Astaxanthin and Peridinin Inhibit Oxidative Damage in Fe^{2+}-Loaded Liposomes: Scavenging Oxyradicals or Changing Membrane Permeability?

Marcelo P. Barros,*,[1] Ernani Pinto,*,† Pio Colepicolo,† and Marianne Pedersén*

*Department of Botany, Stockholm University, SE-10691 Stockholm, Sweden; and †Departamento de Bioquimica, IQUSP, C.P. 26077, 05599-970, São Paulo, Brazil

Astaxanthin and peridinin, two typical carotenoids of marine microalgae, and lycopene were incorporated in phosphatidylcholine multilamellar liposomes and tested as inhibitors of lipid oxidation. Contrarily to peridinin results, astaxanthin strongly reduced lipid damage when the lipoperoxidation promoters— H$_2$O$_2$, *tert*-butyl hydroperoxide (*t*-ButOOH) or ascorbate—and Fe^{2+}:EDTA were added simultaneously to the liposomes. In order to check if the antioxidant activity of carotenoids was also related to their effect on membrane permeability, the peroxidation processes were initiated by adding the promoters to Fe^{2+}-loaded liposomes (encapsulated in the inner aqueous solution). Despite that the rigidifying effect of carotenoids in membranes was not directly measured here, peridinin probably has decreased membrane permeability to initiators (*t*-ButOOH > ascorbate > H$_2$O$_2$) since its incorporation limited oxidative damage on iron-liposomes. On the other hand, the antioxidant activity of astaxanthin in iron-containing vesicles might be derived from its known rigidifying effect and the inherent scavenging ability. © 2001 Academic Press

Key Words: astaxanthin; peridinin; antioxidant; liposome; lipoperoxidation.

Peridinin is an unusual C$_{37}$ carbon skeleton carotenoid with epoxy, hydroxy, and acetate groups on β-rings, an allene moiety and a lactone group conjugated to the π-electron system (Fig. 1) (1). In addition

---

Abbreviations used: BHT, butylated hydroxytoluene; EDTA, ethylenediaminotetraacetic acid; Iron-PCL, Fe^{2+}:EDTA-loaded egg-yolk phosphatidylcholine liposomes; MDA, malondialdehyde; PCL, egg-yolk phosphatidylcholine liposomes; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; *t*-ButOOH, *tert*-butyl hydroperoxide; Trolox, (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

[1] To whom correspondence should be addressed at Departamento de Bioquímica, IQUSP, Bloco 9 superior, C.P. 26077, 05599-970, São Paulo, Brazil. Fax: +55-11-38182170. E-mail: mpbarros@botan.su.se.

to the membrane-bound light harvesting complex of Photosystem II (PSII), dinoflagellates also contain a water-soluble external antenna complex, the peridinin-chlorophyll-protein (PCP). Peridinins in PCP and in model antenna systems effectively transfers electronic excitation to chlorophyll a (88 to 95%) which is able to pass this excitation energy to membrane-bound light-harvesting complexes on PSII (1–4). Recently, Pinto *et al.* (5) have demonstrated that peridinin is the major singlet molecular oxygen [O$_2$($^1\Delta_g$)] quencher in *Lingulodinium polyedra,* despite being less efficient than β-carotene. However, it has not been clearly shown if dinoflagellates contain peridinin molecules on antenna complexes of the photosystems within thylakoid membranes (6).

The ketocarotenoid astaxanthin (Fig. 1) is a red pigment common to several aquatic organisms including algae, salmon, troute, and shrimp (7–9). Several reports indicate that astaxanthin is one of the most effective antioxidant against lipid peroxidation and oxidative stress in many *in vitro* and *in vivo* systems (10–15). It has also been shown that simultaneous depletion of astaxanthin and α-tocopherol influences autoxidative defense, fatty acid metabolism and synthesis of coenzyme thiamine-pyrophosphate in Baltic Sea salmon affected by the M74 syndrome (16–20).

Another relevant property of carotenoids is how these compounds affect fluidity and permeability of natural and artificial membranes. Carotenoids with keto and hydroxy groups on both ends of the molecule (e.g., zeaxanthin, astaxanthin, and canthaxanthin) strongly decrease water and small molecules permeability across the lipid bilayer (21). Thus, in addition to a direct scavenging ability against reactive oxygen species (ROS), some polar carotenoids also inhibit the penetration of oxidative substances and, consequently, the initiation of a lipid peroxidation process.

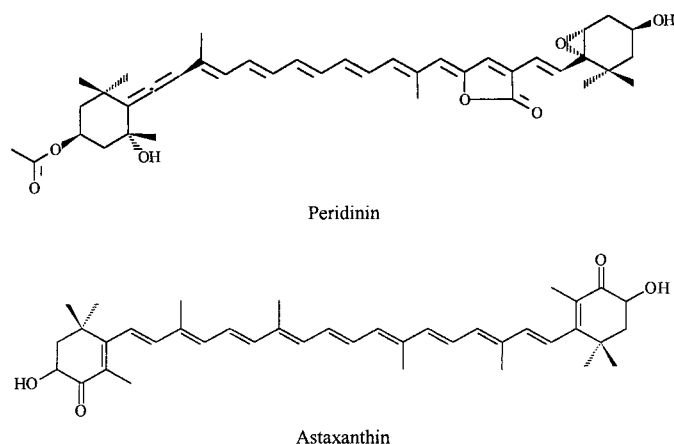The aim of this work is to study the antioxidant activity of astaxanthin and peridinin, two of the most

FIG. 1. Chemical structures of peridinin and astaxanthin.

abundant carotenoids among marine microalgal species. For that purpose, the carotenoids were incorporated into egg-yolk phosphatidylcholine multilamellar liposomes (PCL) and challenged by different ROS which were generated by classical lipoperoxidation initiators. In order to check if the carotenoid antioxidant activity is exclusively or partially derived from its rigidifying effect on membranes, the liposomes were previously loaded with Fe^{2+}:EDTA complexes (Iron-PCL). Thus, to initiate ROS generation in Iron-PCL, the lipoperoxidation agents—H_2O_2, *tert*-butyl hydroperoxide (*t*-ButOOH) and ascorbate—must cross the lipid bilayers and react with the metal ion present inside the vesicles. These experiments were also performed with lycopene and butylated hydroxytoluene (BHT), classical antioxidants, as controls.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma–Aldrich Sweden AB, except $FeSO_4.7H_2O$ and liquid chromatography grade solvents *n*-hexane, chloroform, methanol, and ethanol from Merck Co. (Darmstadt, Germany); ascorbic acid and Perdrogen (H_2O_2 30%) from Riedel-deHaën (Seelze, Germany); and (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Fluka Chemika (Buchs, Switzerland). Peridinin was isolated from *Lingulodinium polyedra* as described by Pinto *et al.* (5). The dialysis membranes were Spectra/Por MWCO 2000 from Spectrum Medical Industries (Los Angeles, CA).

Carotenoid stock solutions. All carotenoids were solubilized in organic solvents previously to their incorporation into egg-yolk phosphatidylcholine liposomes (PCL) and the absorbances of these stock solutions were measured to evaluate their effective concentrations. Peridinin ($\varepsilon^{469} = 85.8 \times 10^3 \ M^{-1} \ cm^{-1}$) was solubilized in chromatography grade methanol while astaxanthin ($\varepsilon^{468} = 125 \times 10^3 \ M^{-1} \ cm^{-1}$) and lycopene ($\varepsilon^{472} = 186 \times 10^3 \ M^{-1} \ cm^{-1}$) were dissolved in purified *n*-hexane (22). The stock solutions were stored at $-80°C$ freezer and protected from light to avoid oxidation.

Preparation of multilamellar liposomes (PCL). In order to prevent aggregate formation and loss of material during the procedure, the carotenoids were isolated from stock solution by flushing the respective organic solvent with a N_2 stream until dryness. After that,

500 μL of chloroform were added to each flask and the egg-yolk phosphatidylcholine solution in $CHCl_3$ was mixed for a final carotenoid:lecithin proportion of 0.5% (25 μM and 5 mM, respectively). Egg yolk phosphatidylcholine was selected for its unsaturated fatty acid content which offers suitable oxidation targets for ROS (23, 24). After brief mixing, chloroform was evaporated by flushing N_2 in a round-bottom flask adapted to a rotavapor apparatus working at a low speed to allow the formation of a homogeneous dried film. The lipid-carotenoid film was stored overnight in the dark under vacuum to eliminate traces of chloroform. The PCL vesicles were prepared by mixing 100 mM phosphate buffer (pH 7.4) to the lipid film followed by strong vortexing for 5 min. The formation of carotenoid aggregates was avoided by preparing the PCL at 40°C, which is high above the transition temperature of 30°C for egg-yolk phosphatidylcholine (25). The suspension was centrifuged at 15,000 rpm for 20 min to eliminate eventual formed aggregates.

Preparation of Fe^{2+}-incorporated multilamellar liposomes (Iron-PCL). The first method tested for Iron-PCL preparation envolved sonication of the lipid-carotenoid film with 100 mM phosphate buffer (pH 7.4) on ice until the dispersion becomes clean (26). However, this classic method of liposome preparation proved to be unefficient for our purposes since it caused a 8.5-fold higher level of lipid oxidation (data not shown). Thus, the Iron-PCL was prepared as PCL: mixing the lipid-carotenoid film with 5 mL of 100 mM phosphate buffer (pH 7.4) plus 5 mM Fe^{2+}:EDTA solution (to a final concentration of 0.1 mM) and strong vortexation. A dialysis procedure was used to eliminate external and loosely bound iron complexes from the liposomes. About 5 mL of uncleaned Iron-PCL were dialysed in Spectra/Por molecularporous membrane (MWCO 2000) at room temperature against 2 L of destilled water for 2 h with smooth agitation by a magnetic stirrer. In the beginning, the liposome suspensions were dialysed against 2 L of 100 mM phosphate buffer (pH 7.4) but this procedure did not efficiently remove the metal ions supposed to be placed outside the liposomes (data not shown). The iron content in the PCL was checked before and after every dialysis process to estimate loss of iron complexes during the procedure.

Induction of lipid peroxidation. Either PCL or Iron-PCL, containing significant concentrations of unsaturated lipids (27), were oxidized by incubation for 45 min at 30°C with 1 mM solution of three different initiators: H_2O_2, *t*-ButOOH or ascorbic acid. To stimulate lipid oxidation in PCL, 0.1 mM Fe^{2+}:EDTA was simultaneously added. Trolox (0.5 mM in 0.1 M phosphate buffer pH 7.4) and 5 μM butylated hydroxytoluene (BHT) were used as controls. Trolox, a water-soluble derivative of α-tocopherol with similar scavenging activity (28), was used as a probe for checking the sites of ROS generation in multilamellar vesicles since it is not supposed to permeate liposome lipid bilayers (Fig. 2).

Measurement of lipoperoxidation extent (TBARS test). After the incubation period, the oxidative reaction was stopped by adding 20 μL of 0.2 M BHT (ethanol solution). To produce the coloured adduct, 350 μL of sample were incubated with 700 μL of 0.375% thiobarbituric acid (TBA) in 0.25 M HCl and 1% Triton X-100 at 100°C for 15 min. After reaching the room temperature, the absorbance of the solutions were measured at 535 nm using malondialdehyde (MDA) as standard (29). Controls for residual absorption of carotenoids at 535 nm were made using 0.25 M HCl plus 1% Triton X-100 solution without TBA.

Iron determination. The iron incorporation in the liposomes was checked before and after the dialysis procedure by a modification of the method described by Bralet *et al.* (30). Aliquotes of 300 μL were taken from the liposome suspensions and 3 μL of Triton X-100 was added to disrupt the vesicles. The samples were added to 50 mM glycine hydrochloride buffer (pH 2.5) with 20 mg/mL ascorbate, 10 mg/mL pepsin and 5 mM 2,2′-bipyridine. After incubation for 2 h at 37°C, the absorbance was measured at 520 nm and results compared to $FeSO_4.7H_2O$ standard curve.
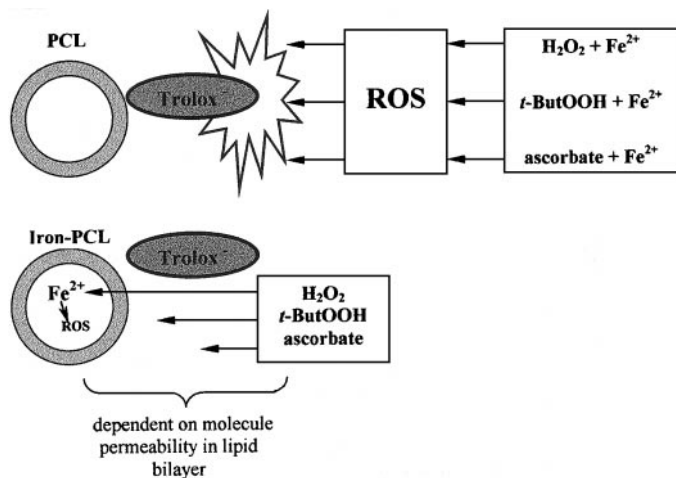
FIG. 2. Trolox scavenging activity against ROS produced by H_2O_2, t-ButOOH and ascorbate in PCL and Iron-PCL.
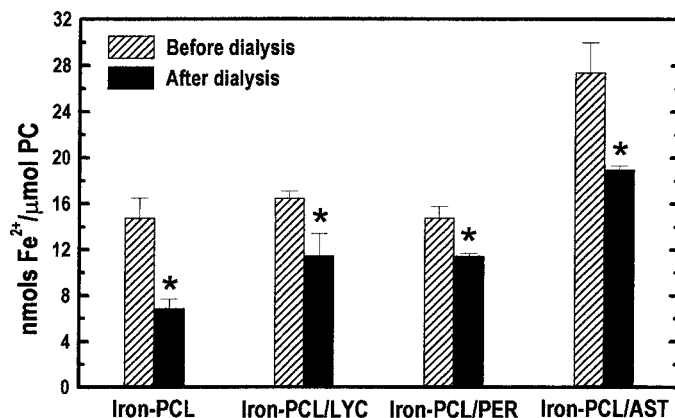

FIG. 3. Iron concentrations in Iron-PCL in the presence or absence of lycopene (Iron-PCL/LYC), astaxanthin (Iron-PCL/AST) or peridinin (Iron-PCL/PER), during a dialysis process (nmols Fe^{2+}/μmol PC). Shown are the means $\pm$ SD of 3 experiments; $*P < 0.05$.

Statistics. Data are presented as means $\pm$ SD (standard deviation) and statistical analysis performed with the Student's t test at significance level of 5%.

RESULTS AND DISCUSSION

Nonloaded Liposomes (PCL)

The TBARS concentration after PCL preparations were (0.169 $\pm$ 0.043 nmol MDA/μmol PC) and (0.209 $\pm$ 0.031 nmol MDA/μmol PC), respectively for PCL and Iron-PCL. As expected, the encapsulation of Fe^{2+}: EDTA complexes in PCL resulted in higher lipid oxidation level (c.a. 25%). The coordination of Fe^{2+} with EDTA does not prevent it to react with ROS and, hypothetically, it would be easier to eliminate (by dialysis) a water-soluble Fe^{2+}:EDTA complex than a membrane-associated Fe^{2+}:phosphatidylcholine chelate (31, 32).

Probably, the osmotic pressure must have led to reorganization of PCL membranes and coalescence of lipid vesicles during the dialysis performed against distilled water (33). Even with distinguished polarity properties, lycopene and astaxanthin induced Fe^{2+}: EDTA elimination from liposomes at the same extent (c.a. 30%). When peridinin was associated, the effect was less intense (23%). On the other hand, a higher loss of iron chelate was measured in carotenoid-free liposomes (53%) (Fig. 3).

Ascorbic acid can behave as a prooxidant since it can reduce Fe^{3+} to Fe^{2+}, a well-known strong promoter of lipoperoxidation (33). However, at millimolar concentrations the ability of ascorbate to scavenge HO$^\bullet$ becomes more significant. Ascorbate is also able to reduce tocopheryl radicals, generated by hydrogen abstraction from α-tocopherol, back to its active antioxidant form. Trolox, with similar scavenging mechanism as α-tocopherol, is supposed to be constantly regenerated

by ascorbate from the Trolox radical form in the aqueous solution (28). Ascorbate is also supposed to be charged at pH 7.4 (ascorbic acid pKa_1 and pKa_2, 4.17 and 11.57, respectively) thus with low permeability throughout membranes.

Butylated hydroxytoluene (BHT) was very efficient in scavenging free radicals generated by all lipoperoxidation agents in PCL even at micromolar range (Fig. 4). This effect was probably due to its higher diffusibility into membranes (34) which would allow this antioxidant to scavenge oxyradicals at several spots through out the lipid bilayer. Trolox, mostly present in the aqueous solution, required millimolar concentrations to inhibit lipid oxidation to the same extention as
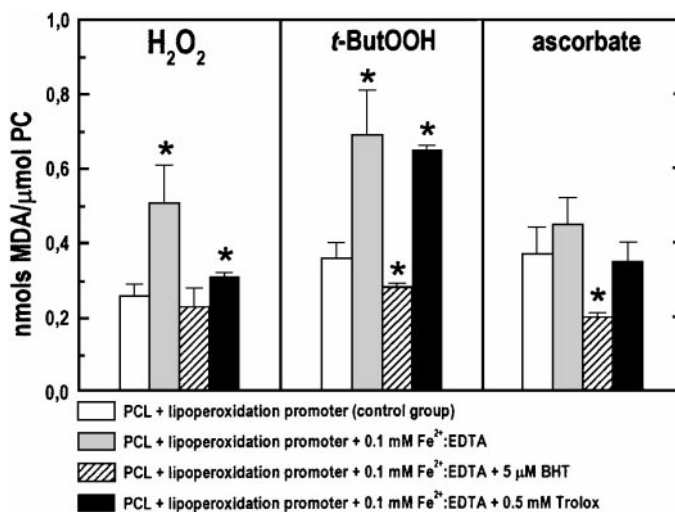

FIG. 4. Effects of BHT and Trolox in lipoperoxidation of PCL in the absence of carotenoids induced by mixing lipoperoxidation promoters—H_2O_2, t-ButOOH, or ascorbate—to chelated Fe^{2+} ions (nmols MDA/μmol PC). Shown are the means $\pm$ SD of 4 experiments; $*P < 0.05$.
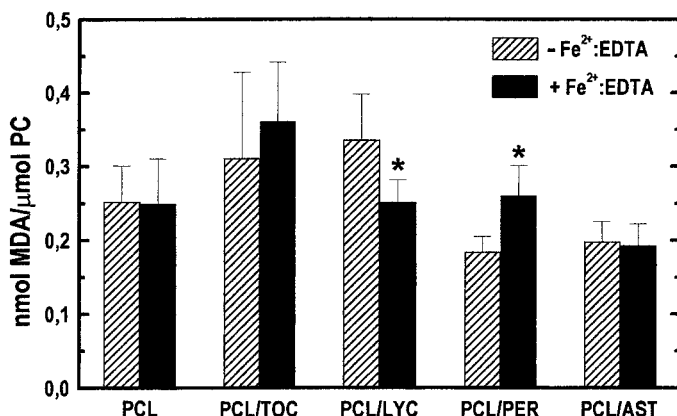
FIG. 5. Levels of TBARS promoted by addition of Fe^{2+}:EDTA in PCL containing α-tocopherol (PCL/TOC), lycopene (PCL/LYC), peridinin (PCL/PER) and astaxanthin (PCL/AST). Shown are the means $\pm$ SD of 4 experiments; $*P < 0.05$.

BHT in H_2O_2/Fe^{2+} system. Higher levels of TBARS were produced by addition of Fe^{2+} and t-ButOOH to PCL: 1.9-fold higher compared to 94% obtained with H_2O_2. When lipoperoxidation process is initiated by t-ButOOH most of the free radicals detected in the lipid bilayer is peroxyl radical (ROO$^{\bullet}$) (35). A significant proportion of alkoxyl radical (RO$^{\bullet}$) and singlet oxygen $[O_2(^1\Delta_g)]$ have also to be considered (23, 26, 35–38). Trolox was not able to efficiently protect the PCL membranes in t-ButOOH-induced peroxidation. Usually, Trolox is more reactive with ROS than BHT, especially concerning peroxyl radicals (39), but the higher permeability of the phenolic compound may have compensated for its lower reactivity.

To evaluate the single effect of iron addition to PCL (with or without carotenoids), the TBARS measurements were also performed in the absence of peroxidation agents. As an extra control, PCL was also prepared containing 25 μM α-tocopherol as described by Palozza & Krinsky (40). As could be observed in Fig. 5, astaxanthin and peridinin were able to inhibit lipoperoxidation before the addition of iron complexes. The addition Fe^{2+}:EDTA to PCL, in the absence of carotenoids, did not change TBARS production although an increase of 25% in MDA content was previously observed after Iron-PCL preparation. The lipid oxidation in astaxanthin- (PCL/AST) and peridinin-incorporated liposomes (PCL/PER) were both, approximately, 25% lower than in PCL although only PCL/AST was insensitive to iron addition. Lycopene was the only carotenoid which reduced (25%) the level of lipoperoxidation after Fe^{2+}:EDTA addition (Fig. 5).

The carotenoid-incorporated liposomes were challenged by ROS produced outside, when initiator and iron complexes were added simultaneously. These results are presented in Fig. 6. The simultaneous addition of ferrous salt and ascorbate resulted in a more moderate increase of TBARS concentration (21%) than

those observed for H_2O_2 and t-ButOOH systems suggesting the previously described dual effect of ascorbate concerning its action against free radicals. It is noteworthy that, usually, iron ions are contaminating ascorbic acid by 0.02% which would allow the initiation of lipid oxidation even without adding Fe^{2+} solution (31).

Astaxanthin proved to be the best antioxidant in all experiments performed with both peroxidation initiator and iron chelate placed outside the PCL, as expected from other authors (10, 17). The ketocarotenoid was the only tested compound to avoid extreme high levels of lipid damage caused by concomitant addition of ferrous ions and H_2O_2, t-ButOOH or ascorbate: respectively, 45, 45, and 33% lower lipid oxidation than PCL added with iron (II). As observed with the experiments without peroxidation agents (Fig. 5), astaxanthin also induced the lowest enhancement of MDA production upon iron ions addition.

No antioxidant activity was found for peridinin when incorporated into PCL and challenged by free radicals produced outside. Actually, peridinin led to intense augmentation of oxidized lipid levels after ferrous ions were added to H_2O_2- and t-ButOOH-treated liposomes: 2.9-fold and 3.5-fold higher, respectively. The TBARS level obtained after incubation of PCL/PER with ascorbate 1 mM in the absence of Fe^{2+} (0.65 $\pm$ 0.14 nmol MDA/μmol PC) was one of the highest of all experiments performed.

Lycopene, under the reaction conditions described here, could not inhibit the lipoperoxidation process in PCL. The effect of chelated iron (II) inclusion to ascorbate-treated PCL/LYC was lower than with other lipid peroxidation agents despite being the highest value measured (0.73 $\pm$ 0.03 nmol MDA/μmol PC). Apolar carotenoids, e.g., β-carotene and lycopene, have
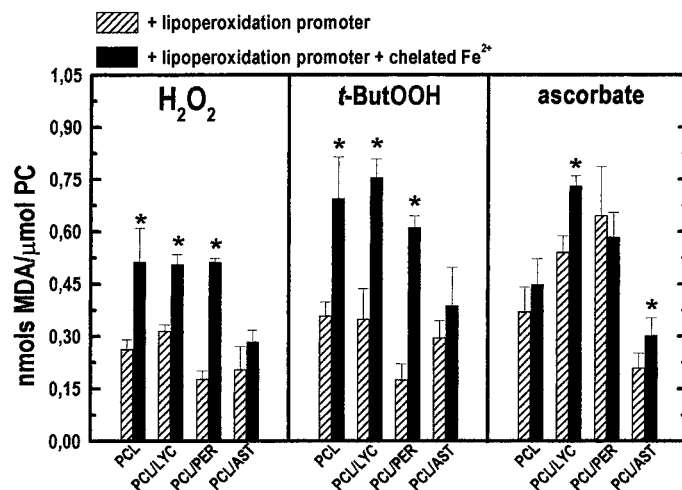

FIG. 6. TBARS levels induced by H_2O_2, t-ButOOH or ascorbate and Fe^{2+}:EDTA in carotenoid-associated PCL. Shown are the means $\pm$ SD of 4 experiments; $*P < 0.05$.
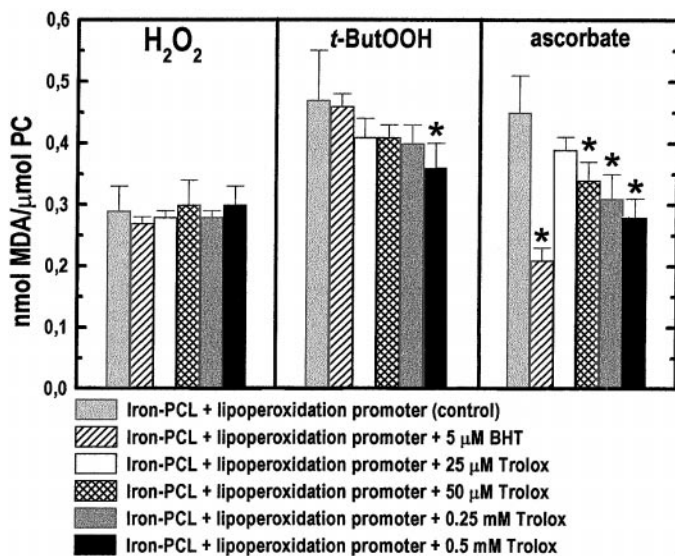
FIG. 7. Effects of 5 μM BHT and 25 μM, 50 μM, 0.25 mM and 0.5 mM Trolox in lipoperoxidation of Iron-PCL (in the absence of carotenoids) induced by adding 1 mM ROS promoters—H_2O_2, t-ButOOH, and ascorbate (nmols MDA/μmol PC). Shown are the means $\pm$ SD of 4 experiments; $*P < 0.05$.

been reported to perturb the acyl chain packing and to increase bilayer permeability (41, 42). In some circumstances, efficient *in vivo* antioxidants like β-carotene and lycopene could also act, or partially offer, a prooxidative effect in lipid peroxidation process masking its antioxidant activity.

Iron-Loaded Liposomes (Iron-PCL)

After the dialysis, an insignificant concentration of Fe^{2+}:EDTA was present outside the PCL. As it is shown in Fig. 7, no significant variation was observed in H_2O_2-generating system when 25 μM, 50 μM, 0.25 mM, or 0.5 mM Trolox were added. This aspect suggests that these oxyradicals were generated in the internal aqueous solution, triggered by the permeation of the easily diffusible molecule, H_2O_2. When both iron (II) and H_2O_2 were added to the external aqueous solution, 0.5 mM Trolox and 5 μM BHT inhibited lipoperoxidation by 40 and 55%, respectively (Fig. 4).

A constant (13%), but not significant, inhibition of MDA production in t-ButOOH-treated Iron-PCL was caused by Trolox in the concentration range from 25 μM to 0.25 mM (Fig. 7). However, 0.5 mM Trolox significantly suppressed lipid peroxidation: 23.6%. Even also being a small and uncharged molecule, t-ButOOH was expected to permeate membranes in a less extention than H_2O_2. Paradoxically, higher lipid oxidation products were measured after incubation of Iron-PCL with t-ButOOH than with H_2O_2. BHT was not able to prevent lipid oxidation in this system although, when peroxyl and alkoxyl were generated out-

side the liposomes (Fig. 4) a 55% lowed MDA content was obtained.

Ascorbate addition to Iron-PCL also resulted in a higher lipid oxidation despite being negatively charged at pH 7.4 and not assumed to penetrate intensely the lipid bilayers. A possible explanation is the 0.02% usual iron contamination of commercial ascorbic acid (31). The effect of Trolox on lipoperoxidation is another indication that the oxidation process was initiated at the outer moiety. In fact, the addition of increasing concentrations of Trolox led to gradual higher protection of the membranes against oxidative damage. Another indication of external action of free radicals is the 53% inhibition of lipoperoxidation in Iron-PCL induced by 5 μM BHT.

As shown in Fig. 8, lipoperoxidation in Iron-PCL was intensely stimulated by the addition of peroxidation promoters—H_2O_2, t-ButOOH and ascorbate, respectively—2.4-, 4-, and 3.8-fold higher than MDA concentrations obtained without promoters (dotted line; 0.12 ± 0.02 nmol MDA/μmol PC). The MDA concentrations found when H_2O_2 and t-ButOOH were added to Iron-PCL were significantly lower than those measured when iron ions and promoter were added simultaneously to the vesicles (Fig. 4). When ascorbate/Fe^{2+}: EDTA was used as lipoperoxidation initiator system, an equivalent MDA concentration was obtained for both types of vesicles: (0.45 ± 0.06) and (0.45 ± 0.07) nmol MDA/μmol PC for, respectively, Iron-PCL and PCL.

Astaxanthin was the more efficient antioxidant since it suppressed the H_2O_2-induced lipoperoxidation in Iron-PCL by 26% (Fig. 8). Peridinin showed a more modest inhibition of lipid oxidation process (17.7%), suggesting that, due to its incorporation into lipid bilayer, it could have limited the permeation of the peroxidation agent, H_2O_2 in these experiments. On
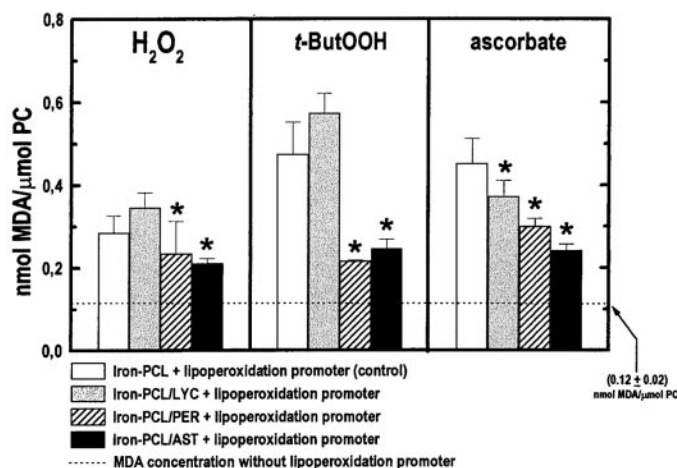

FIG. 8. TBARS levels induced by addition of H_2O_2, t-ButOOH or ascorbate to Iron-PCL in the presence of lycopene (Iron-PCL/LYC), peridinin (Iron-PCL/PER) or astaxanthin (Iron-PCL/AST). Shown are the means $\pm$ SD of 4 experiments; $*P < 0.05$.

the other hand, lycopene showed evidences that it has enhanced membrane permeability to H_2O_2 and t-ButOOH, since increases of c.a. 21% in MDA concentrations (not significant) were observed in both systems. When 1 mM ascorbate was added to Iron-PCL, peridinin significantly limited lipoperoxidation which was comparable to the values obtained with astaxanthin: respectively, 33.5% and 46.4%. Lycopene was only able to protect liposome membranes when ascorbate was used as a promoter of lipid oxidation (17.4% lower than control).

CONCLUSIONS

Carotenoids, especially astaxanthin and zeaxanthin, show high rate constants for reactions with peroxyl radicals (ROO˙) and as a $[O_2(^1\Delta_g)]$ quencher (24, 37). Shimidzu *et al.* (43) developed *in vitro* assays to study the quenching efficiency of several carotenoids from marine organisms against $[O_2(^1\Delta_g)]$ and has evidenced astaxanthin as one of the most efficient. Recently, Pinto *et al.* (5) have demonstrated that peridinin, despite being less efficient than β-carotene, is the major $[O_2(^1\Delta_g)]$ quencher in *Linulodinium polyedra,* mainly due its elevated concentration in this organism. The antioxidant effect of carotenoids is probably also derived from its rigidifying effect on membranes which could lead to a limitation in metal ions or oxidative compound penetration into lipid bilayers (44, 45). On the other hand, hydrophobic carotenoids (e.g., lycopene and β-carotene) make the membranes more fluid and, under some circumstances, more fluid and, under some circumstances, more susceptible to oxidative damage (41). The data reported here suggest that lycopene, under the described reaction conditions, was not able to protect membrane lipids against iron-induced oxidation process. This fact has also been recently pointed out as a possible explanation for the ambiguous action of β-carotene challenged by oxyradicals in different lipid systems (42).

Astaxanthin, as previously demonstrated *in vitro* and *in vivo* (10, 17, 46–50), was able to strongly inhibit the propagation step of lipoperoxidation in all tested systems. It is possible that two combined properties of astaxanthin were responsible for this fact: (i) the rigidifying effect on membrane, which could have limited the penetration of lipoperoxidation promoters—H_2O_2, t-ButOOH and ascorbate—into the liposome membranes (21, 51, 52); and (ii) the inherent antioxidant activity of this ketocarotenoid (53). However, the same explanation is not valid for antioxidant action of peridinin in Iron-PCL assays. Peridinin did not show any antioxidant property when the ROS were produced outside the liposomes. However, when the peridinin-associated vesicles were pre-loaded with Fe^{2+}:EDTA complexes, a significant inhibition of lipoperoxidation was observed (in all tested systems).

Despite that no report about peridinin orientation on lipid bilayers was available in the literature, it is tempting to suggest, by a preliminary analysis of its chemical structure, that this carotenoid might have a vertical or more angular orientation in egg-yolk lecithin liposomes. Thus, it is possible, despite being speculative, that peridinin also shows polar-carotenoid rigidifying effect on membranes and, consequently, its detected inhibitory action on lipid oxidation might be related to a peridinin-induced decrease in the permeation of lipoperoxidation promoters in membranes.

ACKNOWLEDGMENTS

REFERENCES

1. Bautista, J. A., Connors, R. E., Raju, B. B., Hiller, R. G., Sharples, F. P., Gosztola, D., Wasielewski, M. R., and Frank, H. A. (1999) Excited state properties of peridinin: Observation of a solvent dependence of the lowest excited singlet state lifetime and spectral behavior unique among carotenoids. *J. Phys. Chem. B* **103,** 8751–8758.

2. Frank, H. A. (2001) Spectroscopic studies of the low-lying singlet excited electronic states and photochemical properties of carotenoids. *Arch. Biochem. Biophys.* **385,** 53–60.

3. Le Tutour, B., Benslimane, F., Gouleau, M. P., Gouygou, J. P., Saadan, B., and Quemeneur, F. (1998). Antioxidant and prooxidant activities of the brown algae, *Laminaria digitata, Himanthalia elongata, Fucus vesiculosus, Fucus serratus* and *Ascophyllum nodosum. J. Appl. Phycol.* **10,** 121–129.

4. Osuka, A., Kume, T., Haggquist, G. W., Javorfi, T., Lima, J. C., Melo, E., and Naqvi, K. R. (1999). Photophysical characteristics of two model antenna systems: A fucoxanthin-pyropheoporbide dyad and its peridinin analogue. *Chem. Phys. Lett.* **313,** 499–504.

5. Pinto, E., Catalani, L. H., Lopes, N. P., DiMascio, P., and Colepicolo, P. (2000) Peridinin as the major biological carotenoid quencher of singlet oxygen in marine algae *Gonyaulax polyedra. Biochem. Biophys. Res. Commun.* **268,** 496–500.

6. Tracewell, C. A., Vrettos, J. S., Bautista, J. A., Frank, H. A., and Brudvig, G. W. (2001) Carotenoid photooxidation in photosystem II. *Arch. Biochem. Biophys.* **385,** 61–69.

7. Johnson, E. A., and Schroeder, W. A. (1996) Biotechnology of astaxanthin production in *Phaffia rhodozyma. In* Biotechnology for Improved Foods and Flavors (Takeda, G. R., Teranishi, R., and Williams, P. J., Eds.), pp. 39–50, American Chemical Society, Washington.

8. Henmi, H., Hata, M., and Takeuchi, M. (1991). Studies on the carotenoids in the muscle of salmon. Combination of astaxanthin and canthaxanthin with bovine serum-albumin and egg-albumin. *Comp. Biochem. Physiol.* **99B,** 609–612.

9. Boussiba, S. (2000). Carotenogenesis in the green alga *Haematococcus pluvialis:* Cellular physiology and stress response. *Physiol. Plantarum* **108,** 111–117.

10. Woodall, A. A., Britton, G., and Jackson, M. J. (1997). Carote-

noids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta* **1336,** 575–586.

11. Lawlor, S. M., and O'Brien, N. M. (1995). Astaxanthin—Antioxidant effects in chicken-embryo fibroblasts. *Nutr. Res.* **15,** 1695–1704.

12. Schroeder, W. A., and Johnson, E. A. (1995). Singlet oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia-Rhodozyma. J. Biol. Chem.* **270,** 18374–18379.

13. Kobayashi, M., Kakizono, T., Nishio, N., Nagai, S., Kurimura, Y., and Tsuji, Y. (1997). Antioxidant role of astaxanthin in the green alga *Haematococcus pluvialis. Appl. Microbiol. Biotech.* **48,** 351–356.

14. Kurashige, M., Okimasu, E., Inoue, M., and Utsumi, K. (1990). Inhibition of oxidative injury of biological-membranes by astaxanthin. *Physiol. Chem. Phys. Med. NMR* **22,** 27–38.

15. Young, A. J., and Lowe, G. M. (2001) Antioxidant and prooxidant properties of carotenoids. *Arch. Biochem. Biophys.* **385,** 20–27.

16. Lundström, J., Carney, B., Amcoff, P., Pettersson, A., Börjeson, H., Förlin, L., and Norrgren, L. (1999). Antioxidative systems, detoxifying enzymes and thiamine levels in Baltic salmon (*Salmo salar*) that develop M74. *AMBIO* **28,** 24–29.

17. Bell, J. G., McEvoy, J., Tocher, D. R., and Sargent, J. R. (2000) Depletion of alpha-tocopherol and astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defense and fatty acid metabolism. *J. Nutr.* **130,** 1800–1808.

18. Christiansen, R., Glette, J., Lio, O., Torrissen, O. J., and Waagbo, R. (1995). Antioxidant status and immunity in Atlantic salmon, *Salmo salar L.,* fed semi-purified diets with and without astaxanthin supplementation. *J. Fish Dis.* **18,** 317–328.

19. Amcoff, P., Börjeson, H., Landergren, P., Vallin, L., and Norrgren, L. (1999). Thiamine (vitamin B-1) concentrations in salmon (*Salmo salar*), brown trout (*Salmo trutta*) and cod (*Gadus morhua*) from the Baltic sea. *AMBIO* **28,** 48–54.

20. Pettersson, A., and Lignell, Å. (1999). Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. *AMBIO* **28,** 43–47.

21. Wisniewska, A., and Subczynski, W. K. (1998). Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers. *Biochim. Biophys. Acta* **1368,** 235–246.

22. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) Data for Biochemical Research, 3rd ed., Clarendon Press, Oxford.

23. Burton, G. W., and Ingold, K. U. (1984). Beta-carotene—An unusual type of lipid antioxidant. *Science* **224,** 569–573.

24. Truscott, T. G. (1990). The photophysics and photochemistry of the carotenoids. *J. Photochem. Photobiol. B* **6,** 359–371.

25. Saarinen-Savolainen, P., Järvinen, T., Taipale, H., and Urtti, A. (1997) Method for evaluating drug release from liposomes in sink conditions. *Int. J. Pharmac.* **159,** 27–33.

26. Ohyashiki, T., and Nunomura, M. (2000). A marked stimulation of Fe^{3+}-dependent lipid peroxidation in phospholipid liposomes under acidic conditions. *Biochim. Biophys. Acta* **1484,** 241–250.

27. Bondia-Martinez, E., Lopez-Sabater, M. C., Castellote-Bargallo, A. I., Rodriguez-Palmero, M., Gonzalez-Corbella, M. J., Rivero-Urgell, M., Campoy-Folgoso, C., and Bayes-Garcia, R. (1998). Fatty acid composition of plasma and erythrocytes in term infants fed human milk and formulae with and without docosahexaenoic and arachidonic acids from egg yolk lecithin. *Early Hum. Dev.* **53,** S109–S119.

28. Guo, Q., and Packer, L. (2000). Ascorbate-dependent recycling of the vitamin E homologue trolox by dihydrolipoate and glutathione in murine skin homogenates. *Free Radic. Biol. Med.* **29,** 368–374.

29. Fraga, C. G., Leibovitz, B. E., and Tappel, A. L. (1988). Lipid-peroxidation measured as thiobarbituric acid-reactive substances in tissues-slices-characterization and comparison with homogenates and microsomes. *Free Rad. Biol. Med.* **4,** 155–161.

30. Bralet, J., Schreiber, L., and Bouvier, C. (1992). Effect of acidosis and anoxia on iron delocalization from brain homogenates. *Biochem. Pharmacol.* **43,** 979–983.

31. Gutteridge, J. M. C. (1987). Ferrous-salt-promoted damage to deoxyribose and benzoate—The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. *Biochem. J.* **243,** 709–713.

32. Fridovich, I. (1998). Oxygen toxicity: A radical explanation. *J. Exp. Biol.* **201,** 1203–1209.

33. Minotti, G., and Aust, S. D. (1992). Redox cycling of iron and lipid-peroxidation. *Lipids* **27,** 219–226.

34. Lambert, C. R., Black, H. S., and Truscott, T. G. (1996) Reactivity of butylated hydroxytoluene. *Free Radic. Biol. Med.* **21,** 395–400.

35. Tang, L. X., Zhang, Y., Qian, Z. M., and Shen, X. (2000). The mechanism of Fe^{2+}-initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical. *Biochem. J.* **352,** 27–36.

36. Junghans, A., Sies, H., and Stahl, W. (2000). Carotenoid-containing unilamellar liposomes loaded with glutathione: a model to study hydrophobic-hydrophilic antioxidant interaction. *Free Rad. Res.* **33,** 801–808.

37. DiMascio, P., Kaiser, S., and Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.* **274,** 532–538.

38. DiMascio, P., Catalani, L. H., and Bechara, E. J. H. (1992). Are dioxetanes chemiluminescent intermediates in lipoperoxidation? *Free Radic. Biol. Med.* **12,** 471–478.

39. Kaneko, T., Kaji, K., and Matsuo, M. (1994). Protection of linoleic-acid hydroperoxide-induced cytotoxicity by phenolic antioxidants. *Free Radic. Biol. Med.* **16,** 405–409.

40. Palozza, P., and Krinsky, N. (1992). Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. *Arch. Biochem. Biophys.* **297,** 291–295.

41. Castelli, F., Caruso, S., and Giuffrida, N. (1999). Different effects of two structurally similar carotenoids, lutein and beta-carotene, on the thermotropic behaviour of phosphatidylcholine liposomes. Calorimetric evidence of their hindered transport through biomembranes. *Thermochim. Acta* **327,** 125–131.

42. Britton, G. (1995). Structure and properties of carotenoids in relation to function. *FASEB J.* **9,** 1551–1558.

43. Shimidzu, N., Goto, M., and Miki, W. (1996). Carotenoids as singlet oxygen quenchers in marine organisms. *Fisheries Sci.* **62,** 134–137.

44. Subczynski, W. K., Lomnicka, M., and Hyde, J. S. (1996) Permeability of nitric oxide through lipid bilayer membranes. *Free Rad. Res.* **24,** 343–349.

45. Subczynski, W. K., Wisniewska, A., Yin, J.-J., Hyde, J. S., and Kusumi, A. (1994). Hydrophobic barriers of lipid bilayer-membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. *Biochemistry* **33,** 7670–7681.

46. Jyonouchi, H., Sun, S., Tomita, Y., and Gross, M. D. (1995). Astaxanthin, a carotenoid without vitamin-A activity, augments antibody-responses in cultures including T-helper cell clones and suboptimal doses of antigen. *J. Nutr.* **125,** 2483–2492.

47. Bennedsen, M., Wang, X., Willen, R., Wadstrom, T., and Andersen, L. P. (1999). Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. *Immunol. Lett.* **70,** 185–189.

48. Nishino, H., Tokuda, H., Satomi, Y., Masuda, M., Bu, P., Ono-

zuka, M., Yamaguchi, S., Okuda, Y., Takayasu, J., Tsuruta, J., Okuda, M., Ichiishi, E., Murakoshi, M., Kato, T., Misawa, N., Narisawa, T., Takasuka, N., and Yano, M. (1999). Cancer prevention by carotenoids. *Pure Appl. Chem.* **71,** 2273–2278.

49. Stahl, W., Junghans, A., de Boer, B., Driomina, E. S., Briviba, K., and Sies, H. (1998). Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. *FEBS Lett.* **427,** 305–308.

50. Rengel, D., Díez-Navajas, A., Serna-Rico, S., Veiga, P., Muga, A., and Milicua, J. C. G. (2000). Exogenously incorporated ketocarotenoids in large unilamellar vesicles. Protective activity against peroxidation. *Biochim. Biophys. Acta* **1463,** 179–187.

51. Subczynski, W. K., Markowska, E., Gruszecki, W. I., and Sielewiesiuk, J. (1992). Effects of polar carotenoids on dimyristoylphosphatidylcholine membranes—a spin-label study. *Biochim. Biophys. Acta* **1105,** 97–108.

52. Socaciu, C., Lausch, C., and Diehl, H. A. (1999) Carotenoids in DPPC vesicles: Membrane dynamics. *Spectrochim. Acta Part A* **55,** 2289–2297.

53. Goto, S., Kogure, K., Abe, K., Kimata, Y., Kitahama, K., Yamashita, E., and Terada, H. (2001) Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim. Biophys. Acta* **1512,** 251–258.