Relative stability of carotenoid radical cations and homologue tocopheroxyl radicals. A real time kinetic study of antioxidant hierarchy

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Abstract Real time detection following laser flash photolysis of transient carotenoid radical cations and tocopheroxyl radicals formed in chloroform and bleaching of the carotenoids has allowed interaction between carotenoids and tocopherols to be studied. It is found that α-, β-, and γ-tocopherol reduce all the carotenoid radical cations investigated whereas the δ-tocopheroxyl radical can be reduced by lycopene and β-carotene. Astaxanthin, canthaxanthin, and β-apo-8′-carotenal radical cations are scavenged rapidly by all four tocopherol homologues whereas the other carotenoid radical cations react much more slowly with the tocopherols. The results allow the antioxidant hierarchy to be established: α-tocopherol > lycopene ~ β-tocopherol ~ γ-tocopherol > β-carotene > zeaxanthin ~ δ-tocopherol > lutein > echinenone ≫ canthaxanthin ~ β-apo-8′-carotenal > astaxanthin.

Key words: Carotenoid; Tocopherol; Laser flash photolysis; Radical

1. Introduction

One general conclusion emerging from the increasing number of studies of biological response to oxidative stress seems to be that antioxidative protection depends on interaction between antioxidants with differences in standard reduction potentials and with differences in partitioning between aqueous and lipid phases [1–3]. However, both kinetic and thermodynamic properties of antioxidants need to be known in detail for various types of antioxidants in order to understand, on a molecular basis, antioxidant interaction and synergism. Antioxidants may thus act synergistically due to differences in reactivity towards different oxidants thereby yielding a better overall protection in combination than either could individually. Or the synergism may be due to direct interaction between the antioxidants. It was hence proposed [4] that ascorbate was able to reduce the one-electron oxidized vitamin E (tocopheroxyl radical) in lipid/water interfaces thereby recycling vitamin E at the expense of vitamin C. Recently, it was demonstrated that vitamin C is indeed capable of reducing the α-tocopheroxyl radical in micellar and membrane systems [5]. The role of other antioxidants in recycling vitamin E or the antioxidants being recycled by vitamin E is less well understood. Lutein or β-carotene together with γ-tocopherol were found to more efficiently retard oxidation of triglycerides in vegetable oils than γ-tocopherol alone [6,7]. α-Tocopherol and β-carotene in combination were found to provide a higher antioxidative capacity in a membrane system than α-tocopherol or β-carotene alone, and α-tocopherol protected β-carotene from being oxidized at the expense of an increased consumption of α-tocopherol [8,9]. However, until very recently no mechanistic studies of this synergism have been reported. It has more recently been claimed that a number of carotenoids, with the notable exception of astaxanthin, were able to reduce the α-tocopheroxyl radical to α-tocopherol, and it was further proposed that the carotenoid reduction of α-tocopheroxyl radicals was an intermediate step in the recycling of α-tocopherol by vitamin C [10]. However, it was later shown that β-carotene could not reduce the α-tocopheroxyl radical but that in fact the β-carotene radical cation was reduced by α-tocopherol, i.e. that the reverse reaction was dominant, and this result applies to several different solvents [11,12]. Furthermore, the radical of a water-soluble tocopherol homologue could not oxidize β-carotene in HL-60 cells or model systems [13] and α-tocopherol reduced the lycopene radical cation rather than lycopene being able to reduce the α-tocopheroxyl radical [14].

In view of the rather few mechanistic studies of antioxidant interactions, we have undertaken investigations using real time kinetic methods encompassing seven carotenoids: β-carotene, zeaxanthin, lutein, echinenone, canthaxanthin, β-apo-8′-carotenal, and astaxanthin, selected on the basis of differences in structural features, in comparison with the four tocopherol homologues. The results clearly demonstrate the relative reactivity of the one-electron oxidized antioxidants and allow us to establish the antioxidant hierarchy of the selected carotenoids and tocopherol homologues.

2. Materials and methods

β-Carotene, astaxanthin, canthaxanthin, zeaxanthin, echinenone, lutein, and β-apo-8′-carotenal (Fig. 1) were all supplied by Roche A/S (Hvidovre, Denmark) sealed in ampoules under argon and were used without further purification. Chloroform, 'Baker Analyzed' Reagent, from Mallinckrodt Baker B.V. (Deventer, Holland) and α-, β-, γ-, and δ-tocopherol from Merck (Darmstadt, Germany) were used as received.

Laser flash photolysis experiments were carried out with an LKS.50 laser flash photolysis spectrometer from Applied Photophysics Ltd (Leatherhead, UK). The third harmonic at 355 nm of a pulsed Q-switched Nd-YAG laser, Spectron Laser Systems (Rugby, UK), was used for excitation. The intensity of the laser pulse was approximately 55 mJ at 355 nm and the duration of the pulse was around 10 ns. A 1P28 photomultiplier tube from Hamamatsu (Hamamatsu City, Japan) was used to detect transient absorption at wavelengths below 550 nm. Red and near infrared detection was conducted with an S1336-44BK silicon photodiode from Hamamatsu. For red and near infrared measurements, red bandpass filters were used in order to minimize degradation of the carotenoids by the xenon arc lamp used for monitoring, whereas a UV cut-off filter was used for monitoring in the blue-green spectral region. Spectral slit widths were typically 4–5 nm. The samples were excited in 1 cm × 1 cm fluorescence cells from Hellma (Müllheim, Germany). All samples were thermostatted at

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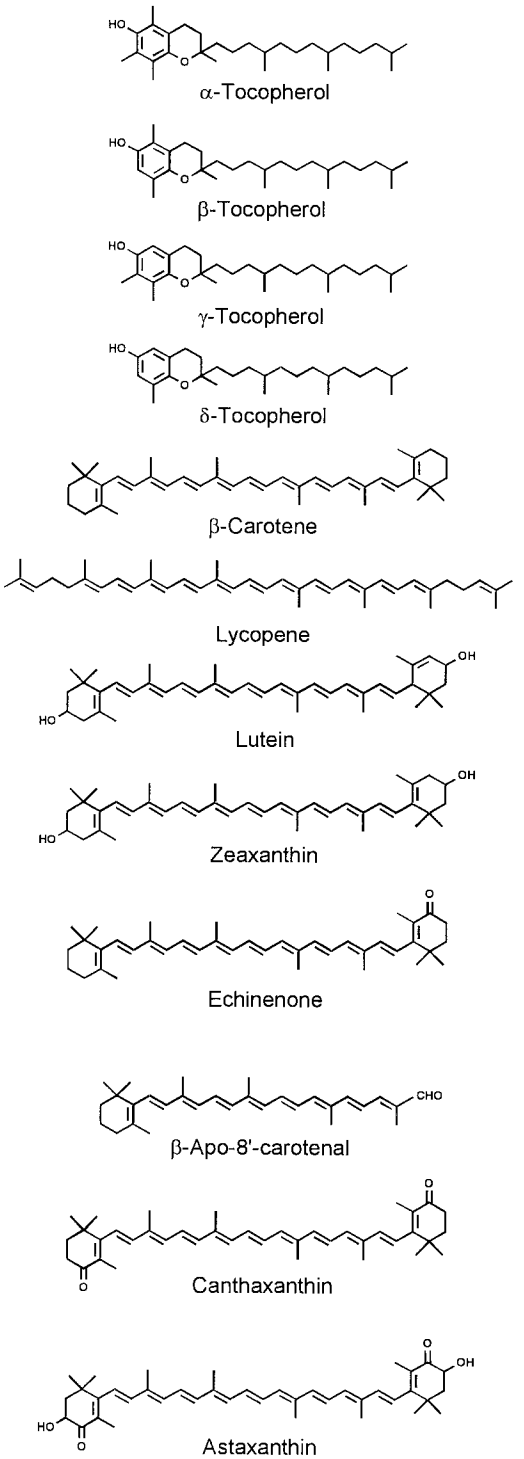

Fig. 1. Structure of the tocopherols and carotenoids investigated.

20.0 ± 0.5°C. Solutions were used the same day they were prepared. Oxygen was found to have no effect on the observed kinetics when comparing kinetic traces from solutions deoxygenated by three freeze-pump-thaw cycles to traces from air-saturated solutions.

3. Results

Laser flash photolysis of carotenoids in chloroform leads to photooxidation of the carotenoids. This oxidation has previously been shown to proceed via carotenoid radical intermediary species [15,16]. The oxidation happens in two steps: (i) an instantaneous bleaching of the carotenoid and formation of a near infrared absorbing transient species upon laser flash photolysis; and (ii) reaction between chloroform radicals, formed as a result of the instantaneous bleaching, and excess carotenoid leading to further bleaching and further formation of the near infrared absorbing transient species. The slow bleaching has been found to be complete in around 1 ms for a number of carotenoids [15,16]. The initially formed near infrared absorbing species decays to another transient species absorbing at a slightly longer wavelength. This latter species was identified as the carotenoid radical cation, whereas the identity of the instantaneously formed species is less certain but has been described to be either a radical adduct between chloroform and carotenoid or a carotenoid radical cation/chloroform radical anion ion pair [15,16]. This previously developed method of generation of carotenoid radical cations using laser flash spectroscopy forms the basis for detailed study of the tocopherol/carotenoid interaction. In the presence of tocopherols, part of the chloroform radicals are scavenged by the tocopherols to give tocopheroxyl radicals. This system thus gives rise to the presence of carotenoids, carotenoid radical cations, tocopherols, and tocopheroxyl radicals in the same solution, and their interaction may hence be studied by following the formation and disappearance of the various species combining different spectral regions for the detection, i.e. around 500 nm for the carotenoid and the near infrared region for carotenoid radical cations.

3.1. Reaction between carotenoids and tocopherols

In the absence of tocopherols, the carotenoid radical cation decays by second-order kinetics to 'stable' products

$$2\ Car^{\bullet+} \rightarrow \text{products} \quad (1)$$

In the presence of any of the four tocopherols, the lifetime of the β-apo-8′-carotenal, astaxanthin, and canthaxanthin radical cations is greatly reduced (Fig. 2B,D,F). The lifetime of the echinenone radical cation is also reduced by the tocopherols (Fig. 2H) but to a smaller extent. This indicates that the carotenoid radical cations react with tocopherol

$$Car^{\bullet+} + TOH \rightarrow Car + TO^{\bullet} + H^{+} \quad (2)$$

thereby decreasing the lifetime of the carotenoid radical cations. According to Eq. 2 the decay of the carotenoid radical cation should be expected to be (pseudo) first-order in a large excess of tocopherol. However, as the time traces show (most notably in Fig. 2B), the decay of the radical cation is biphasic, and the time traces could be fitted to two exponential decay functions. The time traces of the bleaching of the carotenoids show that the bleaching is only partly reversible in the presence of tocopherols (Fig. 2A,C,E,G) whereas it is not in their absence.

The carotenoids without carbonyl groups, i.e. lutein, zeaxanthin, and β-carotene, also react with the tocopherols (Fig. 3) but at a much slower rate than the oxo-xanthophylls. The lifetime of the lutein radical cation is reduced by all four tocopherols, whereas the lifetime of the zeaxanthin radical cation is reduced by all four tocopherols at short times but seems to increase at longer times in the presence of δ-toco-

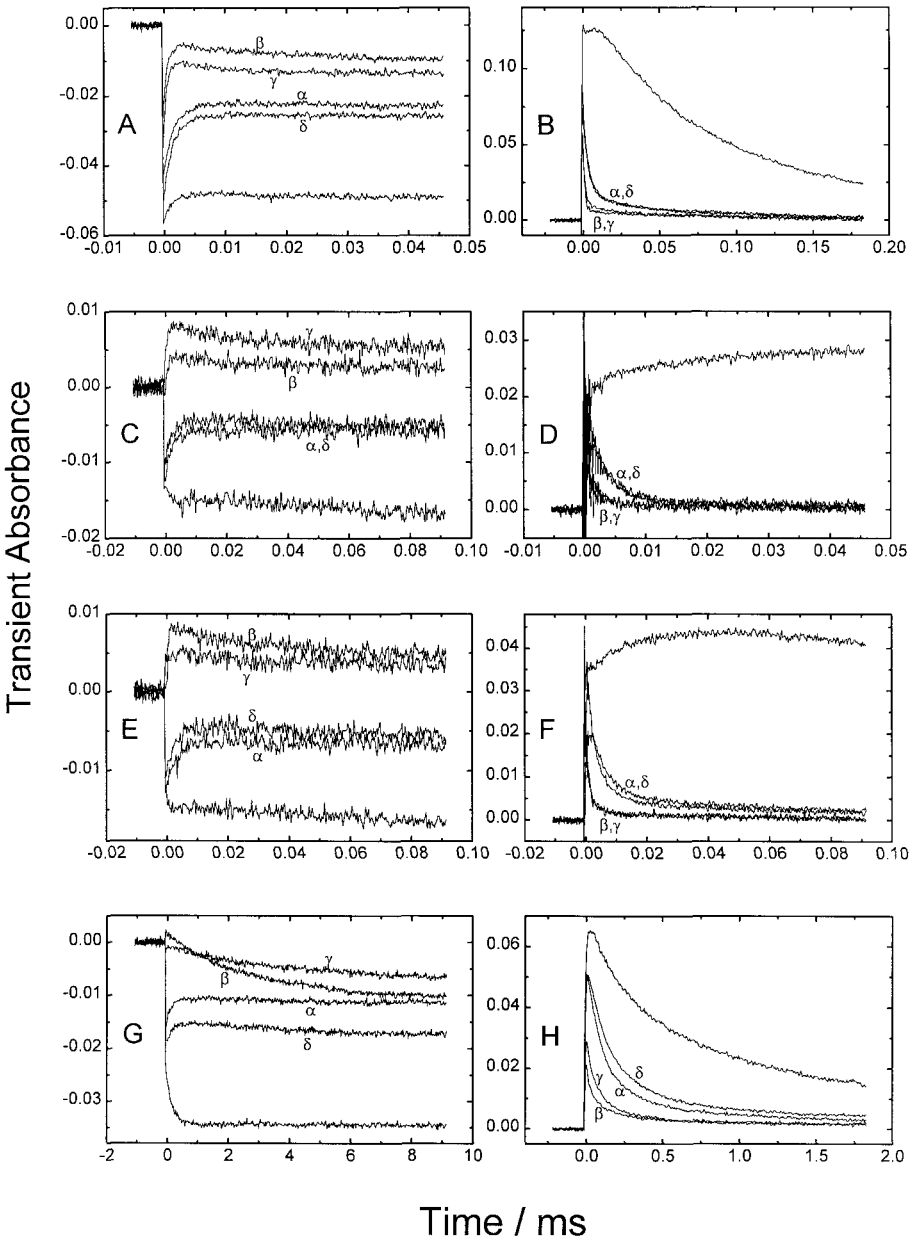

Fig. 2. Time traces of transient absorption following laser flash photolysis at 355 nm of chloroform solutions of 10 μM β-apo-8′-carotenal (A and B), astaxanthin (C and D), canthaxanthin (E and F), and echinenone (G and H) with and without 1.0 mM α-, β-, γ-, or δ-tocopherol. Monitored wavelengths: 520 nm (A and G), 530 nm (C and E), 860 nm (B and H), and 900 nm (D and F). The panels to the left show bleaching of the carotenoids and the panels to the right show decay of the corresponding radical cations.

pherol (Fig. 3D). The lifetime of the β-carotene radical cation is *shorter* in the presence of α-, β-, or γ-tocopherol but *increases* in the presence of δ-tocopherol (Fig. 3F). Again, partial recovery of absorption is observed in the region where the carotenoids absorb (Fig. 3A,C,E). The β- and γ-tocopheroxyl radicals absorb in the same spectral region as the carotenoids, and the bleaching observed on a long time scale (ms) is due to decay of these radicals.

4. Discussion

In a previous study it was found that the carotenes lycopene and β-carotene reacted faster with the chloroform radicals than the hydroxy-xanthophylls zeaxanthin and lutein, and the oxo-xanthophylls astaxanthin, canthaxanthin, and β-apo-8′-carotenal reacted even more slowly [15,16]. In another study the following order of reactivity towards the phenoxyl radical was found: lycopene > β-carotene > zeaxanthin > lutein > echinenone > canthaxanthin ~ β-apo-8′-carotenal > astaxanthin [17,18]. Miller et al. [19] found roughly the same order of reactivity towards the ABTS radical cation.

Lycopene was found to be able to reduce the δ-tocopheroxyl radical [14]

$$\mathrm{Car} + \mathrm{TO}^{\bullet} + \mathrm{H}^{+} \rightarrow \mathrm{Car}^{\bullet+} + \mathrm{TOH} \quad (3)$$

whereas the lycopene radical cation could be reduced by α-tocopherol according to Eq. 2 but at a slow rate compared to

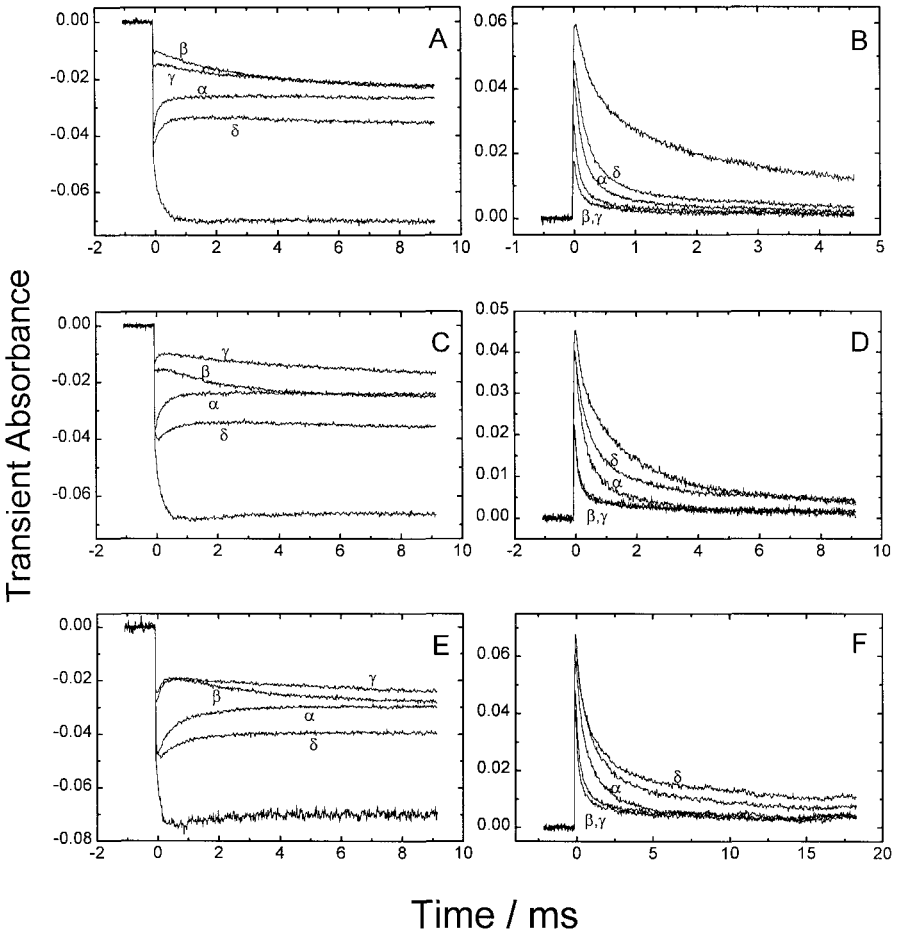

Fig. 3. Time traces of transient absorption following laser flash photolysis at 355 nm of chloroform solutions of 10 μM lutein (A and B), zeaxanthin (C and D), and β-carotene (E and F) with and without 1.0 mM α-, β-, γ-, or δ-tocopherol. Monitored wavelengths: 495 nm (A), 530 nm (C and E), 940 nm (B), 980 nm (D) and 1000 nm (F). The panels to the left show bleaching of the carotenoids and the panels to the right show decay of the corresponding radical cations.

the bimolecular decay (Eq. 1). The lycopene radical cation and the β- or γ-tocopheroxyl radicals were shown to exist in an equilibrium

$$\text{Car} + \text{TO}^{\bullet} + \text{H}^{+} \rightleftarrows \text{Car}^{\bullet+} + \text{TOH} \quad (4)$$

with β- and γ-tocopherol capable of reducing the lycopene radical cation at a high concentration of this species but the reverse reaction taking place at a low concentration of lycopene radical cation [14].

The lifetime of the β-carotene radical cation increases slightly in the presence of δ-tocopherol (Fig. 3F) which is due to reduction of the δ-tocopheroxyl radical by β-carotene (Eq. 3) thereby generating more β-carotene radical cation than that generated directly by reaction with the solvent. However, the increase in lifetime is not as significant as in the case of lycopene [14] showing that lycopene reacts to a larger extent with the δ-tocopheroxyl radical than β-carotene does, and that the lycopene radical cation therefore is more 'stable' than the β-carotene radical cation. α-Tocopherol decreases the lifetime of the β-carotene radical cation (Fig. 3F) according to Eq. 2, and this reaction seems to be of more importance than Eq. 1 in the case of β-carotene than in the case of lycopene [14]. Both β- and γ-tocopherol react at similar rates with all carotenoids which is not surprising considering the similarity of their structures (Fig. 1). β- and γ-tocopherol react (according to Eq. 2) faster with the β-carotene radical cation than the other tocopherols. These two tocopherols were also found to react faster with the chloroform-derived radicals [14] and the lycopene radical cation than α- and δ-tocopherol. However, at longer times the decay of the β-carotene radical cation seems to be slower in the presence of β- or γ-tocopherol than in the absence of these tocopherols

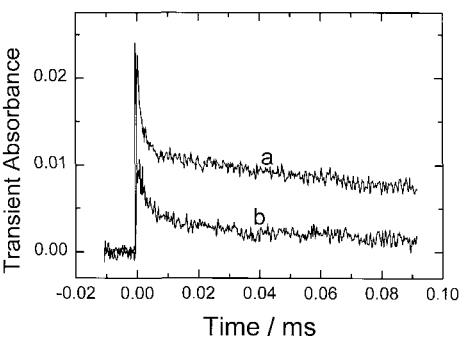

Fig. 4. Time traces of transient absorption at 720 nm following laser flash photolysis at 355 nm of chloroform solutions of 10 μM lutein with (b) and without (a) 1.0 mM β-tocopherol. The fast bleaching is due to decay of the instantaneously formed species and the slower decay is due to formation of the radical cation.

(Fig. 3F), a difference which must be due to Eq. 3, showing that the β-carotene radical cation is in equilibrium with the β- and γ-tocopheroxyl radicals (Eq. 4). However, a comparison of the time traces of decay of the lycopene [14] and the β-carotene radical cations (Fig. 3F) shows that the equilibrium is shifted more to the right (Eq. 4) in the case of lycopene than in the case of β-carotene, in accordance with the higher stability of the lycopene radical cation. Only partial recovery of β-carotene takes place in the presence of tocopherol (Fig. 3E). This is due to the fact that Eq. 1, which does not lead to recovery of the carotenoid, is still important as the tocopherols only lead to small changes in the lifetime of the β-carotene radical cation (Fig. 3F), i.e. Eq. 2 does not compete effectively with Eq. 1. However, it may also be due to the fact that reaction between tocopherol and carotenoid does not proceed solely via Eq. 4, as will be discussed below.

The zeaxanthin radical cation is also reduced at short times by all four tocopherols (Fig. 3D) but at longer times zeaxanthin is able to reduce the δ-tocopheroxyl radical, as evidenced by the apparent slower decay of the radical cation in the presence of δ-tocopherol compared to the decay in its absence. Again, there is evidence that the zeaxanthin radical cation is in equilibrium with the β- and γ-tocopheroxyl radicals (Eq. 4) but the equilibrium seems to be even further shifted to the left than in the case of β-carotene. The lutein radical cation is reduced by all four tocopherols (Fig. 3B) but the time traces indicate that it may be in equilibrium with the δ-tocopheroxyl radical though this reaction is more shifted to the left than in the case of zeaxanthin.

The echinenone radical cation (Fig. 2H) reacts with the four tocopherols similarly to the lutein radical cation (Fig. 3B). However, the β-apo-8′-carotenal (Fig. 2B), astaxanthin (Fig. 2D), and canthaxanthin (Fig. 2F) radical cations react with the four tocopherols at a much higher rate than the other carotenoids. The astaxanthin radical cation is reduced faster than both the canthaxanthin and the β-apo-8′-carotenal radical cations. These three carotenoid radical cations are reduced by each of the four tocopherols at a high enough rate to make Eq. 1 negligible compared to Eq. 2. However, the carotenoids are only partially regenerated (Fig. 2A,C,E) indicating that the tocopherols do not react with carotenoid radical cations according to Eq. 2 exclusively. The most likely explanation is that the tocopherols react with the carotenoid radical cations by two parallel pathways. It has been found that carotenoids react with phenoxyl radicals by two parallel pathways [17,18]: Eq. 2 and another reaction generating a radical adduct

$$\mathrm{Car} + \mathrm{PhO}^{\bullet} \rightarrow [\mathrm{Car}-\mathrm{PhO}]^{\bullet} \quad (5)$$

It is hence possible that tocopherols may react with the carotenoid radical cations not only via Eq. 2 but also according to:

$$\mathrm{Car}^{\bullet+} + \mathrm{TOH} \rightarrow [\mathrm{Car}-\mathrm{TO}]^{\bullet} + \mathrm{H}^{+} \quad (6)$$

which would explain why only partial recovery of carotenoid is observed.

It is likely that the carotenes and the hydroxy-xanthophylls also react with tocopherols according to Eq. 6 though this cannot be confirmed by the time traces alone because in this case the lack of full recovery may be due to the importance of Eq. 1, which does not regenerate the carotenoid, in the decay of these radical cations.

As already mentioned, the time traces of decay of the radical cations of β-apo-8′-carotenal (Fig. 3B), astaxanthin (Fig. 3D), and canthaxanthin (Fig. 3F) does not fit a single first-order decay but rather two first-order processes. Fig. 4 shows the decay of the instantaneously formed species at short times and formation of the radical cation which at longer times leads to an apparent slower decay. Fig. 4 shows that decay of the instantaneously formed species does not seem to be modified appreciably by the presence of tocopherols. The portion of the time traces after a few microseconds (Fig. 2B,D,E) is hence mainly due to decay of the instantaneously formed species and not the radical cation since the instantaneously formed species also absorb in this region [16]. The fact that the tocopherols do not seem to react with the instantaneously formed species may also be the reason why only partial recovery of the carotenoids is observed since the instantaneously formed species has been found to decay to stable products not only via the radical cation (Eq. 1) but also directly [15,16]. Whether Eq. 6 really takes place or the lack of full recovery is due to decay of the instantaneously formed species cannot be decided from the time traces alone.

Comparing the influence of the four tocopherol homologues on the lifetime of the carotenoid radical cations leads to the following order of 'stability' (i.e. lower reactivity) of the carotenoid radical cations and tocopheroxyl radicals: α-tocopherol > lycopene ~ β-tocopherol ~ γ-tocopherol > β-carotene > zeaxanthin ~ δ-tocopherol > lutein > echinenone ≫ canthaxanthin ~ β-apo-8′-carotenal > astaxanthin. This is, as far as the carotenoids are concerned, exactly the same order of reactivity as was observed towards the phenoxyl radical [18]. Based on this hierarchy, one would expect lycopene to be able to reduce other carotenoid radical cations by electron transfer to generate the lycopene radical cation. α-, β-, and γ-tocopherol should be able to recycle all carotenoids investigated in this study by reducing the carotenoid radical cations while δ-tocopherol should be able to recycle all carotenoids except lycopene and β-carotene. However, the tocopherols seem to react with carotenoid radical cations not exclusively via one-electron transfer according to Eq. 2, and their efficiency in recycling carotenoids is hence not 100%.

The antioxidant hierarchy presented here may be used to predict results or explain observations in biological systems. For instance, the order of consumption of antioxidants in LDL was found to be [20]: α-tocopherol > γ-tocopherol > lycopene > lutein/zeaxanthin > β-carotene which, except for lutein/zeaxanthin, is the order expected based on our hierarchy if recycling takes place. The reason for the apparent discrepancy as far as lutein/zeaxanthin are concerned may be due to differences in localization of the carotenoids in LDL. Zeaxanthin and lutein with their polar groups may be expected to be closer to the lipid-water boundary than β-carotene which may be deeply embedded in the lipid fraction and therefore not as prone to oxidation. It was thus found that the polar xanthophylls canthaxanthin and lutein reacted faster with phenoxyl radicals in micelles than β-carotene [13] but the reverse order of reactivity was found in homogeneous solution [18]. Furthermore, β-carotene was found to suppress lipid peroxidation in microsomes much more efficiently in the case of lipophilic peroxyl radicals than in the case of hydrophilic peroxyl radicals [9], again indicating that β-carotene is not located near

the water-lipid boundary. Indeed, zeaxanthin was found to be located at the surface of lipid droplets whereas β-carotene was located in the core [21]. However, the above-mentioned order of consumption is not proof of recycling but could simply be due to a faster rate of reaction with free radicals of the antioxidants consumed fastest. Our results, though, do indicate that γ-tocopherol reacts faster with free radicals (carotenoid and chloroform) than α-tocopherol, and the fact that α-tocopherol is consumed faster could indicate that γ-tocopherol is being recycled by α-tocopherol. Lycopene, like β-carotene, is expected not to be located near the water-lipid boundary but is still consumed faster than lutein/zeaxanthin [20]. This could indicate that lutein and zeaxanthin are being recycled by lycopene. Recycling is more efficient in the case of lycopene than in the case of β-carotene because lycopene is higher in the hierarchy which could explain why lycopene is being consumed faster than lutein/zeaxanthin but β-carotene is not. Recycling of carotenoids and tocopherols as suggested by our hierarchy is hence very likely in biological systems, and carotenoids may play different roles, i.e. as primary scavenger of free radicals and as 'recycler', in preventing lipid peroxidation due to their differences in structure which lead to preferential localization at the core or the surface of lipid-systems.

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References

[1] Chen, H. and Tappel, A.L. (1995) Free Radical Biol. Med. 18, 949–953.
[2] Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996) Free Radical Biol. Med. 20, 933–956.
[3] Mukai, K., Oka, W., Watanabe, K., Egawa, Y., Nagaoka, S. and Terao, J. (1997) J. Phys. Chem. A 101, 3746–3753.
[4] Tappel, A.L. (1968) Geriatrics 23, 97–105.
[5] Bisby, R.H. and Parker, A.W. (1995) Arch. Biochem. Biophys. 317, 170–178.
[6] Haila, K. and Heinonen, M. (1994) Lebensm.-Wiss. Technol. 27, 573–577.
[7] Haila, K., Lievonen, S.M. and Heinonen, M. (1996) J. Agric. Food Chem. 44, 2096–2100.
[8] Palozza, P. and Krinsky, N.I. (1992) Arch. Biochem. Biophys. 297, 184–187.
[9] Palozza, P. and Krinsky, N.I. (1993) Acta Med. Rom. 31, 131–140.
[10] Böhm, F., Edge, R., Land, E.J., McGarvey, D.J. and Truscott, T.G. (1997) J. Am. Chem. Soc. 119, 621–622.
[11] Mortensen, A., Skibsted, L.H., Willnow, A. and Everett, S.A. (1997) Free Radical Res. (in press).
[12] Valgimigli, L., Lucarini, M., Pedulli, G.F. and Ingold, K.U. (1997) J. Am. Chem. Soc. 119, 8095–8096.
[13] Tyurin, V.A., Carta, G., Tyurina, Y.Y., Banni, S., Day, B.W., Corongiu, F.P. and Kagan, V.E. (1997) Lipids 32, 131–142.
[14] Mortensen, A. and Skibsted, L.H. (1997) Free Radical Res. (in press).
[15] Mortensen, A. and Skibsted, L.H. (1996) Free Radical Res. 25, 355–368.
[16] Mortensen, A. and Skibsted, L.H. (1997) Free Radical Res. 26, 549–563.
[17] Mortensen, A. and Skibsted, L.H. (1996) Free Radical Res. 25, 515–525.
[18] Mortensen, A. and Skibsted, L.H. (1997) J. Agric. Food Chem. 45, 2970–2977.
[19] Miller, N.J., Sampson, J., Candeias, L.P., Bramley, P.M. and Rice-Evans, C.A. (1996) FEBS Lett. 384, 240–242.
[20] Esterbauer, H., Puhl, H., Waeg, G., Krebs, A. and Dieber-Rotheneder, M. (1989) in: Vitamin E: Biochemistry and Clinical Applications (Packer, L. and Fuchs, J., Eds.), pp. 649–671, Marcel Dekker, New York.
[21] Borel, P., Grolier, P., Armand, M., Partier, A., Lafont, H., Lairon, D. and Azais-Braesco, V. (1996) J. Lipid Res. 37, 250–261.