Interactions between Carotenoids and the $CCl_3O_2^•$ Radical

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Abstract: The reactions of $CCl_3O_2^•$ (a model of alkyl peroxyl radicals which can be selectively generated in nanoseconds) with a range of carotenoids (β-carotene, septapreno-β-carotene, canthaxanthin, astaxanthin, zeaxanthin, and lutein) in the heterogeneous micellar environment, aqueous 2% Triton X-100, have been studied by pulse radiolysis. For all carotenoids investigated two reaction products, absorbing in the near-infrared region, are observed and assigned to the carotenoid radical cation and an addition radical. With the exception of astaxanthin, the carotenoid radical cation formation is biexponential and the slower component matches the first-order decay of the addition radical. In the case of astaxanthin, no radical cation is formed directly by reaction with $CCl_3O_2^•$, it is formed exclusively from the decay of the addition radical. The results are discussed in terms of the antioxidant properties of carotenoid pigments.

Introduction

Carotenoids (Car), in particular β-carotene, are well-known as ubiquitous free radical quenchers.[1–3] Many are present in the normal diet and when taken in larger quantities as supplements have proved effective in the prevention of skin tumours[4,5] and other types of cancer.[6,7] The role of β-carotene in the prevention of cancer may be related to its ability to trap oxy-radical species[1,8,9] which otherwise may cause DNA damage within cells, leading to carcinogenesis.[10] In addition, carotenoids are also believed to exert a protective effect with respect to heart disease and age-related macular degeneration. While the interactions of carotenoids with singlet oxygen are well-studied[11–13] and known to involve electron exchange energy transfer, relatively little is known of the interactions of carotenoids with oxy radicals.

Burton and Ingold[2] studied the interaction of β-carotene with peroxyl radicals, $RO_2^•$, arising from lipid peroxidation, and suggested formation of an addition radical ($Car\text{-}RO_2^•$). We have recently compared the reactivity of the superoxide anion radical, $O_2^{•-}$, with β-carotene and its open chain analogue lycopene[14] and have observed different behavior with these two carotenoids. We suggested that an addition radical is produced in the case of β-carotene, whereas lycopene undergoes reversible electron transfer with $O_2^{•-}$. Consistent with this we have shown that the radical anion of β-carotene readily transfers an electron to lycopene in hexane ($k = 4 \times 10^{10}\ M^{-1}\ s^{-1}$) whereas the reverse reaction was not observed. Clearly, carotenoids exhibit marked differences in their redox potentials.

In this paper we report results on the interaction of the (trichloromethyl)peroxyl radical, $CCl_3O_2^•$, a model of alkyl peroxyl radicals, with β-carotene and several other carotenoids (for the structures of carotenoids investigated, see Figure 1). Carbon tetrachloride is known to cause various types of tissue injury, in particular hepatoxicity,[15] via the $CCl_3O_2^•$ peroxyl radical, and it is therefore of interest to consider the protective role of carotenoids. The reaction of $CCl_3O_2^•$ with β-carotene has been suggested to involve electron transfer to generate the carotene radical cation, $Car^{•+}$.[8,16] We have previously characterized the spectra of several such radical cations in hexane[17] and report additional data here. The emphasis of the work now reported, however, concerns a more biologically relevant environment, where β-carotene and other carotenoids are solubilized into Triton X-100 micelles.

Materials and Methods

Water was re-distilled from alkaline permanganate. Triton X-100 (TX-100) was supplied by Fluka, and carbon tetrachloride, acetone, and 2-propanol were supplied by Aldrich. Phosphate buffer (10^{-3} M) was prepared using Analar grade Na_2HPO_4 and NaH_2PO_4 from B.D.H. Sodium bromide was obtained from Fisons. All carotenoids were used

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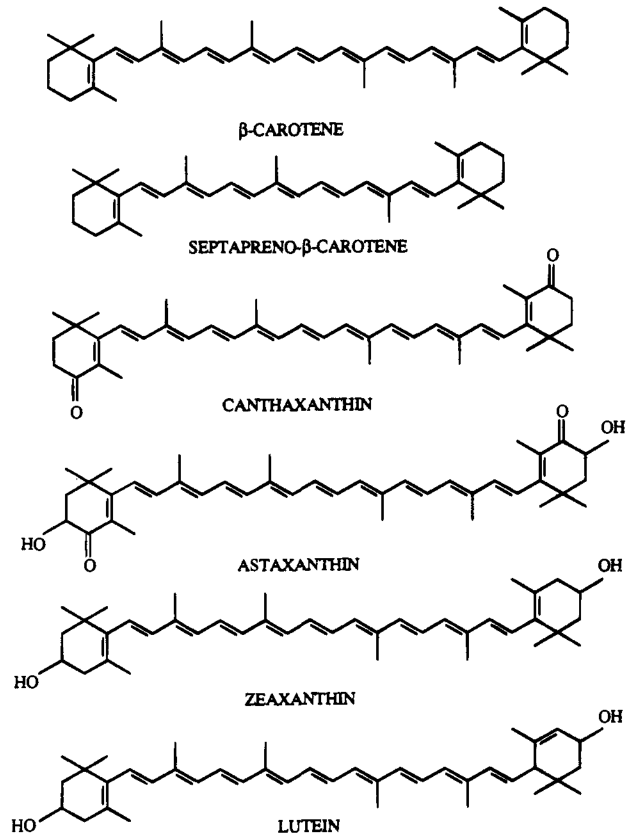

Figure 1. Structures of carotenoids used.

as supplied by Hoffmann-La Roche. Hexane was spectroscopic grade from Rathburn.

The pulse radiolysis experiments were carried out with a 9–12 MeV Vickers linear accelerator either as previously described[18,19] using a single monitoring wavelength for each 20–500 ns pulse or using a system that records data for ten wavelengths for each individual pulse.[20] In both cases, solutions were studied using quartz flow-through cells with an optical path length of 2.5 cm.

In the experiments reported, two solvent systems were used. In the first experiment, carotenoids were solubilized in buffered water using the neutral detergent TX-100, as previously described.[21] In the second, the carotenoids were dissolved directly in hexane.

The oxidizing species, $Br_2^{\bullet -}$, is formed in N_2O saturated aqueous solutions containing 0.05 M sodium bromide according to the sequence[21,22]

$$H_2O \rightsquigarrow e^-_{aq} + OH^\bullet$$

$$e^-_{aq} + N_2O + H_2O \rightarrow N_2 + OH^\bullet + OH^-$$

$$OH^\bullet + Br^- \rightarrow Br^\bullet + OH^-$$

$$Br^\bullet + Br^- \rightarrow Br_2^{\bullet -}$$

In the presence of carotenoid, electron transfer yields the carotenoid radical cation via the following reaction

$$Br_2^{\bullet -} + Car \rightarrow 2Br^- + Car^{\bullet +}$$

In order to prepare the (trichloromethyl)peroxyl radical, $CCl_3O_2^\bullet$, the bromide was omitted and carbon tetrachloride (10^{-2} M), 2-propanol (2 M), and acetone (1 M) were added.[23] In the presence of air, $CCl_3O_2^\bullet$ is produced by the following reaction sequence:

$$OH^\bullet + (CH_3)_2CHOH \rightarrow H_2O + (CH_3)_2C^\bullet OH$$

$$H^\bullet + (CH_3)_2CHOH \rightarrow H_2 + (CH_3)_2C^\bullet OH$$

$$e^-_{aq} + (CH_3)_2CO \rightarrow (CH_3)_2CO^{\bullet -} \xrightleftharpoons{H^+} (CH_3)_2C^\bullet OH$$

$$(CH_3)_2C^\bullet OH + CCl_4 \rightarrow (CH_3)_2CO + CCl_3^\bullet + H^+ + Cl^-$$

$$CCl_3^\bullet + O_2 \rightarrow CCl_3O_2^\bullet$$

In hexane, carotenoid radical cations are formed by positive charge transfer from solvent cations, and N_2O saturation results in prevention of radical anion formation by electron capture.[22] In the absence of carotenoid, no transient absorption in the near-infrared was observed for any of the systems described above.

Results and Discussion

Figure 2a shows the transient spectra recorded at various times following the reaction of $CCl_3O_2^\bullet$ with β-carotene in aqueous 2% TX-100, pH 7. Similar results were obtained in the range pH 3–7. At short time delays (~30 μs) the spectrum exhibits two peaks at 820 and 920 nm. The two absorption peaks display differing formation and decay kinetics (see later) suggesting that they belong to separate species (see Figure 2a). The corresponding transient absorption spectra at various times obtained using septapreno-β-carotene, canthaxanthin, astaxanthin, zeaxanthin, and lutein in place of β-carotene are also given in Figure 2. It can be seen that like β-carotene, all the carotenoids studied give a spectrum with two distinct peaks with different kinetics. To help assign these absorption bands experiments were carried out on the reaction of carotenoid with $Br_2^{\bullet -}$ using aqueous 2% TX-100 as solvent. These systems are expected to give rise exclusively to the corresponding carotenoid radical cations. Only the radical cation of β-carotene has previously been reported in TX-100,[21] and the spectrum we report here for β-carotene is in full agreement with this earlier work. The radical cation spectra in TX-100 for all the carotenoids studied are given in Figure 3. Table 1 compares the radical cation absorption maxima in aqueous TX-100 with those in hexane. It can be seen that there is a marked hypsochromic shift in the aqueous environment reflecting stronger solvation of the radical cation in the polar medium. In a previous paper,[25] using the time-resolved resonance Raman technique, we have shown that the β-carotene triplet state in TX-100 exhibits similar resonance Raman spectra to that in benzene, suggesting the carotene is situated in the core of the micelle. The present results, on the carotene radical cations in TX-100 compared with hexane, contrast with the above results on the triplet state. This may suggest that the carotene radical cation is formed in the micellar outer regions where its properties are influenced by the bulk media, that is, there is more than one environment for the carotenoids in micelles. If TX-100 is indeed a useful model of the carotenoid in a cellular membrane, such a range of sites may have biological consequences. Certainly, in a heterogenous

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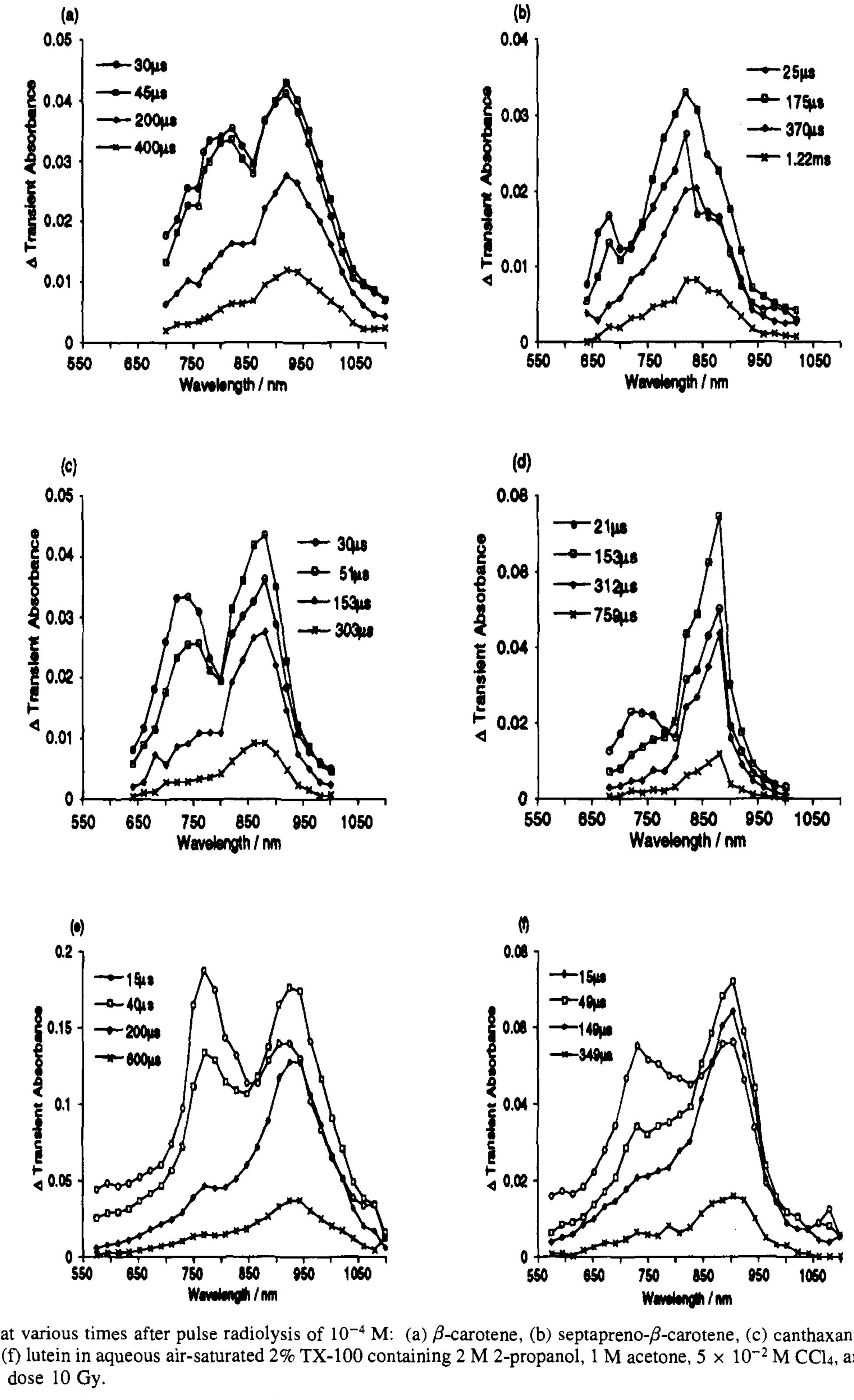

Figure 2. Spectra at various times after pulse radiolysis of 10^{-4} M: (a) β-carotene, (b) septapreno-β-carotene, (c) canthaxanthin, (d) astaxanthin, (e) zeaxanthin, and (f) lutein in aqueous air-saturated 2% TX-100 containing 2 M 2-propanol, 1 M acetone, 5×10^{-2} M CCl_4, and 10^{-3} M phosphate buffer, pH 7. Pulse dose 10 Gy.

cellular system, carotenoids would be expected to have more than one microenvironment.

By comparison of the carotenoid radical cation spectra, given in Figure 3, with the transient absorption maxima given in Figure 2, we assign the longer wavelength peaks obtained from the reaction of $CCl_3O_2^{\bullet}$ with these carotenoids to the corresponding radical cations:

$$CCl_3O_2^{\bullet} + Car \rightarrow CCl_3O_2^{-} + Car^{\bullet +}$$

The nature of the other species absorbing at shorter wavelengths is not clear. In previous work, Willson[16] reported the spectrum obtained from the interaction of $CCl_3O_2^{\bullet}$ with β-carotene in 50/50 water—*tert*-butyl alcohol as solvent. The spectrum showed two distinct peaks (~920 and ~780 nm), and furthermore the yield of radical cation was only 50% of that expected from the radiation dose used, implying an additional process was also occurring such as a radical addition. Although no assignment of the spectral peak at 780 nm was made, the observations are consistent with the spectra reported here.

The unassigned peaks for the six carotenoids studied in the present work and that of Willson *et al.* may be due to an addition radical, rather like that proposed by Burton and Ingold[2] for $RO_2^{\bullet}$

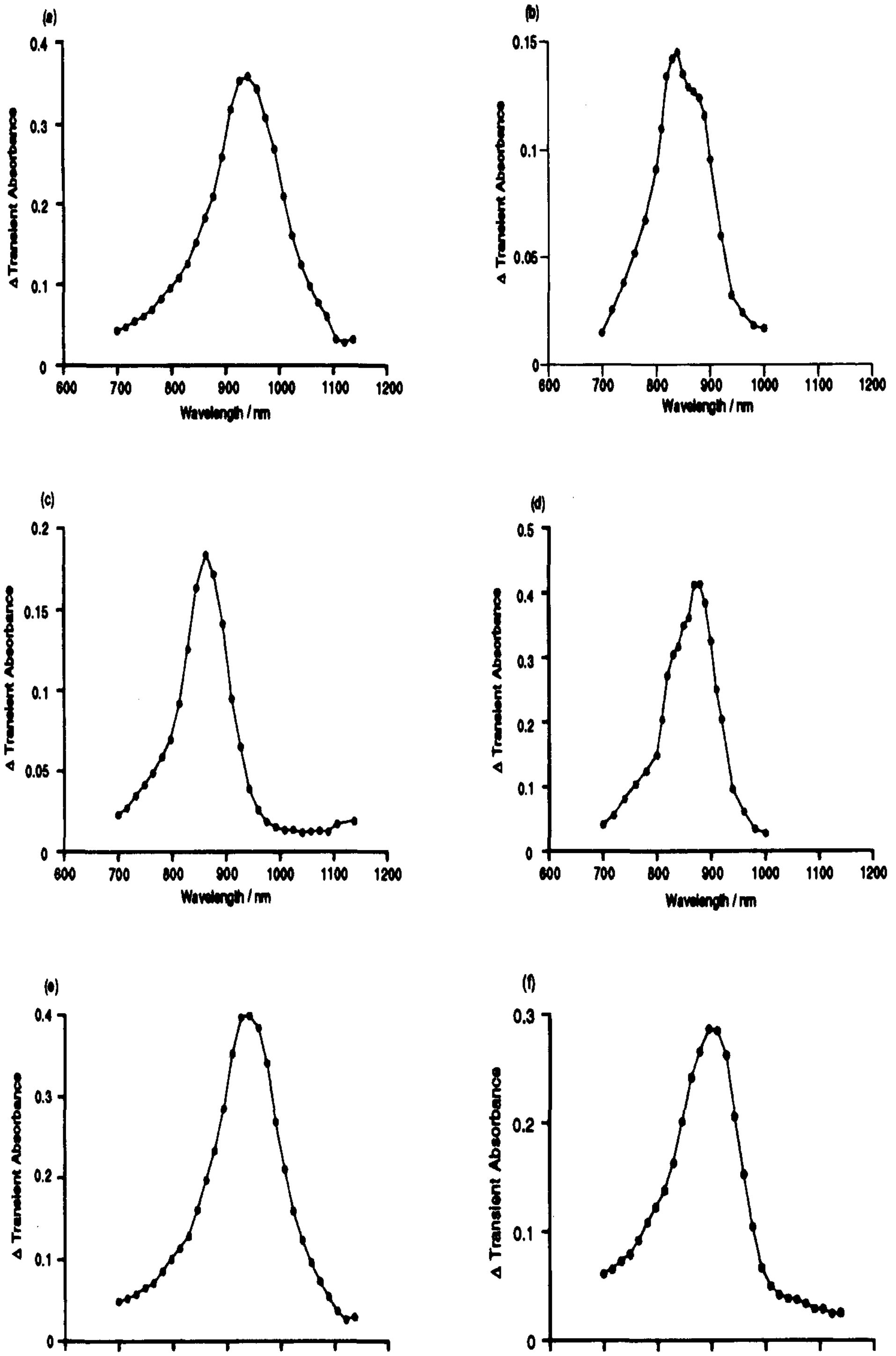

Figure 3. Maximum transient absorptions after pulse radiolysis of 10^{-4} M (a) β-carotene, (b) septapreno-β-carotene, (c) canthaxanthin, (d) astaxanthin, (e) zeaxanthin, and (f) lutein in aqueous N_2O-saturated 2% TX-100 containing 10^{-1} M NaBr + 10^{-3} M phosphate buffer, pH 7. Pulse dose 10 Gy.

and by ourselves for $O_2^{•-}$.[14] Another example of such radical addition is that of the nitrate radical ($NO_3^•$) adding to (short) conjugated polyenes.[26] Our observations of virtually identical data throughout the pH range 3−7 discounts the assignment of the 820-nm peak as the neutral radical (Car•) converting by proton addition to the radical cation, although such neutral radicals and their protonation equilibria have been discussed in dichloromethane.[27] The formation of a carotenoid dication is also unlikely since we are initiating one-electron reactions.

Examination of the spectra in Figure 2 shows that the radical cation peak grows in as the shorter wavelength peak decays. In fact, within experimental error (see Table 2), the rate of decay of the addition radical matches the rate of formation of the radical cation (see, for example, Figure 4 for lutein). This is strong evidence that the short wavelength species (the addition radical) decays to the radical cation. As can be seen from Table 2 all the carotenoid radical cations studied, except astaxanthin, are formed by two processes, a fast initial step ($k = 2.2 \pm 0.4 \times 10^5\ s^{-1}$) and a slow step ($k = 1.2 \pm 0.2 \times 10^4\ s^{-1}$), with the

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Table 1. Absorption Maxima for Various Carotenoid Cations in Aqueous 2% TX-100 and Hexane

carotenoid (n)[d]	λ_{max} of radical cation (nm) TX-100[a]	hexane
β-carotene (11)	936	1040[c]
septapreno-β-carotene (9)	850	915[b]
canthaxanthin (11)	862	960[b]
astaxanthin (11)	875	940[a]
zeaxanthin (11)	936	1040[a]
lutein (10)	900	973[a]

[a] This study. [b] Reference 24. [c] Reference 17. [d] n is the number of conjugated C—C double bonds.

Table 2. First-Order or Pseudo-First-Order Rate Constants, k ($\pm$20%), for the Radical Processes Involving $CCl_3O_2^{\bullet}$ and 10^{-4} M Carotenoid

	k/s^{-1}			
carotenoid	adduct growth $\times 10^5$	adduct decay $\times 10^4$	initial cation growth $\times 10^5$	final cation growth $\times 10^4$
β-carotene	2.0	1.5	2.2	1.0
septapreno-β-carotene	2.0	2.3	1.8	1.4
canthaxanthin	2.1	1.9	2.4	1.5
astaxanthin	1.4	1.5		1.5
zeaxanthin	1.9	1.4	1.9	1.0
lutein	2.9	1.3	2.6	1.1

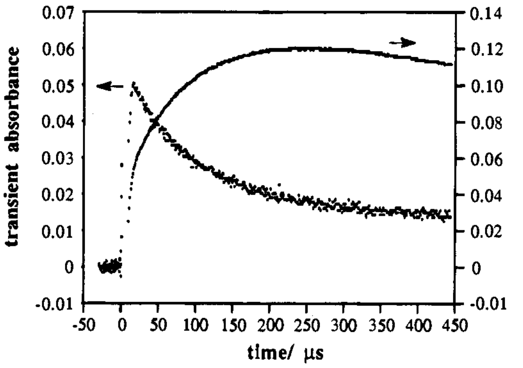

Figure 4. Kinetic absorption traces observed at 730 nm (left axis) and 905 nm (right axis) following pulse radiolysis of 10^{-4} M lutein in aqueous air-saturated 2% TX-100 containing 2 M 2-propanol, 1 M acetone, 5×10^{-2} M CCl_4, and 10^{-3} M phosphate buffer, pH 7. The traces show respectively the decay of the lutein·$^{\bullet}CCl_3O_2^{\bullet}$ addition radical (730 nm) and the concomitant growth of the lutein radical cation (905 nm). On both traces, the absorption changes in the first 5 μs are due to the primary reaction of the carotenoid with $CCl_3O_2^{\bullet}$.

slow step being equal (within experimental error) to the initial rate of decay of the adduct ($k = 1.8 \pm 0.5 \times 10^4$ s^{-1}). For astaxanthin it seems that the radical cation is formed exclusively from the adduct. It is interesting to note that it is this carotenoid that has been proposed recently to be the most effective at suppressing the peroxidation of methyl linoleate.[28]

Previous work on other oxidizing radical systems ($OH^{\bullet}$ addition to phenols) has shown that radical addition processes may be readily followed by elimination reactions.[29] Presumably, in the present work, the adduct is a less thermodynamically stable species than the corresponding fully conjugated radical cation.

Table 2 (columns 1 and 3) also shows that, apart from astaxanthin, the initial fast formation of both the radical cation and the shorter wavelength adduct is equal. This is expected since they both follow the decay of the $CCl_3O_2^{\bullet}$ radical.

Table 3. Molar Absorption Coefficients (ϵ) and Second-Order Rate Constants, $2k$ ($\pm$20%), for the Decays of the Radical Cations of Carotenoids in 2% TX-100

carotenoid	$10^4\epsilon$/M^{-1} cm^{-1} [a]	$10^7 2k$/M^{-1} s^{-1}
β-carotene	8.7 (936 nm)	6.3
septapreno-β-carotene	3.7 (850 nm)	14.0
canthaxanthin	6.7 (862 nm)	4.0
astaxanthin	3.0 (875 nm)	3.3
zeaxanthin	4.1 (936 nm)	1.8
lutein	6.7 (900 nm)	2.3

[a] Absorbed doses were determined from the transient $(SCN)_2^{\bullet-}$ formation in air-saturated aqueous 10^{-2} M KSCN solutions, using $G = 0.30$ μM/Gy and $\epsilon_{500nm} = 7100$ M^{-1} cm^{-1}.[30] The molar absorption coefficients were based on a yield of $Br_2^{\bullet -}$ radicals of 0.62 μM/Gy.[31]

The rates of decay of the carotenoid radical cation show that they follow pseudo-first-order kinetics in the presence of the rather large concentrations of 2-propanol and acetone, but mainly second-order kinetics in the absence of these solutes. In Table 3 we report the second-order rate constants and molar absorption coefficients for the carotenoid radical cations in TX-100. As can be seen, the non-oxy-substituted carotenoids (β-carotene and septapreno-β-carotene) show the fastest second-order decay, possibly suggesting a role for steric hindrance in such second-order processes.

Clearly a more definite assignment of the short wavelength species is desirable and as a control experiment we investigated the effect of removing oxygen from the system. This leads to generation of carbon-centered $CCl_3^{\bullet}$ radicals rather than oxy-centered $CCl_3O_2^{\bullet}$ radicals. In the absence of oxygen only the radical cation of the carotenoids is formed. Since adducts have been observed with $O_2^{\bullet-}$,[14] and, for much shorter polyenes, with $NO_3^{\bullet}$ [26] and now with $CCl_3O_2^{\bullet}$, this suggests that oxy-containing radicals are required for adduct formation.

In conclusion, we report that all carotenoids studied react readily with the peroxyl radical arising from CCl_4 and there is no significant difference in reaction rates. However, astaxanthin does behave rather differently from the other carotenoids studied in that the initial reaction is exclusively radical addition. Overall our results suggest that such carotenoids could indeed exert a protective effect with respect to the damage by $CCl_3O_2^{\bullet}$ and peroxyl radicals in general in man. We are extending our investigations to a wider range of oxygen-containing carotenoids (astaxanthin contains 4 oxygen atoms) and oxy radicals.

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