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Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability

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Abstract

The ability of carotenoids to protect egg-yolk phosphatidylcholine (EYPC) lipids against oxidation by peroxyl radicals generated from azo-initiators was studied. In homogeneous organic solution, all the carotenoids tested ameliorated lipid peroxidation by AMVN, but none was as effective as α-tocopherol. β-Ring carotenoids showed a correlation between protective effect and rate of carotenoid destruction. β,β-Carotene and zeaxanthin, which react with peroxyl radicals at similar rates, gave a similar degree of protection in organic solution. The reactivity and protective ability of the 4,4′-diketocarotenoids, astaxanthin and canthaxanthin was less. Carotenoids incorporated into ordered membrane systems (EYPC liposomes) displayed different protective efficacies. Zeaxanthin and β-cryptoxanthin were more effective than β,β-carotene against oxidation initiated in the aqueous and lipid phases. Astaxanthin and canthaxanthin afforded less protection to the liposomal lipids. Lycopene was destroyed most rapidly but was least effective as an antioxidant. Located in the hydrophobic inner core of the bilayer, the hydrocarbons lycopene and β,β-carotene would not be in a position to readily intercept free-radicals entering the membrane from the aqueous phase. Carotenoids with polar end groups span the bilayer with their end groups located near the hydrophobic–hydrophillic interface where free-radical attack from AAPH first occurs. Hydrogen abstraction from C-4 may be one of the mechanisms of carotenoid antioxidant activity in this system. The chemical reactivity of a carotenoid is not the only factor that determines its ability to protect membranes against oxidation. The position and orientation of the carotenoid in the bilayer is also of importance. © 1997 Elsevier Science B.V.

Keywords: Carotenoid; Antioxidant; Free radical; Lipid peroxidation; Liposome

1. Introduction

Evidence suggests that degenerative disorders such as cancer, cardiovascular disease, cataractogenesis and macular degeneration are mediated via oxidative damage to tissues [1]. Epidemiological surveys suggest individuals with low intakes of antioxidants have higher risks of developing these diseases [2–4]. The

Abbreviations: AAPH: 2,2′-azobis(2-amidinopropane hydrochloride); AMVN: 2,2′-azobis(2,4′-dimethylvaleronitrile); EYPC: Egg yolk phosphatidylcholine; LDL: Low density lipoprotein; PCOOH: Phospholipid hydroperoxide

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antioxidant properties of vitamin E (tocopherol) and vitamin C (ascorbate) are well known, but other dietary components such as the carotenoids are under investigation for similar actions. Several reports suggest that increased intake and serum levels of carotenoids are correlated with reduced risk of some cancers, cardiovascular and ocular diseases [5–9]. Such a protective role is usually attributed to a proposed action of the carotenoids as antioxidants, but other mechanisms, such as up-regulation of gap-junction protein expression have been described [10–13]. Many studies have shown that β,β-carotene exhibits protective effects against lipid peroxidation mediated by free radicals or singlet oxygen, in organic solution, liposomes, liver microsomes and cells in culture [14–36]. β,β-Carotene is not the only carotenoid present in human food. other carotenoids are obtained from the diet, notably lycopene, lutein and zeaxanthin, in quantities at least as great as β,β-carotene [37]. These carotenoids are normally present in plasma and their concentrations may exceed that of β,β-carotene. These other carotenoids may also be possible protective antioxidants [38].

To have antioxidant properties a carotenoid must be able to prevent or compete with the oxidation reactions that cause damage to lipids and other cellular components. It is well known that carotenoids react rapidly with oxidizing agents and free radicals. Reaction courses have been inferred from the identification of carotenoid products detected in small quantities at the end of an oxidation reaction in which most of the carotenoid has disappeared [39,40]. Under any given set of oxidizing conditions a range of different products can be detected, arising from a variety of oxidation reactions that affect different parts of the molecule, e.g., cleavage of the polyene chain, epoxidation of endocyclic double bonds or reactions at allylic positions. Mechanisms that have been proposed for these reactions include the addition of peroxyl radicals to the polyene chain, electron capture by the pigment and hydrogen abstraction from allylic positions [14,41–43]. The consensus view is that at low oxygen concentrations carotenoids can be effective antioxidants, whereas at higher oxygen concentrations they are less effective and may promote oxidation [14,44].

All carotenoids are susceptible to oxidation, but not all at the same rate or by the same reactions. Details of structure determine which reactions predominate for a particular carotenoid under a given set of conditions. Although most work on the antioxidant action of carotenoids has dealt primarily with β,β-carotene, studies have been reported which compare the action of different carotenoids. In a recent paper, we report a study which was to relate reactivity to structure for a variety of carotenoids in reaction with peroxyl radicals generated via the thermal decomposition of azo-compounds [43]. We now report the ability of these carotenoids to inhibit lipid peroxidation induced by peroxyl radicals, both in homogeneous organic solution and in EYPC liposomes. The results are discussed in terms of the structure and chemistry of the carotenoids concerned.

2. Materials and methods

2.1. Chemicals

Carotenoids were a gift from Hoffmann–La Roche, Basel. Their structures are shown in the earlier paper [43]. Purity of carotenoids was confirmed by HPLC. Egg-yolk phosphatidylcholine (99% +) (EYPC) and α-tocopherol were purchased from Sigma. 2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH) and 2,2′-azobis(2,4′-dimethylvaleronitrile) (AMVN) were purchased from Park Scientific, Northampton, UK. All solvents were of HPLC grade and degassed prior to use in the HPLC mobile phase. Chloroform was stabilised by the addition of 2% ethanol (v/v).

2.1.1. Oxidation of EYPC by AMVN-derived peroxyl radicals in homogeneous organic solution: Effect of carotenoids or α-tocopherol on phospholipid hydroperoxide (PCOOH) formation

Carotenoids or α-tocopherol (0.5 μmol), first dissolved in appropriate solvents for quantitation by UV–vis spectrophotometry, were evaporated to dryness under nitrogen. EYPC (50 μmol) was diluted with chloroform (5 ml) and added to the dried substances. The phospholipid/antioxidant mixtures were redissolved by vigorous vortex mixing for at least 1 min. No evidence of carotenoid aggregates was observed by UV–vis spectrophotometry. To limit isomerization or oxidation, the samples were stored on ice under nitrogen in the dark prior to the reaction.

AMVN (10 mM) was freshly prepared in chloroform and kept on ice in the dark prior to incubation. AMVN was added to the EYPC–antioxidant solution to give the following final concentrations; AMVN (5 mM), carotenoids or α-tocopherol (0.05 mM) and EYPC (5 mM) The samples were incubated under air in the dark at 37°C. Aliquots of samples were removed for measurement of PCOOH formation.

2.1.2. Oxidation of EYPC liposomes by AAPH-derived and AMVN-derived peroxyl radicals: Effect of carotenoids and α-tocopherol on PCOOH formation

EYPC liposomes were prepared according to Lim et al. [36] at a final concentration of 5 mM EYPC containing 1 mol% carotenoid or α-tocopherol. AMVN in chloroform was added to a final concentration of 0.5 mM, prior to removal of solvent during liposome preparation. When AAPH was used, this was added to a final concentration of 10 mM. Samples were incubated under air in the dark in a thermostatted water bath at 50°C. Incubation at this temperature creates a high rate of peroxyl radical production, removing the need for long incubation times when background oxidation could occur. This higher temperature is reported not to impair the efficiency of the antioxidants nor affect membrane structure [45].

2.1.3. Determination of PCOOH formation in homogeneous organic solution and liposomes

The method of Lim et al. [36] was used to measure PCOOH formation. A Shimadzu L6A chromatograph was used with a Shimadzu SPD-6A detector coupled to a Shimadzu C-R5A peak integrator. A Spherisorb 25 cm × 4.6 mm silica column, particle size 5 μm, was used as the stationary phase, with an isocratic mobile phase consisting of 85% methanol in water at a flow rate of 1.8 ml min^{-1} at ambient temperature. Detection was by UV absorption at 234 nm, with the detector sensitivity set at 0.16 AU full scale deflection. Samples (20 μl) drawn directly from the solution or liposomal incubations were injected directly onto the column. PCOOH was quantified by comparison with a PCOOH standard prepared by overnight incubation at 37°C of EYPC liposomes (5 mM) with AAPH (10 mM). The PCOOH was separated by HPLC, the PCOOH collected, redissolved and quantified by UV–vis spectrophotometry at 234 nm, using a molar extinction coefficient of 30 000 [46]. PCOOH eluted as a single broad peak with a retention time around 3–4.5 min and was separated from the solvent front. Absolute amounts of PCOOH formed in such experiments can vary, so in each case a matched control was prepared without antioxidants for comparison as suggested by Stocker et al. [47].

2.1.4. Determination of the residual carotenoid content in homogeneous organic solutions or liposomes after reaction with peroxyl radicals

The change in carotenoid concentration in the homogeneous organic solutions was monitored by following the loss of absorbance of the carotenoid at its λ_{max} by UV–vis spectrophotometry. Carotenoids in liposomal preparations (1 ml) were assayed after extraction with ethanol:hexane:diethyl ether (1:1:1 v/v) (2 ml). The amount of residual pigment was expressed relative to the initial pigment concentration, determined similarly.

2.2. Statistical analysis

Student's *t*-test was used to assess significance between groups and was accepted at $p < 0.05$. Data are presented as means ± standard error.

3. Results

3.1. Inhibition of lipid peroxidation in organic solution by carotenoids

The ability of carotenoids to protect EYPC against lipid peroxidation by AMVN-derived peroxyl radicals was examined first in homogeneous organic solution. At the concentrations used the carotenoids were all in solution; UV–vis absorption spectra showed no evidence of molecular aggregation or insoluble microcrystals. In the absence of carotenoids, lipid hydioperoxides accumulated at a linear rate of 2.8×10^{-8} M s^{-1}. The effects of carotenoids or α-tocopherol on the time course of PCOOH accumulation are shown in Fig. 1, and the relative inhibitory effects of the different carotenoids are assessed in Fig. 2 by comparing the amounts of PCOOH formed after 1 h. Destruction of carotenoids during the reactions was also measured (Fig. 3).

All the carotenoids limited significantly lipid peroxidation. With α-tocopherol, there was a clear lag period (induction period) of 1–2 h during which little or no lipid peroxidation occurred. β,β-carotene and zeaxanthin reduced but did not completely limit PCOOH formation, but the rate of PCOOH formation in the presence of these pigments did not attain the rate seen in the absence of any antioxidant within 4 h. All the carotenoids suffered rapid destruction. After 1 h, only 10–20% of the original carotenoid remained.

A clear correlation between inhibition of lipid peroxidation and destruction of carotenoid during the

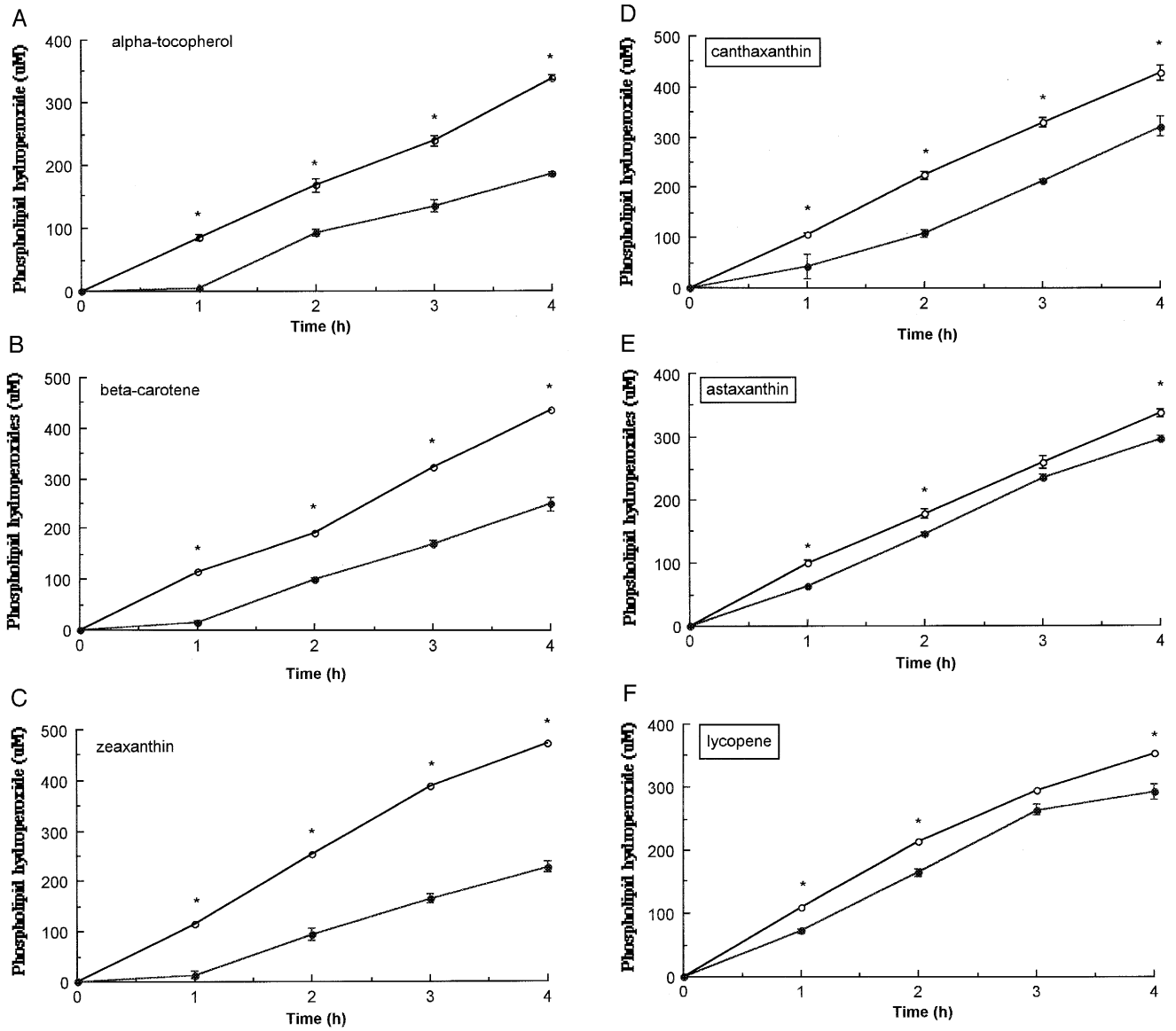

Fig. 1. Rate of formation of phospholipid hydroperoxides by the reaction of EYPC (5 mM) with peroxyl radicals generated via AMVN in homogenous organic solution, in air at 37°C, in the presence (●) or absence (○) of α-tocopherol or carotenoids (1 mol%). (A) α-tocopherol; (B) β,β-carotene; (C) zeaxanthin; (D) canthaxanthin; (E) astaxanthin; (F) lycopene. Each point represents the mean of at least four experiments ± standard error. In this and subsequent figures, when not visible, error bars lie within data symbols. Values significantly different: * $P < 0.05$.

lag phase was observed for β-ring carotenoids. β,β-carotene, zeaxanthin and canthaxanthin gave the greatest protection and showed the greatest level of destruction. Astaxanthin was destroyed much more slowly and afforded much less protection. The protective abilities of β,β-carotene and zeaxanthin in homogeneous organic solution were not significantly different ($P > 0.05$). Lycopene, an acyclic carotenoid, was destroyed most rapidly but afforded the least protection.

3.2. Inhibition of AMVN-derived lipid peroxidation in liposomes by carotenoids

The fact that carotenoids are able to protect against lipid peroxidation in homogeneous solution does not prove that they have antioxidant properties in vivo, where they are components of structurally ordered systems such as cell membranes or lipoproteins. The effects of carotenoids on the formation of PCOOH in EYPC liposomes were therefore determined. The carotenoids were studied at the same concentration relative to EYPC in solution (1 mol%), but the carotenoids were incorporated into the liposome bilayer structure, where they are located in the hydrophobic core of the bilayer.

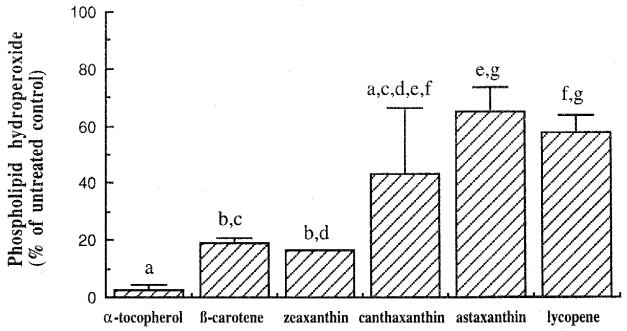

Fig. 2. Comparison of the effects of α-tocopherol and carotenoids (1 mol%) on the formation of phospholipid hydroperoxides by reaction of EYPC (5 mM) with peroxyl radicals generated via AMVN in homogenous organic solution, in air at 37°C. The data show the amount of lipid hydroperoxide formed after 1 h in the presence of α-tocopherol or carotenoid as a percentage of that formed in a parallel control experiment without additive. Data represent means of at least 4 experiments ± standard error. Pairs of superscripts denote that the difference between the antioxidants is not significant ($P > 0.05$), otherwise all differences are significant at $P < 0.05$.

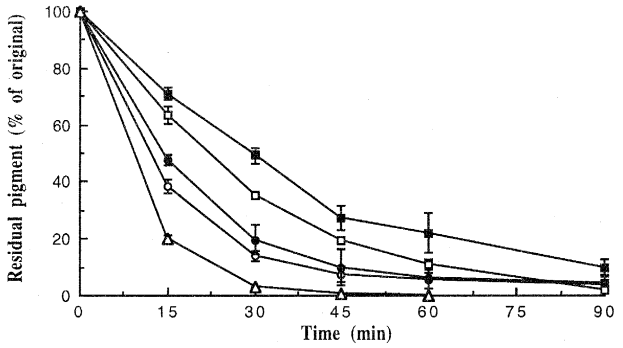

Fig. 3. Comparison of the rates of destruction of carotenoids (1 mol%) during the oxidation of EYPC (5 mM) by AMVN in homogenous organic solution at 37°C in air. The results are expressed as the percentage of the original carotenoid remaining at the given time. β,β-Carotene (○); zeaxanthin (●); canthaxanthin (□); astaxanthin (■); lycopene (△). Data represent means of at least 4 experiments ± standard error.

In the first series of experiments, AMVN was used. This hydrophobic azo-initiator intercalates into the hydrophobic inner core of the liposome and generates peroxyl radicals in this region. In the absence of any antioxidant, PCOOH were formed at a linear rate of 1.8 ± 0.07 μM s^{-1} ($n = 32$). All carotenoids except lycopene reduced the rate of PCOOH formation (Figs. 4 and 5). Zeaxanthin was as effective an antioxidant as α-tocopherol and both zeaxanthin and β-cryptoxanthin were more effective than β,β-carotene as measured by the percentage inhibition of PCOOH formation compared with control (Fig. 5). Canthaxanthin and astaxanthin were not as protective as β,β-carotene, zeaxanthin or β-cryptoxanthin. The monoketone echinenone had a greater protective effect than the diketones. The inhibitory effect of lycopene was the least but still significant ($P < 0.05$). The level of destruction of carotenoids under these conditions were much lower than in solution (Fig. 6). The hydrocarbons, β,β-carotene and lycopene, underwent greater degradation than most of the xanthophylls. Degradation of the hydroxyxanthophylls was significantly greater than ketocarotenoids ($P < 0.05$).

The effects of β,β-carotene and zeaxanthin were examined at different concentrations (Fig. 7). At each

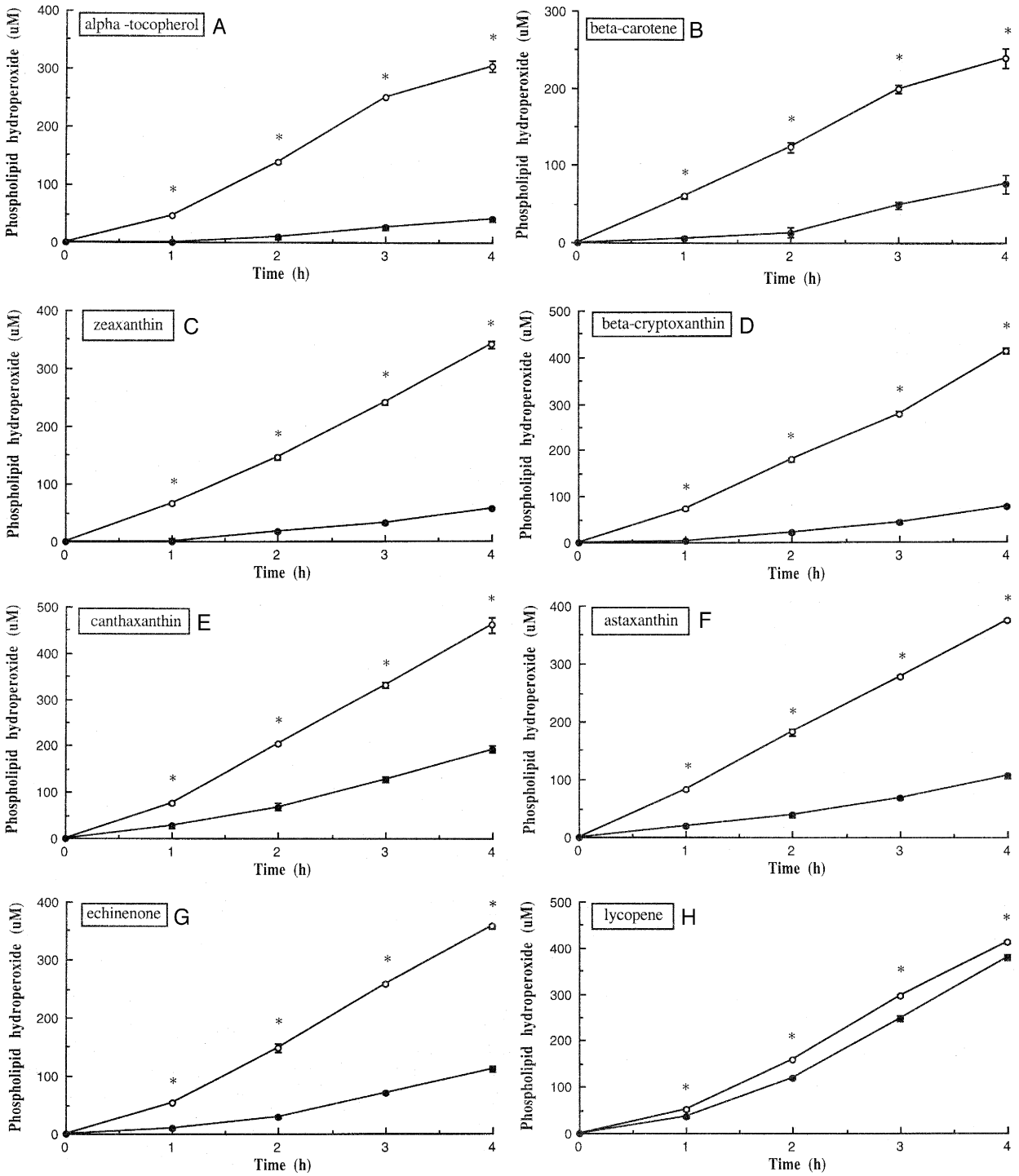

Fig. 4. Rate of formation of phospholipid hydroperoxides by the reaction of EYPC (5 mM) with peroxyl radicals generated via AMVN in liposomes, in air at 50°C, in the presence (●) or absence (○) of α-tocopherol or carotenoids (1 mol%). (A) α-tocopherol; (B) β,β-carotene; (C) zeaxanthin; (D) β-cryptoxanthin; (E) canthaxanthin; (F) astaxanthin; (G) echinenone; (H) lycopene. Each point represents the mean of at least four experiments ± standard error. Values significantly different: * $P < 0.05$.

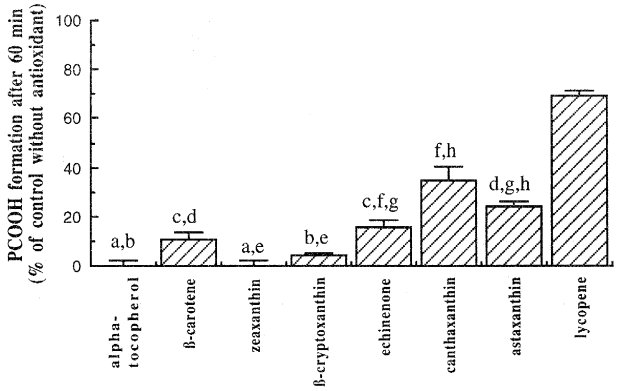

Fig. 5. Comparison of the effects of α-tocopherol and carotenoids (1 mol%) on the formation of phospholipid hydroperoxides by reaction of EYPC (5 mM) with peroxyl radicals generated via AMVN in liposomes, in air at 50°C. The data show the amount of lipid hydroperoxide formed after 1 h in the presence of α-tocopherol or carotenoid as a percentage of that formed in a parallel control experiment without additive. Data represent means of at least 4 experiments ± standard error. Pairs of superscripts denote that the difference between the antioxidants is not significant ($P > 0.05$), otherwise all differences are significant at $P < 0.05$.

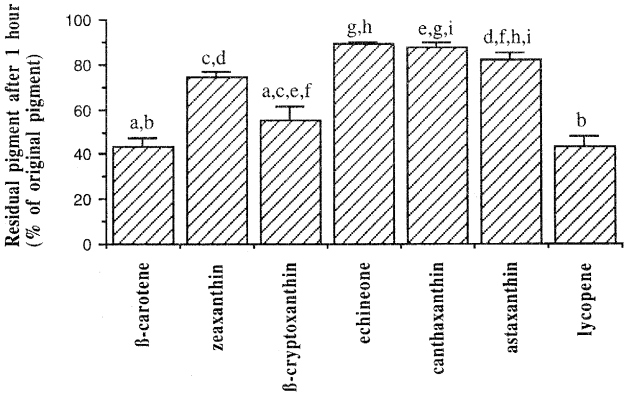

Fig. 6. Destruction of carotenoids (1 mol%) during the oxidation of EYPC (5 mM) by AMVN in liposomes at 50°C in air. The results are expressed as the percentage of original carotenoid remaining after 1 h. Data represent means of at least 4 experiments ± standard error. Pairs of superscripts denote that the difference between the antioxidants is not significant ($P > 0.05$), otherwise all differences are significant at $P < 0.05$.

concentration, zeaxanthin reduced PCOOH formation to a significantly greater degree than did β,β-carotene. At 1 or 2 mol% zeaxanthin relative to EYPC, PCOOH formation was less than 10% of that in the control, whereas with β,β-carotene at the same concentration it was about 40%. Even at 0.1 mol% of zeaxanthin, significant inhibition of lipid peroxidation was seen.

3.3. Inhibition of AAPH-derived lipid peroxidation in liposomes by carotenoids

The azo-initiator AAPH is water-soluble and forms peroxyl radicals in the aqueous phase. Evidence obtained suggested that the location and orientation of both pigment and initiator was important in deterring protective ability in membranes, so the ability of carotenoids to prevent lipid peroxidation induced by AAPH was also investigated. In the absence of antioxidant, PCOOH accumulated at a rate of 5.2 ± 0.04 $\mu M\ s^{-1}$ ($n = 32$). The effects of α-tocopherol and carotenoids on this rate are illustrated and compared in Figs. 8 and 9 and the rates of carotenoid destruction under these conditions are shown in Fig. 10. In this case, α-tocopherol, β-cryptoxanthin and zeaxanthin gave significantly more protection than β,β-carotene, as measured by percentage inhibition of PCOOH formation. Canthaxanthin afforded less protection but was still significant. Astaxanthin and lycopene did not inhibit lipid peroxidation to any great

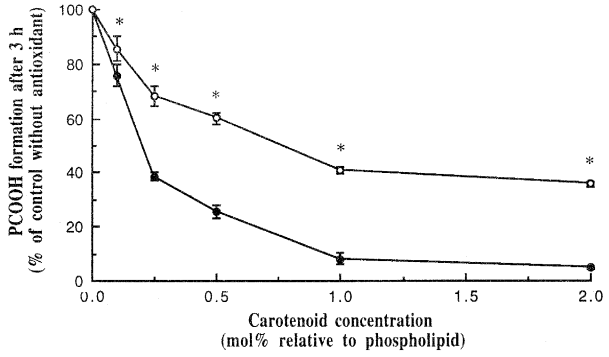

Fig. 7. Effect of different concentration of β,β-carotene (○) or zeaxanthin (●) on the formation of PCOOH in EYPC liposomes (5 mM) after 3 h incubation in air at 50°C in the presence of AMVN. The amount of PCOOH formed in the presence of carotenoid is expressed as a percentage of that formed in the control, without carotenoid. Data represent means of at least 4 experiments ± standard error.

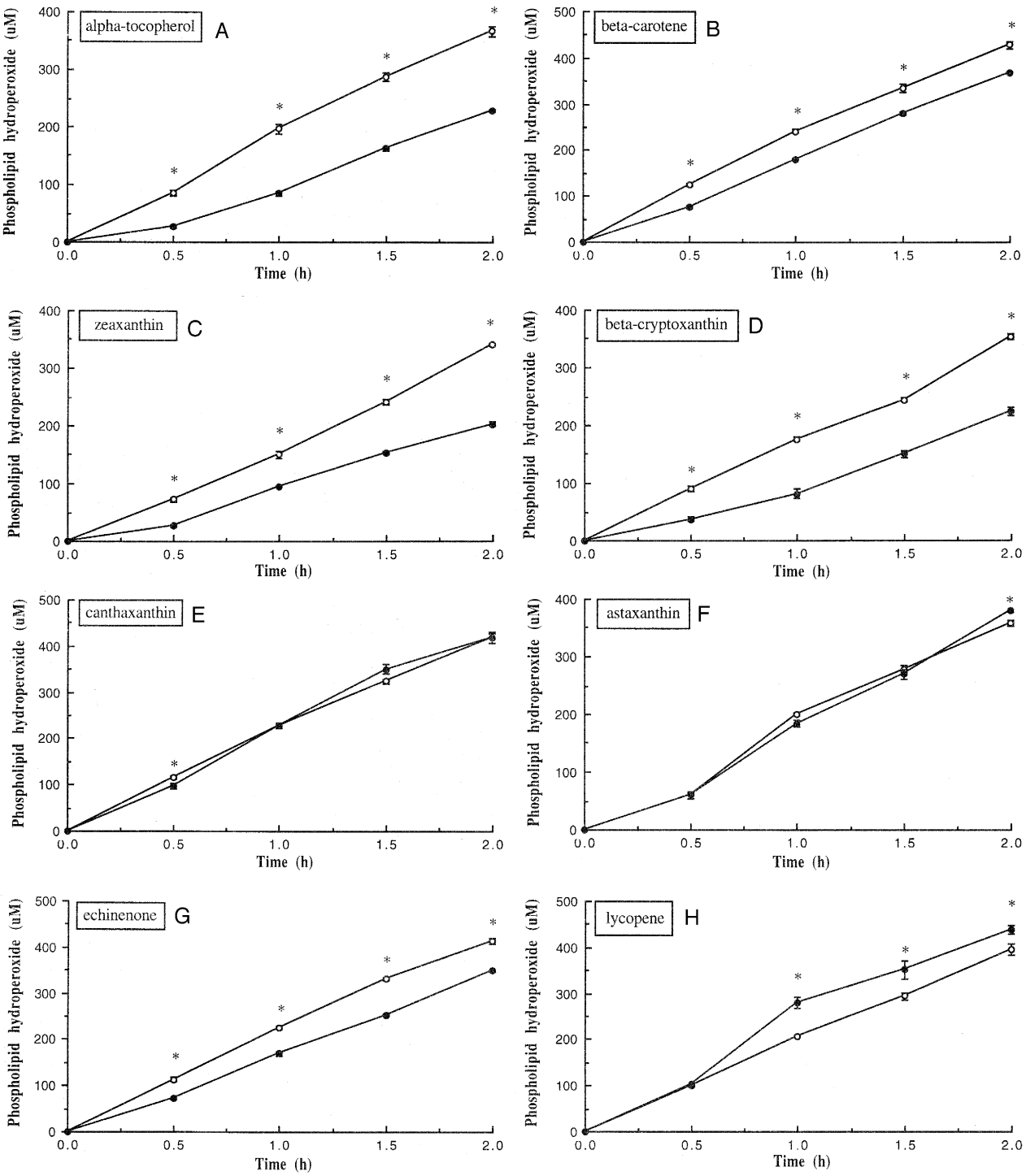

Fig. 8. Rate of formation of phospholipid hydroperoxides by the reaction of EYPC (5 mM) with peroxyl radicals generated via AAPH in liposomes, in air at 50°C, in the presence (●) or absence (○) of α-tocopherol or carotenoids (1 mol%). (A) α-tocopherol; (B) β,β-carotene; (C) zeaxanthin; (D) β-cryptoxanthin; (E) canthaxanthin; (F) astaxanthin; (G) echinenone; (H) lycopene. Each point represents the mean of at least four experiments ± standard error. Values significantly different: * $P < 0.05$.

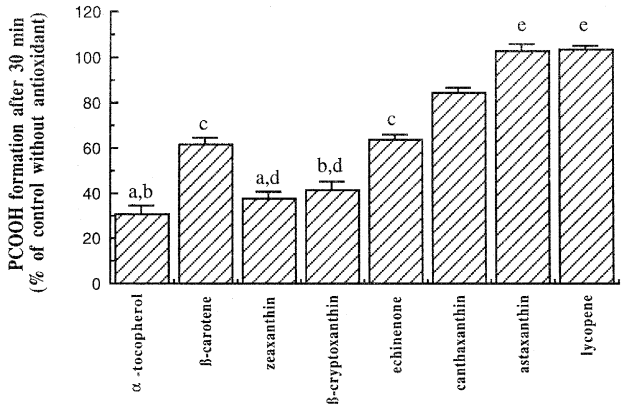

Fig. 9. Comparison of the effects of α-tocopherol and carotenoids (1 mol%) on the formation of phospholipid hydroperoxides by reaction of EYPC (5 mM) with peroxyl radicals generated via AAPH in liposomes, in air at 50°C. The data show the amount of lipid hydroperoxide formed after 30 min in the presence of α-tocopherol or carotenoid as a percentage of that formed in a parallel control experiment without additive. Data represent means of at least 4 experiments ± standard error. Pairs of superscripts denote that the difference between the antioxidants is not significant ($P > 0.05$), otherwise all differences are significant at $P > 0.05$.

extent. Lycopene exhibited a significant pro-oxidant effect after 60 min incubation; this was not seen for astaxanthin. Zeaxanthin and β-cryptoxanthin underwent significantly more destruction than other carotenoids in this system ($P < 0.05$).

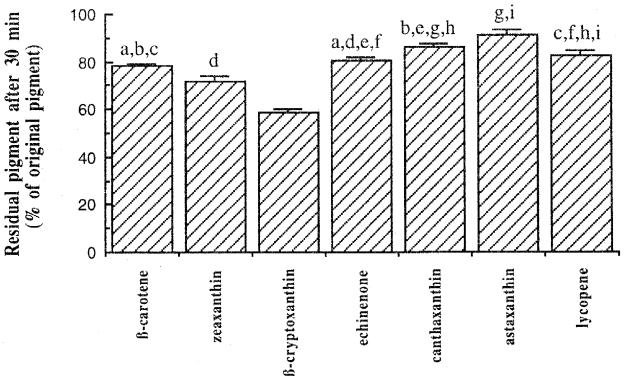

Fig. 10. Destruction of carotenoids (1 mol%) during the oxidation of EYPC (5 mM) by AAPH in liposomes at 50°C in air. The results are expressed as the percentage of original carotenoid remaining after 30 min. Data represent means of at least 4 experiments ± standard error. Pairs of superscripts denote that the difference between the antioxidants is not significant ($P > 0.05$), otherwise all differences are significant at $P < 0.05$.

4. Discussion

Carotenoids are effective lipid-soluble antioxidants, but the mechanism of action is not known. Peroxyl radical addition to, and electron capture by, the carotenoid polyene chain are proposed as mechanisms [14,42]. We have also suggested that hydrogen abstraction from the allylic positions may occur [43]. Any or all of these processes may underlie the antioxidant action of carotenoids, but could also lead to a pro-oxidant effect if the carotenoid radical(s) formed react with oxygen to produce carotenoid peroxyl radicals. Which pathway predominates depends on oxygen tension; higher oxygen tensions increase the chances of forming carotenoid peroxyl radicals and hence of autooxidation or a prooxidant effect [14]. Carotenoids differ in their reactivity towards peroxyl radicals and may therefore differ in their ability to act as antioxidants. We discuss the current findings in the light of our previous work and that of others.

All the carotenoids examined reduced the (AMVN-generated) peroxyl radical-mediated formation of PCOOH from EYPC, both in solution and in liposomes. The protective effect of all the carotenoids in solution was less than α-tocopherol. With the exception of the acyclic carotenoid lycopene, the efficiency of protection by the carotenoids was positively correlated with carotenoid-radical reactivity. Carotenoids and α-tocopherol act as antioxidants by reacting more rapidly with peroxyl radicals than do the unsaturated acyl chains of the EYPC. In the process carotenoids are destroyed and, once consumed, lipid peroxidation proceeds unchecked.

In all experiments, lycopene differed in protective ability compared with other carotenoids. Lycopene showed a high rate of destruction but poor protection against lipid peroxidation. When liposomes containing lycopene were stressed with AAPH, a significantly greater rate of PCOOH formation was observed than in controls. Lycopene may thus promote chain reactions rather than inhibit them in this system. For other carotenoids in these systems, the rates of their antioxidant reactions with peroxyl radicals appear to exceed those of autooxidative processes. The results obtained with homogeneous solutions and those obtained with liposomes showed significant differences, particularly with β,β-carotene and zeaxanthin. These compounds have the same chro-

mophore and, in solution, their reactivities towards peroxyl radicals are very similar. Their protective effects against lipid peroxidation in homogeneous solution are not significantly different, but in EYPC liposomes stressed with AMVN the inhibitory effect of zeaxanthin is significantly greater than β,β-carotene ($P < 0.05$). A greater difference occurs when AAPH is used to produce peroxyl radicals in the aqueous phase. The major difference between experiments undertaken with AMVN in homogeneous solution and liposomes was the physical nature of the system, i.e., the homogeneous distribution of phospholipids in solution and their ordered structure in liposomes. Resonance Raman spectroscopic studies indicate that β,β-carotene is restricted to the hydrophobic core of the bilayer [48]. β,β-Carotene, and presumably lycopene, would thus be susceptible to attack by peroxyl radicals generated in this area, (i.e., from AMVN, which intercalates in the hydrophobic core of the liposomes), but are less accessible to radicals generated in the aqueous phase from AAPH. Zeaxanthin, a dihydroxy carotenoid, spans across DMPC bilayers [49] and adopts a similar orientation in EYPC bilayers with the polar hydroxyl groups located close to the hydrophobic–hydrophillic interface of the lipid bilayer (Woodall et al., unpublished data). Zeaxanthin is thus held in close proximity and approximately parallel to the lipid acyl chains. This is the optimum position to provide protection against peroxyl radicals at all 'depths' in the hydrophobic phase. Zeaxanthin would also be able to intercept peroxyl radicals entering the lipid bilayer from the aqueous phase. These could react by hydrogen abstraction from the allylic positions at C-4 of zeaxanthin, or by peroxyl radical addition to the 5,6 C=C bond in the β-ring. The increased protective effect of β-cryptoxanthin (a monohydroxy carotenoid) compared with β,β-carotene against lipid peroxidation may also be due to such an observed orientational effect (Woodall et al., unpublished data).

Astaxanthin adopts a similar orientation to zeaxanthin in membranes. The protective effect of astaxanthin was less than zeaxanthin, however, and reflects the lower reactivity and protective ability of astaxanthin in solution. There are differences between astaxanthin and zeaxanthin in electronic charge distribution along the chromophore, caused by the electron withdrawing effect of the C=O groups in astaxanthin [43]. The greatest difference lies in the end groups where the CH_2 group at C-4 and C-4′ of zeaxanthin may undergo hydrogen abstraction, which is not possible with astaxanthin. This may be particularly significant in liposomes, where the β-ring would be the first part of astaxanthin encountered by aqueous peroxyl radicals.

Some reports suggest that the most effective carotenoids in preventing lipid peroxidation are the diketo compounds, canthaxanthin and especially astaxanthin. Miki [50] found astaxanthin to be a more effective antioxidant than α-tocopherol against lipid peroxidation. Palozza and Rrinsky [51] also demonstrated this when astaxanthin was added to microsomal membrane preparations. It is not clear with the microsomal system whether the added carotenoid intercalates into the lipid bilayer or is adsorbed to the surface of the membrane, a process which could be favoured by astaxanthin's two hydroxyketo end groups. However, the liposomal system also has disadvantages. The concentration of carotenoid incorporated in liposomes in this study was much greater than normally occurs in a natural membrane, and the systems were subjected to attack by high concentrations of free radicals over a short time period. Our studies therefore provide only comparative information on the relative ability of the carotenoids to protect model membranes against lipid peroxidation, and do not attempt to reflect all the factors seen in vivo.

Our results agree with those of Lim et al. [36] that, in liposomes, zeaxanthin is a significantly more effective antioxidant than β,β-carotene or canthaxanthin. β-cryptoxanthin, another dietary carotenoid, is also more effective than β,β-carotene in model membranes, suggesting that the biophysical interaction of the carotenoid with the bilayer is an important determinant of antioxidant activity. Our results with astaxanthin differ from those of Lim et al. [36]. They used a temperature of 37°C to generate free radicals from the azo-initiators at comparatively low rates of radical production (4.58×10^{-9} and 2.29×10^{-8} M s^{-1} for AMVN and AAPH, respectively), which led to long lag periods (up to 24 h) and long time courses of up to 48 h. In our work, a temperature of 50°C was used to generate free radicals at a more rapid rate, and the mean control rates of lipid peroxidation were faster (1.8×10^{-8} and 5.2×10^{-8} M s^{-1} for AMVN

and AAPH, respectively). The higher temperature was used to achieve rates of peroxidation comparable to those in the solution experiments. This difference in rates of generation of peroxyl radicals could account for the difference in results. The liposomal bilayer is a dynamic system and carotenoids have some mobility within this structure. Over the periods of incubation used by Lim et al. [36], significant aggregation of carotenoid molecules within the bilayer may have occurred. Carotenoid aggregation may reduce the potential for radical-pigment interaction and may also favour carotenoid-carotenoid autooxidation in the aggregate. In addition, during long periods of incubation at 37°C in the presence of oxygen, the generation rate of radicals is low and the concentration of oxygen high relative to the concentration of radicals formed so there could be significant autooxidation of carotenoids which would lower their effective concentration as liposomal antioxidants. Carotenoids such as canthaxanthin and astaxanthin, which are less reactive towards free radicals, would less readily autooxidise compared with β,β-carotene and would be present for longer periods to intercept free radicals. In our system, the opportunity for background autooxidation of carotenoids is limited by shorter incubation tunes which would generate higher concentrations of radicals relative to the oxygen concentration. Despite the differences in detail, the combined data from both studies suggest a significant protective ability of the dietary hydroxycarotenoids zeaxanthin and β-cryptoxanthin over β,β-carotene.

5. Conclusions

This work provides further evidence that carotenoids protect unsaturated lipids and membrane structures against damage caused by peroxyl radicals. In homogeneous solution, the effectiveness of a particular carotenoid at preventing lipid peroxidation is related to its chemical reactivity towards peroxyl radicals. Carotenoids that have similar protective ability and reactivity in solution (e.g., β,β-carotene and zeaxanthin) differ in their ability to protect liposomes against lipid peroxidation. The position and orientation of the carotenoid in the membrane are additional important factors. The differences in behaviour identified in the current work and that of others cited suggest β,β-carotene should not be the only dietary carotenoid considered as a potentially beneficial antioxidant. Some other carotenoids, notably zeaxanthin and β-cryptoxanthin, could be more effective antioxidants because of their favourable location and orientation in membrane bilayers. In particular, the work presented here and by others [36] demonstrating the increased protective ability of zeaxanthin compared with β,β-carotene provides one possible explanation why the human macula may have evolved to isolate zeaxanthin from the plasma in preference to β,β-carotene and lycopene. This information may be relevant in studies examining the role of carotenoids in the prevention of age-related macular degeneration.

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