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Exogenously incorporated ketocarotenoids in large unilamellar vesicles. Protective activity against peroxidation

D. Rengel, A. Díez-Navajas, A. Serna-Rico, P. Veiga, A. Muga, J.C.G. Milicua *

Department of Biochemistry and Molecular Biology, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain

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Abstract

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, though astaxanthin showed stronger activity. For these experiments, different amounts of xanthophylls were exogenously added to previously made LUVs, non-incorporated pigment being afterwards removed. Differential scanning calorimetry assays with L-β,γ-dimyristoyl-α-phosphatidylcholine LUVs demonstrated that xanthophylls incorporated as described interact with the lipid matrix becoming interspersed among the phospholipid molecules.

Keywords: Carotenoid; Liposome; Antioxidant; Lipid peroxidation; Lipid-carotenoid interaction

1. Introduction

Carotenoids are probably the most widespread natural pigments. They are also essential in several important biological processes. They can be found in microorganisms regulating, among other functions, membrane fluidity, in plants, in either photosynthetic or non-photosynthetic tissues, and providing the coloration of many plant and animal species, e.g. insects, fish or birds. Apart from these and other well-described functions of carotenoids, other roles or actions have been assigned to these natural pigments in recent years. Many of these newly discovered actions of carotenoids are related to their preventive properties against several diseases affecting humans, e.g. cataracts, cancer and atherosclerosis [1,2], that seem to be associated with their putative antioxidant properties. Experiments evaluating the antioxidant properties of different carotenoids, both in vivo and in vitro, have been reviewed and discussed by several authors [3–6]. However, the experimental evidence provided up to now is less than definitive. Much either depends on the system and on the assay conditions, or is even contradictory.

Since Burton and Ingold studied the antioxidant effect of β-carotene [7], it has been shown that, under certain conditions, carotenoids act as chain-breaking antioxidants in organic solutions [8–12,41]. It has also been found that the antioxidant properties of carotenoids are tightly dependent on the O_2 pressure in the system, the chemical structure of the pigment itself and the presence of other antioxidant molecules [1]. Model lipid peroxidation systems into which carotenoids can be exogenously incorporated have been

* Corresponding author. Fax: +34-94-4648500; E-mail: gbpgomij@lg.ehu.es

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studied. Cell microsomal fractions [13–15] and multilamellar vesicles (MLVs) have been used most often [16–22,42]. Results have been, again, extremely dependent on the system assayed, as derived from the comparison of the antioxidant properties of different molecules incorporated into cell or model membranes.

To date, no conclusive pattern for the antioxidant role of carotenoids has been defined. Many features either remain unclear, e.g. the influence of the chemical structure of the pigment, or are extremely dependent on the experimental conditions.

Liposomes are also a very useful model 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. Carotenoids are believed to act as fluidity regulators in the biological membranes of many organisms. Thus, different authors have used artificial membranes to describe the influence of carotenoids on the bilayer structure and dynamics, as well as the orientation of the pigments in the membrane [23–30]. MLVs were used in those studies, while unilamellar vesicles, either large (LUVs) or small (SUVs), have seldom been examined [31].

In this study we evaluate the antioxidant properties of canthaxanthin and astaxanthin (see Fig. 1), two ketocarotenoids that differ only by two hydroxyl groups in positions 3 and 3′. In order to assay the antioxidant role of both pigments, we incorporated them into LUVs, a very reliable type of liposomes that have not been used so far in this type of experiments. We also describe a fast method to incorporate large amounts of carotenoid into preformed liposomes while maintaining the integrity of the lipid bilayer, followed by an easy and efficient way to eliminate the unavoidable non-incorporated carotenoid from the preparations.

2. Materials and methods

2.1. Chemicals

Carotenoids were a kind gift from Hoffman-La Roche (Basel, Switzerland). Egg yolk phosphatidylcholine (PC) and L-β,γ-dimyristoyl-α-phosphatidylcholine (DMPC) were obtained from Lipid Products (Redhill, UK). HEPES was purchased from Boehringer Mannheim (Mannheim, Germany). 2-Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and $CuCl_2$ were from Merck (Darmstadt, Germany). Cyclohexane was from Lab-Scan (Dublin, Ireland). Panreac (Barcelona, Spain) supplied tetrahydrofuran (THF), EDTA, and the other chemicals.

2.2. Preparation of liposomes

A known amount of PC dissolved in chloroform-methanol was evaporated under a N_2 stream. Solvent traces were removed by evaporation under vacuum for 2 h. The dry lipid film was then resuspended in 100 mM HEPES, pH 7.4 to a final concentration of 15 mM phospholipid. Following the extrusion method [43], LUVs with an approximate diameter of 100 nm were obtained. Vesicle size was measured by quasi-elastic light scattering (QELS) in a Malvern Zeta-Sizer 4 Spectrometer. Phospholipid concentration in the liposome preparations was determined by the phosphorus detection method described by Bötcher [32]. The procedure was identical for DMPC liposomes, used in the differential scanning calorimetry (DSC) measurements. The lipid was maintained above its transition temperature during the extrusion and afterwards until the sample was placed in the calorimeter. The same HEPES buffer was used for sample dilutions in all cases.

2.3. Carotenoid incorporation into liposomes

The carotenoid solutions (4 mM) were prepared in redistilled THF. The solvent was redistilled in order to eliminate the BHT traces added to commercial preparations. The xanthophyll concentration required for each experiment was achieved by addition of consecutive doses of 50 μl of these carotenoid solutions to 1 ml of liposomes (10 mM phospholipid concentration). THF was immediately evaporated under a N_2 stream after each dose whilst the mixture was homogenised by vortexing. Then non-bound carotenoid was removed from the samples by means of two consecutive centrifugations in a Beckman TLA 45 rotor at $33\,000 \times g$ for 15 min, at 4°C. Before the second centrifugation, the supernatant was transferred to a fresh tube. Finally, both vesicle size and inorganic phosphorus were measured as described in

Section 2.2. The amount of liposome-bound carotenoid was also quantified.

2.4. Estimation of carotenoid concentration

Carotenoid concentration in solution was spectrophotometrically measured, after dilution of 5 μl of the xanthophyll sample in 5 ml cyclohexane. Liposome-bound carotenoid was determined by acetone-hexane extraction, following the protocol by Gómez and Milicua [33]. The extinction coefficients ($E_{1\%}$) used were 2100 for astaxanthin at 474 nm and 2250 for canthaxanthin at 470 nm.

2.5. Liposome peroxidation and MDA assay

Peroxidation was carried out at 37°C and it was initiated by Cu^{2+}. For each assay 1 ml of liposomes (2 mM PC) was used. Then 10 μl of 10 mM $CuCl_2$ was added, so that the final concentration of Cu^{2+} in the preparations was 100 μM. A 100 μl aliquot was removed from the mixture every hour. Oxidation in the samples was stopped by addition of 5 μl of 20 mM EDTA, pH 7.5, and 5 μl of 10 mM BHT prepared in water and ethanol, respectively. Malondialdehyde (MDA) equivalents were then determined according to the protocol described by Esterbauer and Cheeseman [34]. Liposome size and carotenoid concentration [33] were measured for each aliquot.

2.6. DSC

LUVs of DMPC were prepared, and carotenoid was incorporated into them as described in Sections 2.2 and 2.3, at a temperature well above the gel→liquid crystal transition temperature (T_m) of the lipid (see below). Liposomes were diluted to a final concentration of 1 mM PC. The lipid and the buffer were placed in the sample and reference holder, respectively, of a Microcal high sensitivity scanning calorimeter MC-2. Two scans were carried out with each sample from 10°C to 45°C at 0.75°C/min, resulting in two identical scans in every case. Data analysis was carried out using the Origin software from Microcal.

3. Results

3.1. Carotenoid incorporation into liposomal membranes

The immediate evaporation of the solvent under a N_2 stream allowed extensive carotenoid incorporation into preformed LUVs. Removal of the non-incorporated carotenoid by means of centrifugation is an easy procedure, causing neither loss nor dilution of the lipid. Liposomes treated in this way maintained their original size during the whole process (see Table 1). Larger amounts of added pigment led to non-homogeneously sized liposome dispersions and higher concentrations of the xanthophylls in THF resulted in poorer yields and easier disruption of the bilayers (data not shown).

Under our conditions, the yield of xanthophyll incorporation into previously prepared egg yolk lecithin LUVs was approximately 50% for astaxanthin and 20% for canthaxanthin, as shown in Table 1. As the incorporation of both pigments was carried out under the same experimental conditions, the varia-

Table 1
Incorporation of canthaxanthin and astaxanthin into previously prepared LUVs (10 mM PC)

mol%$_i$	Canthaxanthin			Astaxanthin		
	mol%$_f$	f/i (×100)	Size (nm)	mol%$_f$	f/i (×100)	Size (nm)
0	–	–	102 ± 1.2	–	–	102 ± 1.2
5	1.01 ± 0.19	20.2	105 ± 2.3	2.45 ± 0.23	49.0	104 ± 2.4
7.5	1.46 ± 0.21	19.5	106 ± 1.8	3.61 ± 0.30	48.1	103 ± 2.7
10	1.90 ± 0.24	19.0	105 ± 2.9	4.70 ± 0.32	47.0	107 ± 2.9
15	2.83 ± 0.24	18.9	108 ± 3.7	6.97 ± 0.27	46.5	106 ± 2.7

The table shows the amount of carotenoid added to the preparations (expressed as mol%$_i$), the carotenoid amount measured after the elimination of the non-incorporated pigment (mol%$_f$) and the ratio between both data (f/i). The diameters, expressed in nm, of the different liposomes are shown as well. Data are averages ± S.D. ($n = 5$).

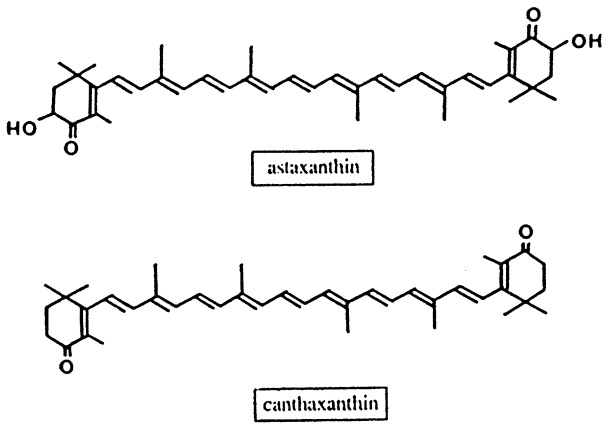

Fig. 1. Chemical structures of astaxanthin and canthaxanthin.

tion in the yield must be due to their structural differences. When the carotenoid dissolved in THF is diluted into the aqueous liposome suspension, it is likely that many aggregation states of the pigment are transiently produced, not all of them leading to membrane incorporation. Astaxanthin is less hydrophobic than canthaxanthin, so that lower states of aggregation would be favoured and the smaller aggregates would be preferentially incorporated.

Our DSC assays reveal that both ketocarotenoids disturb the bilayer matrix, once they have been incorporated into the liposomes (see Table 2 and Fig. 2). This is indicated by a decrease in the enthalpy (ΔH) of the gel→liquid crystal phase transition of DMPC in the presence of the pigment. A similar conclusion may be reached from the drop in cooperativity of the phase transition, expressed as an increase in the transition width ($T_{1/2}$).

2.8 mol% of incorporated astaxanthin and 2.7 mol% of canthaxanthin produce similar changes in the DMPC thermogram. Both xanthophylls perturb the bilayer and interact with the hydrophobic moiety of the membrane.

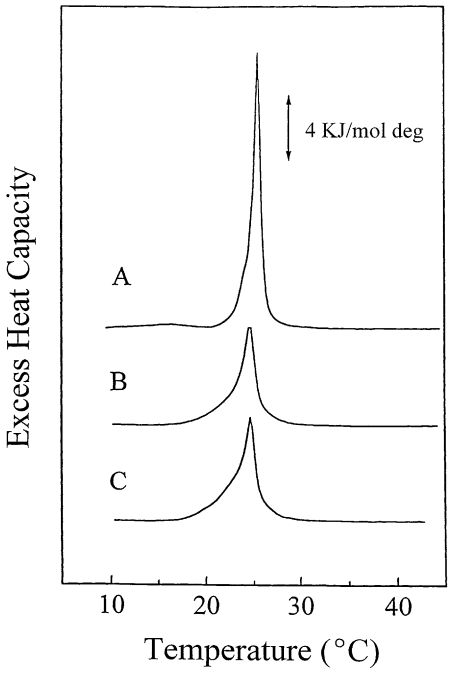

Fig. 2. DSC thermograms obtained from DMPC-made LUVs without carotenoid (A), with 2.83 mol% astaxanthin (B) and 2.73 mol% canthaxanthin (C). DMPC concentration was 1 mM in all cases.

3.2. Phospholipid oxidation in xanthophyll-containing bilayer

In our system, antioxidant activity of both astaxanthin and canthaxanthin can be observed when incorporated into the bilayers. In agreement with most previous data, the preventive role we observed for canthaxanthin and astaxanthin in PC LUVs is defined by the extension of the lag period in lipid oxidation (Fig. 3). As a matter of fact, the time required to reach half-maximal oxidation shows a linear increment with the amount of xanthophyll incorporated (Fig. 4). This increment is higher in the case of liposomes with astaxanthin. However, comparison of the antioxidant properties of canthaxan-

Table 2
Thermodynamic parameters of the gel-to-fluid transition of DMPC LUVs in the absence or presence of carotenoid

	ΔH (kJ/mol)	T_m (°C)	$T_{1/2}$
Control (pure DMPC)	24.7	25.4	0.7
2.83 mol% astaxanthin	18.4	24.7	1.9
2.73 mol% canthaxanthin	20	24.6	1.9

ΔH, transition enthalpy; T_m, midpoint transition temperature; $T_{1/2}$, width at mid-height of the transition.

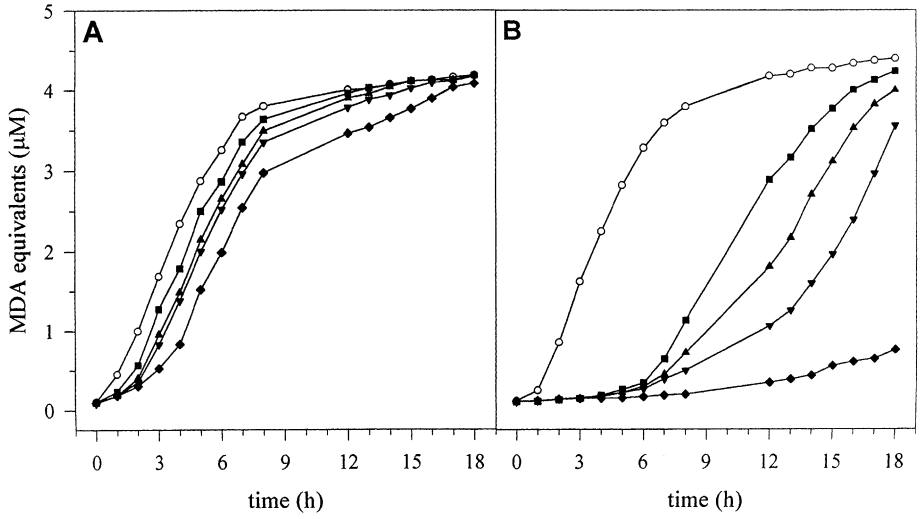

Fig. 3. Oxidation of LUVs of PC with canthaxanthin (A) and astaxanthin (B), initiated by Cu^{2+}. Filled symbols show the results obtained with liposomes with different amounts of carotenoid: 1.01 mol% canthaxanthin and 2.45 mol% astaxanthin (■); 1.46 mol% and 3.61 mol% (▲); 1.9 mol% and 4.7 mol% (▼) and 2.83 mol% and 6.97 mol% (◆). Open circles represent liposomes without carotenoid.

thin and astaxanthin must take into account the differences in the incorporation of both carotenoids. Nevertheless, according to our results, a smaller antioxidant capacity can be assigned to canthaxanthin. On the one hand, the maximum rate in peroxide formation is delayed for 4 h when 2.8 mol% of canthaxanthin is incorporated into the membranes, while 2.5 mol% of astaxanthin is enough to delay the end of the lag period for approximately 6 h (Fig. 3). On the other hand, the increase in the time required for half-maximal peroxidation in liposomes with canthaxanthin is approximately 1.1 h per mol% of carotenoid in all samples, while in liposomes with different amounts of astaxanthin the increase of time is 2.5 h per mol% (Fig. 4).

Liposome size was measured from aliquots while lipid peroxidation was being carried out and no variation was observed in any case at any reaction time (data not shown).

3.3. Carotenoid bleaching

The most salient feature of the oxidative degradation of carotenoids is their bleaching. The different ways in which astaxanthin and canthaxanthin are bleached in our samples are represented in Fig. 5. The amount of canthaxanthin drops drastically in the first 4 h of the reaction (Fig. 5A), when the peroxide formation rate in canthaxanthin-containing liposomes is highest in every case. Up to 70–75% of the pigment is degraded in the first 4 h and only 10% remains in the suspensions after 8 h of oxidation. Moreover, the reactivity of canthaxanthin against Cu^{2+}-initiated oxidation, measured as the percentage of pigment lost during the reaction, is the same in every case, regardless of the amount of carotenoid incorporated into the liposomes. The be-

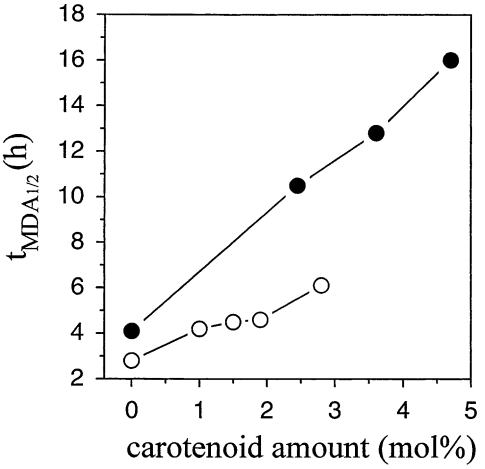

Fig. 4. Time needed to reach half-maximum lipid oxidation in assayed liposome preparations. Figure shows experiments with astaxanthin (●) and canthaxanthin (○).

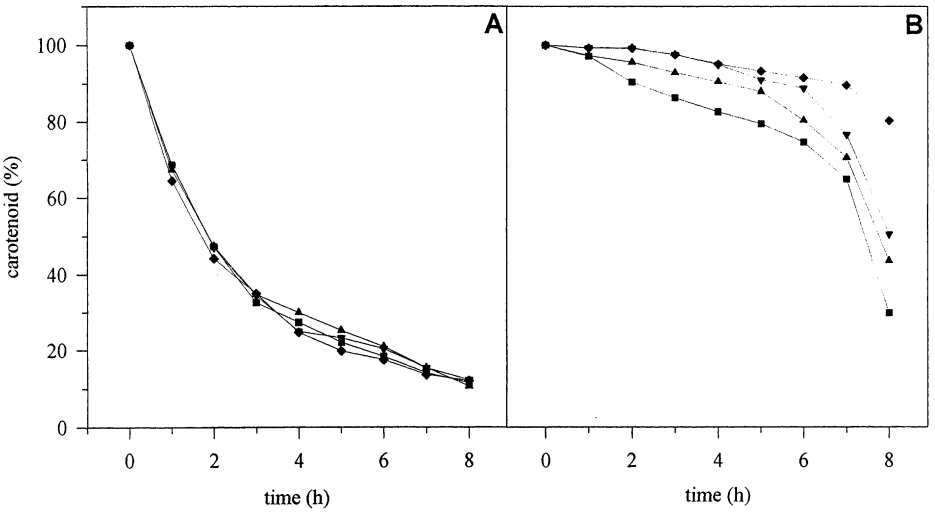

Fig. 5. Colour bleaching during the Cu^{2+}-initiated oxidation of LUVs of PC containing canthaxanthin (A) or astaxanthin (B). Symbols represent different amounts of carotenoid: 1.01 mol% canthaxanthin and 2.45 mol% astaxanthin (■); 1.46 mol% and 3.61 mol% (▲); 1.9 mol% and 4.7 mol% (▼) and 2.83 mol% and 6.97 mol% (◆).

haviour of astaxanthin is different (Fig. 5B). The amount of this pigment in the preparations does not diminish drastically in the first 7 h of the reaction, and only then fast peroxide formation occurs in most cases. Furthermore, at variance with canthaxanthin, the higher the amount of astaxanthin, the slower its degradation in the preparations. The behaviour of astaxanthin fits that described by Esterbauer and co-workers for endogenous antioxidants, including carotenoids in human serum lipoproteins [41], as well as the behaviour described by several authors for these molecules in model membranes or in solution [12,19]. The faster bleaching of canthaxanthin is in agreement with the lower protective action against Cu^{2+}-induced oxidation seen in Fig. 3.

4. Discussion

Biological membranes, mainly microsomes, have often been used to test the antioxidant properties of carotenoids and other molecules [13–15]. Artificial membranes or liposomes are being increasingly used as a model system for these studies, allowing control of lipid composition, making pigment incorporation into the bilayer easier and avoiding interferences in the carotenoid-lipid interaction. Among liposomes, LUVs, the most reliable artificial membranes, have not yet been used to evaluate the antioxidant role of carotenoids. Up to now MLVs have been most commonly used in these studies, being easier to prepare and allowing the simultaneous incorporation of the carotenoid and the phospholipid into the bilayer [16–22,42]. But in many cases the non-incorporated carotenoid was not cleared out from the preparations and the bilayer structure was not experimentally verified either. The combination of analytical and calorimetric data demonstrates that the method described in this work leads to the effective incorporation of up to nearly 3 mol% of canthaxanthin and 7 mol% of astaxanthin into preformed LUVs, without liposome size modification (Tables 1 and 2 and Fig. 2). The difference in the yield of the incorporation of both pigments might be due to the higher hydrophobicity of the canthaxanthin. It has been reported that, in general terms, the more polar a carotenoid is, the more perpendicular its orientation with respect to the plane of the bilayer [23–31]. The hydroxyl groups of astaxanthin might allow the pigment to establish hydrogen bonds with the lipid carbonyl groups, leading to a more perpendicular position immediately after its incorporation. Nevertheless, different orientations have been observed for similar carotenoids depending on the lipid used to prepare the membranes and the incorporation method followed [26,31,38,39].

After their incorporation into DMPC-made LUVs,

both xanthophylls disturb the bilayer and interact with the hydrophobic moiety of the lipid, as revealed by our DSC data (Table 2 and Fig. 2). The decrease in the enthalpy of the phase transition of the lipid and the increase of the transition width support this view. These two phenomena have been previously described for cholesterol [35] and for intrinsic membrane proteins [36,37]. Chatuverdi and Ramakrishna-Kurup [23] found similar effects after the incorporation of lutein into dipalmitoylphosphatidylcholine MLVs. However, our data do not provide direct information on the orientation of the pigments in the bilayer.

The antioxidant role of carotenoids has been a rather controversial issue. Burton and Ingold demonstrated the protective behaviour of the pigment when O_2 pressure was low [7]. However, β-carotene was a rather ineffective antioxidant under high O_2 pressure conditions. Other groups achieved similar results, which revealed the importance of oxygen tension in the antioxidant properties of carotenoids on solution ([1] and references therein). However, Hatta and Frei [40] demonstrated, after their Cu^{2+}-initiated oxidation assays, that consumption of the exogenously incorporated β-carotene and α-tocopherol into lipoproteins was independent of O_2 pressure. Apart from the presence of varying amounts of oxygen in the preparations, a factor that might be involved in the antioxidant capability of the carotenoids in solution is the electronic configuration of the pigment itself. For instance, the importance of the presence of keto groups in positions 4 and 4′ to increase the antioxidant efficiency of the carotenoid has been reported [5,9]. Those keto groups, present in the two xanthophylls that we have assayed, cause substantial changes in the electron density along the polyene chain, especially near the end rings, where the free radicals are most likely to attack. Also according to those studies, the hydroxyl groups in positions 3 and 3′ of astaxanthin do not affect the reactivity of this pigment against free radicals, since those chemical groups do not modify the length of the conjugate diene chain. In the system used by Terao the carotenoids, the hydrophobic oxidative reagent and the target molecules (methyl linoleate) were free in solution [9]. In this environment, the presence of hydroxyl groups in the pigment might not be relevant when the antioxidant abilities of different xanthophylls are compared. However, the higher polarity of hydroxyl group-containing carotenoids might gain relevance when further physical interactions among the different molecules in the system assayed are taking place, as occurs when liposomes in aqueous suspension are used.

From our results, in fact, a higher antioxidant activity can be assigned to astaxanthin when compared to the effect of similar amounts of canthaxanthin (Figs. 3 and 4). Miki observed that astaxanthin and canthaxanthin in solution were better quenchers and scavengers than α-tocopherol [11]. In agreement with our results, he demonstrated that astaxanthin was the carotenoid with the strongest antioxidant activity, suggesting a 'super vitamin E' role for this xanthophyll, though most other authors have failed to find differences in the activity of different ketocarotenoids. As a matter of fact, the bleaching of astaxanthin from our samples followed the pattern described in the literature for different antioxidants in different systems [12,19,41], while canthaxanthin bleaches following a different pattern (Fig. 5). Incorporation into the bilayer is likely to influence the ability of both xanthophylls to act as antioxidants. Apolar carotenes have been reported to perturb the acyl chain packing and to increase bilayer permeability [23,28,29]. It might happen that canthaxanthin played this role in our system, thus reducing its antioxidant properties due to a higher accessibility of the lipids to the external oxidative agent.

In conclusion, we were able to incorporate significant amounts of carotenoids into previously prepared LUVs, without disrupting the bilayers. We achieved a higher yield of incorporation with the hydroxycarotenoid astaxanthin. Our results at comparable carotenoid:lipid ratios suggest a stronger antioxidant activity for astaxanthin as compared to canthaxanthin. These differences could be due to their distinct chemical structure and to differences in the carotenoid-phospholipid interaction that are not reflected in the calorimetric studies. Further biophysical studies are necessary to check the orientation of both carotenoids after their incorporation into the phospholipid bilayer and their interaction with the surrounding lipids.

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