruder (Thermostat Membrane Extrusion Equipment, Lipex Biomembranes Inc., Vancouver, Canada) through polycarbonate filters of 0.4, 0.2 and 0.1 μm. Light scattering microscopy (LSM) was used to measure the SUV sizes in all cases.

To characterise the carotenoid concentration and stability before and after incorporation, UV-Vis spectra of the liposomal suspension and of the carotenoids released from the liposomes in an ethanol solution were taken on a Shimadzu UV-2102 PC Scanning Spectrophotometer. From UV-Vis spectra (190–550 nm), the following parameters were determined: initial concentration of the carotenoid added to the phospholipid mixture (IC); final concentration of carotenoid found in SUVs (EC). By calculation, the incorporation yield (IY) is determined as the ratio between EC and IC.

The lipid concentration of each type of liposomes was determined by the Steward method [26].

The size of the suspended liposomes was determined by a special type of photon correlation spectroscopy, namely the three-dimensional cross-correlation technique [27]. This was necessary because all liposomal samples were optical turbid and had to be investigated in their original state. This is possible with the three-dimensional cross-correlation technique we applied, which in contrast to conventional light scattering instruments guarantees reliable information about the samples even for high turbidity levels.

2.3. Insertion of carotenoids into SUV liposomes

Aliquots of 400 μl PL-SUV liposomes (containing 2 mM phospholipids, as described above) were mixed and incubated for 1 h at 37 °C with 10, 20 and 40 μl solutions of carotenoids dissolved in DCM, containing 7.8 nmol BC, 10.8 nmol LUT and 10.4 nmol ZEA, respectively. The mixture was vortexed for 10 min and centrifuged at 12 000 rev./min, the supernatant was collected and submitted to LSM and UV-Vis measurements, as described previously.

To characterise properly the ability of each carotenoid to be inserted into the SUV bilayer by correlation with the molar carotenoid/lipid ratio in the bilayer, we define the parameter 'insertion factor' (IF). IF was calculated as the ratio R_f/R_i, where R_i represents the initial carotenoid/lipid ratio used in the experiment, and R_f, the final carotenoid/lipid ratio.

2.4. Fluorescent probes and fluorescence measurements

Laurdan was dissolved in DMF giving a stock solution of 1 mM. SUVs prepared as described earlier and containing 0.05 mg lipid were suspended in 5 ml Tris 50 mM (pH 7.4). A 6.4-μl ethanol solution of 1 mM Laurdan was added and incubated for 1 h at 25 °C. The lipid/probe ratio was 500. The emission spectra were recorded between 375 and 600 nm at $\lambda_{exc}=350$ nm, and the excitation spectra between 300 and 420 nm at $\lambda_{em}=440$ nm.

DiI-C_{18} was dissolved in EtOH for a stock solution of 1 mM. Each liposome suspension, containing 0.05 mg lipid, was suspended in 5 ml Tris 50 mM (pH 7.4). A 20-μl ethanol solution of 1 mM DiI-C_{18} was added and incubated for 1 h at 25 °C. The emission spectra were recorded between 560 and 660 nm at $\lambda_{exc}=550$ nm and the excitation spectra between 450 and 570 nm at $\lambda_{em}=580$ nm.

The C_6-NBD-PC (a fluorescent phospholipid

Table 1
Size measurements of liposomes prepared from a mixture of phospholipids and carotenoids and extruded through a 0.1-μm filter

Liposomes containing	$a \pm s^a$ (nm)	% light transmission	Carotenoid/lipid ratio
–	39±7	62.8	–
BC	54±10	15.5	0.15
LUT	43.4±5	54.3	0.97
ZEA	57.2±9	40.6	0.69

Abbreviations: BC, β-Carotene; LUT, lutein; ZEA, zeaxanthin; *a*, mean diameter of liposomes, Δ*a*; s^a uncertainty of mean value a; %, transmission of laser light (628 nm).

analogue) was dissolved in a mixture of CHL/MeOH—2:1, evaporated under nitrogen and then dissolved in EtOH at 1 mM final concentration (stock solution). This stock solution was diluted up to 0.2 mM. A liposome suspension of 0.05 mg lipid in 10 ml Tris 50 mM (pH 7.4) was added to a 20-μl ethanol solution of 0.2 mM C_6-NBD-PC and incubated for 1 h at 37 °C. The emission spectra were recorded between 485 and 650 nm at $\lambda_{exc} = 470$ nm and the excitation spectra between 300 and 520 nm at $\lambda_{em} = 530$ nm.

Fluorescence measurements were performed with a computer-controlled Perkin Elmer LS-50 Luminescence Spectrometer equipped with a thermostatic cuvette (Julabo Labortechnik, Seelbach, Germany). The scan speed was 300 nm/min and the slits were fixed at 5 nm.

To measure the effects of C_6-NBD-PC on membranes, we calculated its insertion efficiency by its fluorescence intensity at 337, 470, 530 and 535 nm, respectively.

3. Results

3.1. Determination of SUV size

We studied the size of carotenoid containing liposomes which had been prepared from the mixture of phospholipids (PL) and a carotenoid. Table 1 represents the mean size of the liposomes as determined by the three-dimensional light scattering cross-correlation technique. The particle size distribution appeared to be very broad The mean values of the carotenoid-doped liposomal diameter were approximately 50 nm, slightly but not significantly higher than the control liposomes, and without significant modifications linked to carotenoids. Remarkable is the low light transmission rate for the BC-containing liposomes (15%), compared to the other liposomes (transmissions above 40%). In the case of BC, this transmission correlates with the formation of larger structures of aggregated liposomes after the extrusion, up to structures of approximately 450 nm. Due to these structures, the light scattering effect is intensified. Light transmission rate and light scattering effect are inversely related.

A correlation can be seen between the carotenoid/lipid ratios in the liposomes and the light transmission rate (Table 1). BC which is least incorporated and gives the lowest carotenoid/lipid ratio is aggregated and exerts a high light scattering effect, while LUT, which is best incorporated, induces the highest carotenoid/lipid ratio and the lowest light scattering effect among the doped liposomes.

In contrast to the carotenoid-containing liposomes which have been prepared from the common mixture, the carotenoids had no effect on the vesicle size when the prepared pure liposomes were incubated with a carotenoid. However, in the latter case the carotenoid incorporation yields were very much lower, to be seen from the IY values presented in Tables 2 and 3.

3.2. Incorporation yields of carotenoids or cholesterol into liposomes which have been prepared from a mixture of phospholipids and a carotenoid or cholesterol

Fig. 1 shows the UV-Vis absorption spectra of these liposomes in Tris solution and after the carotenoid release in an ethanol solution. From the absorption intensities recorded at 450 nm, we confirmed that carotenoids were inserted into the liposomes and that they showed a hypsochromic effect. From the absorption spectra of the ethanol extract we calculated the quantity (effective concentrations, EC) for each carotenoid in these liposomes.

Table 2
Mean values obtained for initial concentration (IC), final concentration (EC) and incorporation yields (IY) of the phospholipids in control liposomes (PL) and in liposomes which have been prepared from a mixture of phospholipids and β-carotene (BC) or lutein (LUT) or zeaxanthin (ZEA) or different cholesterol concentrations ($CHOL_1 < CHOL_2$), respectively

Preparation	PL	BC	LUT	ZEA	$CHOL_1$	$CHOL_2$
IC (mol%)	10.7	2.6	2.8	2.5	10	40
EC_{PL} (μmol/ml)	6.6	4.2	10.3	10.3	9.4	10.5
$EC_{Car\ or\ CHOL}$ (mol%)	–	0.3	1.4	0.95	10	40
IY_{PL}	0.6	0.4	1.0	1.0	0.35	0.5
$IY_{Car\ or\ CHOL}$	–	0.1	0.5	0.4	1.0	1.0

For details see Section 2.

Table 3
Carotenoids (BC, LUT, ZEA) inserted into prepared liposomes: IC, initial concentration of carotenoid; R_i, initial ratio carotenoid/lipid; EC, final concentration of carotenoid; and R_i, final ratio carotenoid/lipid; IY, incorporation yield; IF, insertion factor ($= R_f/R_i$)

Carotenoid inserted	IC (nmol/ml)	EC (nmol/ml)	R_i	R_f	IY	IF
BC	783	34	0.065	0.122	0.043	1.88
LUT	1080	36	0.135	0.127	0.033	0.94
ZEA	1045	34	0.088	0.125	0.033	1.42

For details see Section 2.

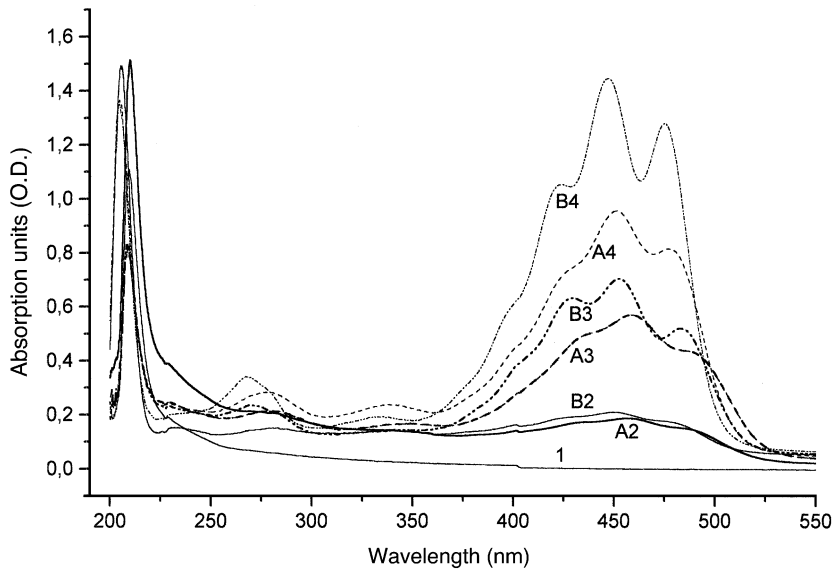

Fig. 1. UV-Vis spectra of liposomes which have been prepared from a mixture of phospholipids and one of the carotenoids β-carotene, lutein or zeaxanthin and after extraction into ethanol. A, liposomes containing carotenoids; B, ethanol extract of liposomes; 1, control liposomes; 2, β-carotene; 3, lutein; 4, zeaxanthin.

Table 2 presents the mean values for initial concentration (IC), final effective concentrations (EC_s) and incorporation yields (IY_s) in the liposomes which have been formed from the mixture of phospholipids with two different cholesterol concentrations (CHOL1, CHOL2) or a carotenoid. Starting with 10.7 mol% initial concentration, the final PL incorporation yield was only 0.6. In the presence of CHOL1 and CHOL2 the PL IY values were even lower: 0.35 and 0.5, respectively, while the CHOL molecules were totally integrated ($IY_{CHOL} = 1$).

When BC was incorporated, its IY was 0.1 while the IY of PL was 0.4. LUT and ZEA (IY = 0.4 and 0.5) gave more ordered liposomes, with an IY of PL = 1.

We see that phospholipids are incorporated into SUV liposomes with incorporation yields of only approximately 0.6, whereas in the presence of LUT and ZEA the phospholipid incorporation is complete (IY = 1.0). In the presence of BC and both cholesterol concentrations the phospholipids incorporate with yields in the range of 0.35–0.5. The polar carotenoids (LUT and ZEA) have higher incorporation yields (IY = 0.5 and 0.4, respectively) than BC (IY = 0.1) while CHOL is completely incorporated (IY = 1). Conclusively, LUT and ZEA are best incorporated into the liposomes, i.e. they cause the highest carotenoid/lipid ratio in the liposomes whereas BC causes the lowest one.

3.3. Insertion ability of carotenoids into prepared liposomes

The incorporation yield of a carotenoid into prepared liposomes is much lower (Table 3) than that which is obtained when liposomes are formed originally from the mixture of phospholipids and a carotenoid (Table 2). The difference is approximately a factor of 15 for LUT and ZEA, and approximately a factor of 2 only for BC, indicating that the non-polar BC can enter easier into prepared liposomes than xanthophylls.

The final effective concentrations (EC) of the carotenoids is about the same for all of them. Although the insertion yield is low, the insertion factor indicates that the insertion of carotenoids into the prepared liposomes is most efficient for BC and lowest for LUT. The insertion factor for BC and ZEA is higher than one (1.88 and 1.42, respectively). From this we conclude that during the procedure of incorporation more phospholipids are lost from the assay than carotenoids. Only lutein keeps the relation with the phospholipids balanced.

3.4. Fluorescence spectra of the liposomes prepared from a mixture of phospholipids and a carotenoid or cholesterol

Fig. 2a,b shows the emission spectra (a) and the excitation spectra (b) of Laurdan incorporated into the liposomes.

It should be noticed that the incorporations induce a slight hypsochromic shift of the Laurdan main emission peak. Carotenoids, at most LUT and ZEA, cause a strong quenching of the Laurdan emission, mainly in the 478-nm emission range. In the Laurdan excitation spectra (Fig. 2b) we observe a slight bathochromic effect and an increase of the $I_{384/356}$ emission ratio. A significant quenching of fluorescence intensity, strongest for LUT and ZEA, can be seen here, too.

Fig. 3 shows the emission spectra of the dye DiI-C_{18} incorporated into the liposomes. In this case the incorporations cause a slight bathochromic effect on the DiI-C_{18} fluorescence. The fluorescence emission of DiI-C_{18} is increased by the carotenoids and quenched by cholesterol. This appears just inverse to the modulation of the Laurdan fluorescence emission under the same conditions.

Fig. 4a,b shows the emission spectra (a) and the excitation spectra (b) of C_6-NBD-PC incorporated into the liposomes. The carotenoids exert a strong fluorescence quenching effect, strongest by LUT and ZEA, while the higher cholesterol concentration (CHOL2) enhances the fluorescence emission and causes a hypsochromic effect.

4. Discussion

Although carotenoids are minor constituents of animal membranes, they modulate membrane

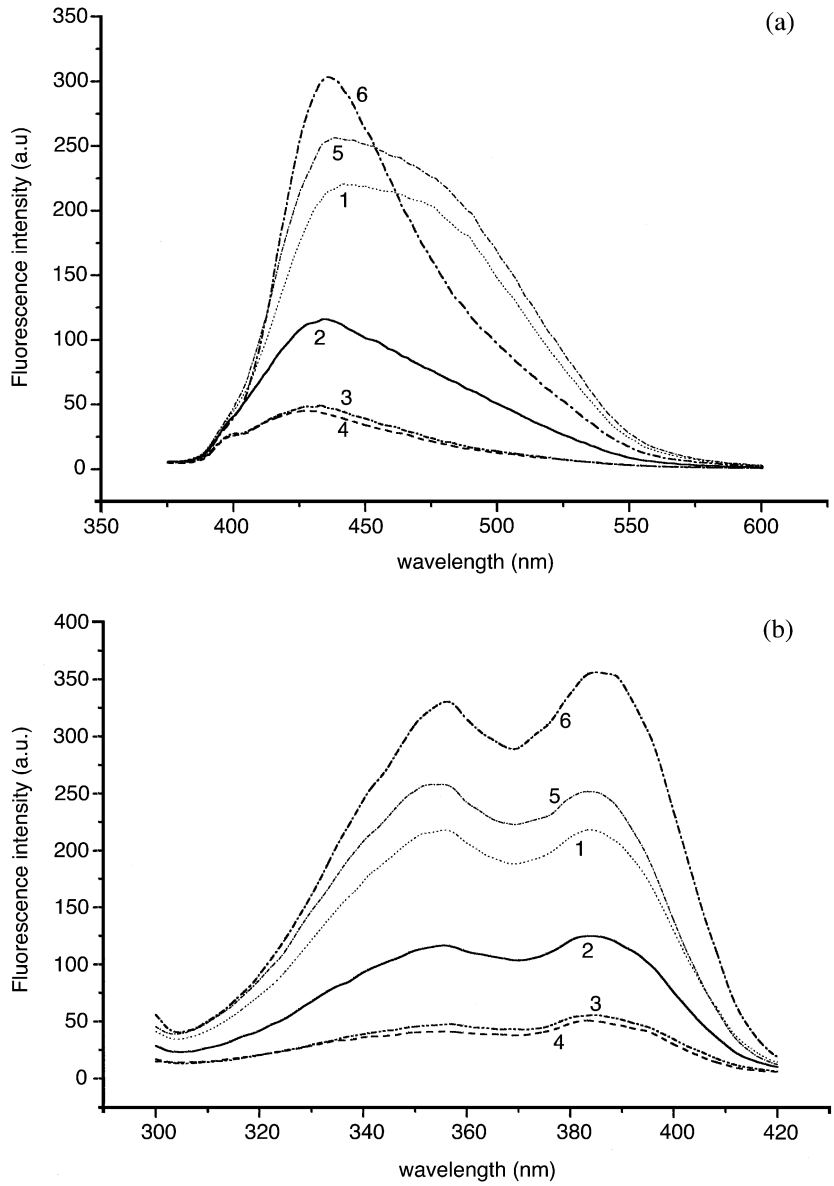

Fig. 2. (a and b). Emission spectra at $\lambda_{exc}=350$ nm (a) and excitation spectra at $\lambda_{obs}=292$ nm (b) of Laurdan incorporated into liposomes which have been prepared from a mixture of phospholipids and a carotenoid or cholesterol. 1, control liposomes; liposomes containing: 2, β-carotene; 3, lutein; 4, zeaxanthin; 5 and 6, low and high cholesterol content, respectively. For details see Section 2.

structure and dynamics. The molecules we studied most were β-carotene, zeaxanthin and lutein. A large range of concentrations (1–20 mol%), the higher ones mainly in NMR and EPR measurements has been applied.

Relevant results were:

- β-Carotene is located preferentially in the non-polar regions and has pronounced fluidising

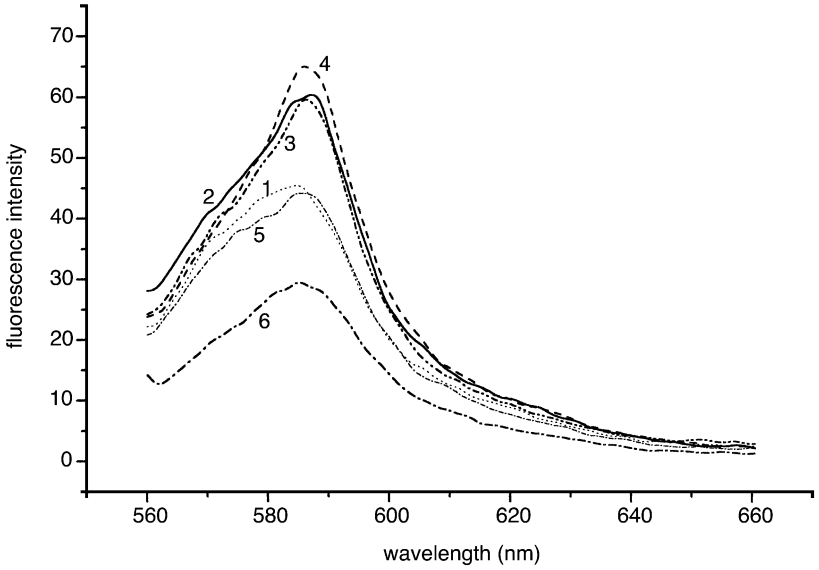

Fig. 3. Emission spectra ($\lambda_{exc}=550$ nm) of the dye DiI-C_{18} incorporated into liposomes which have been prepared from a mixture of phospholipids and a carotenoid or cholesterol. 1, Control liposomes; liposomes containing: 2, β-carotene; 3, lutein; 4, zeaxanthin; 5 and 6, low and high cholesterol content, respectively. For details see Section 2.

effects in the membrane gel state, increasing the motional freedom of head-groups [9].

- Zeaxanthin, but not β-carotene, rigidifies the membrane in the hydrophobic core as well in the polar region [11]. Aggregation of carotenoids in mono-molecular layers has been reported to occur yet at low concentrations (1–5 mol%) depending on the membrane phase. Lutein forms aggregates even easier [21,28], increases the hydrophobicity in the interior membrane and decreases it in the polar region, especially in membranes which are rich in unsaturated fatty acids like EYPC [12,15,16]. The ordering effect is strong in membranes which are rich in saturated fatty acids like DPPC. The authors conclude that carotenoids regulate the fluidity in a similar way as cholesterol, being a better hydrophobic barrier in unsaturated membranes [13,14].

Our previous studies intended to quantify the incorporation yields of carotenoids into liposomal or natural membranes, and to evaluate membrane dynamics (phase transition, fluidity, micropolarity and anisotropy) by application of fluorescent dyes [22–25]. The carotenoids BC, LUT, ZEA, LYC, CTX, ASTA incorporated into MLVs or SUVs caused no significant changes of membrane fluidity as measured by the pyrene excimer method, but a change of the pyrene relative micropolarity was indicated from the pyrene monomer emission spectra. The carotenoid incorporation into membranes seems to be governed not only by the carotenoid polarity but also by their ability to change membrane anisotropy [22]. The competition between carotenoids and cholesterol has been investigated with respect to their modulating effect on membrane mechanics. Incorporated carotenoids caused an enhanced membrane anisotropy even at concentrations 80 times lower than cholesterol, indicating that they can act as very sensitive modulators of membrane structure and dynamics [24]. In microsomes as well as in liposomes membrane anisotropy seems to be the limiting barrier for the insertion of carotenoids into membranes [25].

Our present data contribute further aspects on the insertion of carotenoids into unilamellar membrane bilayers and their effects. Carotenoid-incorporated liposomes have been prepared

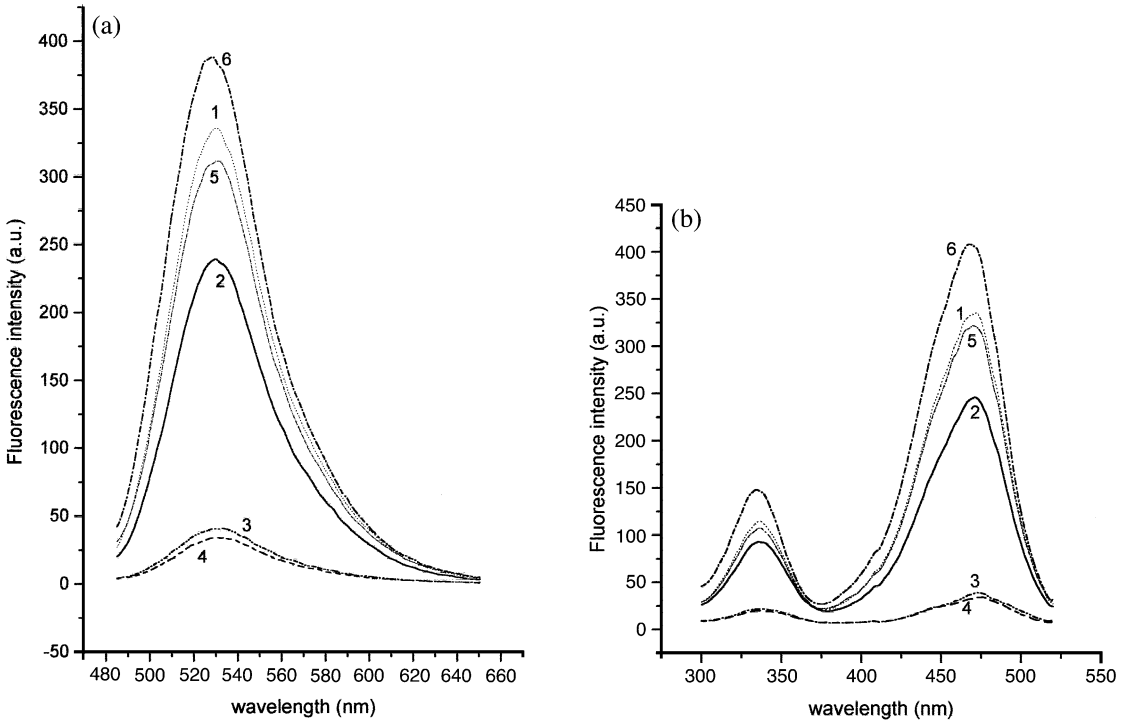

Fig. 4. (a and b). Emission spectra at $\lambda_{exc}=470$ nm (a) and excitation spectra at $\lambda_{obs}=530$ nm (b) of the dye C_6-NBD-PC incorporated into liposomes which have been prepared from a mixture of phospholipids and a carotenoid or cholesterol. 1, Control liposomes; liposomes containing: 2, β-carotene; 3, lutein; 4, zeaxanthin; 5 and 6, low and high cholesterol content, respectively. For details see Section 2.

– either from a mixture of phospholipids containing EYPC, DPPE and DPPS in a molar ratio of 17:5:3 and 2.5 mol% of a carotenoid or cholesterol at 10 or 40 mol%;
– or the pure liposomes have been prepared and then they have been incubated with a carotenoid or cholesterol.

(1). Well-defined and rather homogeneous liposomal vesicles are obtained by extruding them through a 0.1-μm filter. These vesicles show a broad size distribution at approximately 50 nm, without significant modifications linked to carotenoids, and only a slight increase against the control. Apart from the other carotenoids, BC increased the light scattering by a post-extrusion aggregation of liposomes, despite its low incorporation rate.

(2). It is remarkable that both xanthophylls, LUT and ZEA, increase the incorporation yield of phospholipids from 0.6 in control liposomes to 1.0 (Table 2), whereas BC decreases it to 0.4. From this it can be concluded that the xanthophylls are 'phospholipid sucking' carotenoids in contrast to BC. Previously we showed a comparatively high stability of LUT and ZEA in microsomes [25].

No differences in vesicle sizes was observed when carotenoids were inserted into control liposomes. This is not surprising considering the very low integration yields of carotenoids in this preparation mode (Table 3). Nevertheless, also in this case the relative insertion factor of LUT is twice that of BC, and ZEA is in between.

(3). From a membrane biological view it is interesting to look at competitive properties of carotenoids and cholesterol. Previously we found that carotenoids can affect membrane structure and dynamics at lower concentrations than cholesterol does [24]. We hypothesised that carotenoids regu-

late the membrane fluidity in procaryota as does cholesterol in eukaryota (Rohmer cited in [13]). From our present incorporation studies of carotenoids and cholesterol into liposomes (Table 2) we notice that:

- CHOL (in the concentrations applied here) insertion is total (IY = 1) but the phospholipid integration into these liposomes (IY = 0.35–0.5) is lower than into control liposomes (IY = 0.6). Looking at LUT and ZEA instead of CHOL we find just the reverse situation (Table 2). It means that cholesterol distracts phospholipids from the vesicle while LUT and ZEA do not. Contrarily, BC and phospholipids do hardly adapt to each other to form liposomal vesicles. Their incorporation yield as only partners in the formation of liposomes is IY = 0.1 for BC and IY = 0.4 for the phospholipids.
- When carotenoids are inserted by incubation into prepared liposomes, the incorporation is generally considerably lower (Table 3) than in the case where the liposomes have prepared from the original mixture. For BC the incorporation yield is only approximately two times lower, while for LUT and ZEA it is approximately 15 times lower. BC can enter easier into prepared liposomes. A comparison of the two methods to prepare liposomes with incorporated carotenoids, let us conclude that the 'preparation from the mixture' technique disadvantages BC incorporation, while LUT and ZEA give more compact and richer vesicles.

(4). Fluorescence labels are generally adequate tools to investigate membrane properties [17,29,30]. A fluorescent carotenoid (bis-dehydro-β-carotene-2-carboxylic acid), was extracted from a psychrotrophic *Micrococcus roseus* strain and bound to liposomes but its quantum yield was so low, that for the moment no practical tools to study fluorescent carotenoids in membranes are offered [31]. Recently, 0.2–1.0 mol% thermozeaxanthins (novel carotenoid–glucoside esters from the thermophilic bacterium, *Thermus thermophilus*) were studied by measuring the leakage of calcein, used as a fluorescent dye entrapped in large unilamellar EYPC liposomes (LUVs). Thermozeaxanthins stabilised the liposomes in a large temperature range from 30 to 80 °C, as only 2.6% of the entrapped calcein leaked out in contrast to 10% release from the control liposomes [32].

The fluorescent membrane probe Laurdan is located in the glycerol backbone of the lipid layer (Fig. 5). It displays spectral sensitivity to the phospholipid phase state, based on dipolar relaxation phenomena, which originate from its sensitivity to the environmental polarity caused by water penetration into the lipid bilayer [33]. As the relaxation rate of water molecules is comparable to the fluorescence lifetime of Laurdan in the membrane liquid crystalline state, the Laurdan emission peaks are red-shifted while in the gel phase when water rotation is restricted, the emission peaks are observed at shorter wavelengths [30].

Significant differences between carotenoid and cholesterol-incorporated vesicles are obtained from the shapes of the emission and excitation spectra. Liposomes consisting of EYPC, DPPE and DPPS in the molar ratio of 17:5:3 have a higher fluidity than DPPC liposomes. Carotenoid or cholesterol-containing liposomes express higher rigidities than control liposomes. The low concentration of cholesterol (10 mol%) has nearly no effect on the liposomes. The interpretation is consistent with the assumption that carotenoids, like high concentrations of cholesterol, inhibit water penetration into the membrane.

(5). The lipophilic membrane probe dialkylcarbocyanines DiI-C_{18} is anchored in the non-polar region of the membrane by its alkyl moieties. The fluorescence sensitive part (two indoline ring systems) is like a connecting bridge (Fig. 5). The long axis of the fluorophore extends parallel to the membrane surface and the alkyl chains protrude perpendicularly into the lipid interior. The spectral emission peaks (Fig. 3) are shifted towards shorter wavelengths, caused by a more fluid membrane (LC phase). The excitation spectra (not shown) are as sensible to fluidity changes as the emission spectra are.

The carotenoids enhance the DiI-C_{18} fluorescence, suggesting that they bring the fluorophore into a more hydrophobic environment, i.e. a deeper insertion of the non-polar fluorophore. To the contrary, cholesterol quenches DiI-C_{18} fluores-

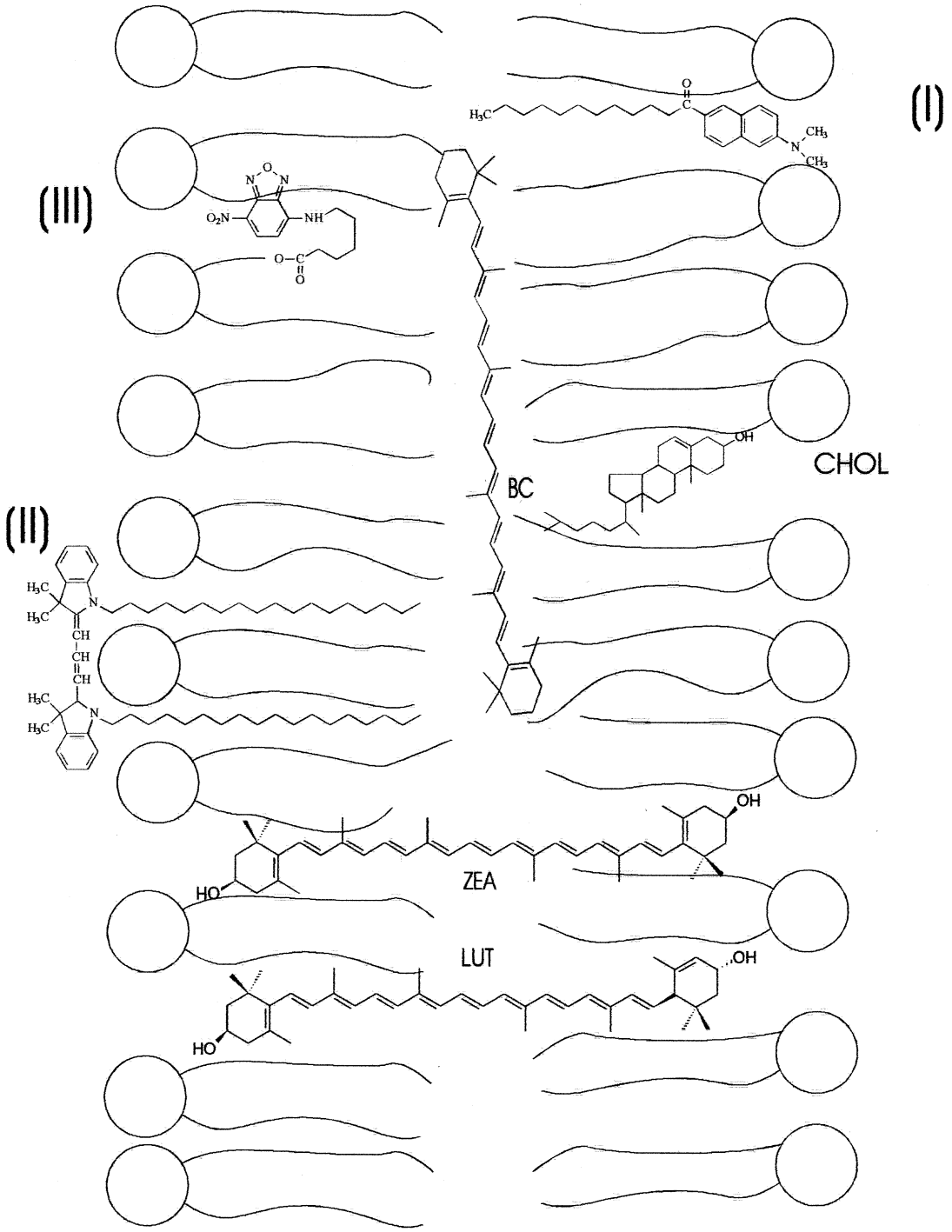

Fig. 5. Specific regions of the membrane bilayer where the molecules we used can insert: Laurdan (I); DiI-C_{18} (II); and C_6-NBD-PC (III). Carotenoid (BC, LUT, ZEA) dimensions and possible locations are drawn in relation to the bilayer dimension.

cence because it creates a more hydrophilic environment.

(6). Fluorescent lipid analogues have been used to visualise membrane effects. In particular the short β-fatty acid chain (C_6) bearing a Nitro-BenzoxaDiazole (NBD), a C_6-NBD, linked to a phospholipid moiety (–PC, or –PE, –PS). It has been the most popular analogue for the investigation of membrane structures by directly visualising the NBD fluorophore in the polar region of the membrane. NBD 'loops back' to the head-group region (Fig. 5) and is useful for the detection of membrane asymmetry changes via internalisation pathways or trans-membrane traffic [34]. At high concentration (more than 0.5 M) NBD is self-quenching in lipid vesicles.

We applied low concentrations of C_6-NBD-PC, useful to investigate the polar region of the membrane. The emission and excitation spectra show modifications of the C_6-NBD-PC fluorescence (Fig. 4). The fluorescence intensity relations appear to be similar to those obtained from the fluorescent label Laurdan. They may follow the same quenching mechanism. In the case of C_6-NBD-PC no spectral shift appears may be reasoned by its low molecular polarisability.

The fluorescence labels provide sensitive and reliable tools to detect effects from carotenoids incorporated into membranes and to discriminate the membranes according to the special carotenoid used. The spectral characteristics of the fluorescent labels applied in this study remain to be evaluated in more detail with respect to the mechanisms how they interact with the membrane-bound carotenoids. These investigations are ongoing.

The main aspect of this study was to compare two different ways of carotenoid incorporation: (i) formation of liposomes from an original mixture of the phospholipid and a carotenoid; and (ii) incubation of purely prepared liposomes with the carotenoid. For comparison in some experiments the carotenoid has been replaced by cholesterol. The preparation method (ii) leads to very low incorporation yields for carotenoids and thus is not favourable for experiments where high incorporation yields are required. However, method (ii) may more resemble natural conditions where carotenoids enter membranes. Under this aspect it is interesting to notice that the insertion yield of β-carotene is lower by a factor of 2, whereas it is approximately 20 for lutein and zeaxanthin. We would like to interpret this as a potential of β-carotene to enter and to cross membranes better than the others. With respect to the human retinal epithelial cells where lutein and zeaxanthin are located in an exceptionally high concentration, it brings us to the hypothesis that this is obtained biosynthetically on the level of membrane formation and not by a later insertion. Just those carotenoids which may enter an existing membrane more easily, like β-carotene and lycopene, decompose rapidly [23] and do not accumulate a considerable stationary concentration.

Three-dimensional light scattering measurements reveal only small vesicle size changes, but the light transmission rates differ considerably. All carotenoid-containing liposomes cause a stronger light scattering than the control liposomes. However, this is less pronounced for those carotenoids (like lutein) which integrate most readily into the membrane and even with a high concentration. Three-dimensional light scattering measurements can be used easily to determine the 'membrane structure adapted' carotenoid integration. Considering liposomes as model membranes we conclude that the efficiency of carotenoid insertions into membranes depends considerably on the special carotenoid and on the way it is inserted.

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