Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants

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Abstract The comparative mechanisms and relative rates of nitrogen dioxide ($NO_2^{\bullet}$), thiyl ($RS^{\bullet}$) and sulphonyl ($RSO_2^{\bullet}$) radical scavenging by the carotenoid antioxidants lycopene, lutein, zeaxanthin, astaxanthin and canthaxanthin have been determined by pulse radiolysis. All the carotenoids under study react with the $NO_2^{\bullet}$ radical via electron transfer to generate the carotenoid radical cation ($Car^{\bullet+}$). In marked contrast the glutathione and 2-mercaptoethanol thiyl radicals react via a radical addition process to generate carotenoid-thiyl radical adducts [RS-Car]$^{\bullet}$. The $RSO_2^{\bullet}$ radical undergoes both radical addition, [RSO_2-Car]$^{\bullet}$ and electron abstraction, $Car^{\bullet+}$. Both carotenoid adduct radicals and radical cations decay bimolecularly. Absolute rate constants for radical scavenging were in the order of $\sim 10^7$–10^9 M^{-1} s^{-1} and follow the sequence $HO(CH_2)_2S^{\bullet} > RSO_2^{\bullet} > GS^{\bullet} > NO_2^{\bullet}$. Although there were some discernible trends in carotenoid reactivity for individual radicals, rate constants varied by no greater than a factor of 2.5. The mechanism and rate of scavenging is strongly dependent on the nature of the oxidising radical species but much less dependent on the carotenoid structure.

Key words: Carotenoid; Thiyl radical; Nitrogen dioxide radical; Sulphonyl radical; Pulse radiolysis

1. Introduction

Although there is considerable epidemiological evidence linking a high dietary intake of carotenoids to a decreased risk of certain cancers [1–3], a number of clinical trials have shown that supplementation with β-carotene is not only ineffective but also induces adverse effects [4–6]. In vitro carotenoids are able to inhibit the oxidation of low-density lipoproteins (LDL) [7–9] and lipids in model systems [10–12]. Although it has been known for some time that β-carotene is an efficient peroxyl radical scavenger [13], the exact mechanism(s) by which carotenoids scavenge free radicals has only been recently addressed [14]. Pulse radiolysis has demonstrated that the carotenes and xanthophylls rapidly scavenge the halogenated peroxyl radical ($CCl_3OO^{\bullet}$) [15–17] and that the reactivity of superoxide ($O_2^{\bullet-}$) is higher with lycopene than β-carotene [18]. Free radicals which can initiate the chain of lipid peroxidation can be scavenged by β-carotene by two, not necessarily exclusive mechanisms. β-Carotene scavenges $NO_2^{\bullet}$ [19] and $CCl_3OO^{\bullet}$ [17] radicals mainly by electron transfer to generate the carotenoid radical cation. In marked contrast, $RS^{\bullet}$ [19] and $O_2^{\bullet-}$ radicals [20] react exclusively via a radical addition process to generate adduct radicals. The sulphonyl radical ($RSO_2^{\bullet}$), generated by conjugation of the $RS^{\bullet}$ radical with molecular oxygen, is scavenged by both electron transfer and radical addition processes [19]. Laser photolysis studies with phenoxyl and chloroform-derived radicals confirm the ability of β-carotene to scavenge free radical species via parallel electron transfer and addition processes [21,22]. Evidence for β-carotene radical adducts and radical cations has also been provided by product analysis in model systems [23,24]. Other studies have identified a hierarchy in carotenoid scavenging ability where the carotenes appeared to be more efficient scavengers than the xanthophylls [25–27].

In the present investigation the comparative mechanisms and relative rates of $NO_2^{\bullet}$, $RS^{\bullet}$, and $RSO_2^{\bullet}$ radical scavenging by the carotene lycopene, and the xanthophylls lutein, zeaxanthin, astaxanthin, and canthaxanthin have been investigated by pulse radiolysis. The mechanism(s) and rates of scavenging are mainly dependent on the nature of the oxidising radical species but much less dependent on the carotenoid structure.

2. Materials and methods

2.1. Materials

Lycopene, astaxanthin, canthaxanthin, zeaxanthin, and lutein were all supplied by Roche A/S, Denmark. *tert*-Butanol (HPLC grade), anhydrous toluene, 2-mercaptoethanol, methanesulphonyl chloride, and glutathione were obtained from the Sigma-Aldrich Company Ltd (Poole, UK). Potassium nitrate and tetrachloromethane were from BDH Chemicals Ltd (Lutterworth, UK) and were of analar grade.

2.2. Pulse radiolysis

The pulse radiolysis technique is a useful means of characterising carotenoid radicals and their reactions on μs–ms timescales [28]. The facility at the Gray Laboratory Cancer Research Trust has already been described and utilises optical detection of carotenoid radicals and bleaching of the carotenoid ground-state absorption to determine rates and mechanisms of free radical scavenging [19]. In this study 500 ns pulses of 6 MeV electrons were used to deliver doses of up to 5 Gy as determined by thiocyanate dosimetry [29]. Near infrared detection of carotenoid radicals was performed using a tungsten lamp and a Si photodiode and bleaching of the carotenoids by a photomultiplier utilising a xenon lamp.

Solutions of 10 μM carotenoid (to allow adequate light transmission in the 300–500 nm spectral region) in *tert*-butanol/water mixtures (60:40%, v/v) were made from stock solutions of carotenoids in toluene. To each of these solutions were added either potassium nitrate (0.1 M), methanesulphonyl chloride (0.1 M), 2-mercaptoethanol (20 mM), glutathione (2 mM) or tetrachloromethane (0.1 M). The pH of solutions containing potassium nitrate was adjusted to around 5 by adding dilute perchloric acid. The solutions containing 2-mercaptoethanol and glutathione were bubbled with nitrous oxide (N_2O) and solutions of methanesulphonyl chloride and potassium nitrate were degassed with nitrogen. Lycopene was found to aggregate in a

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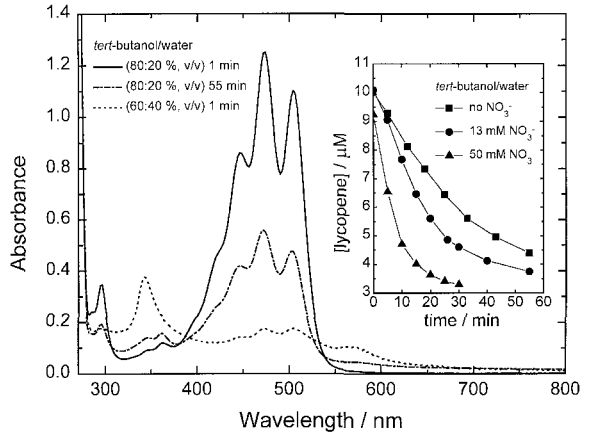

Fig. 1. Absorption spectra showing the aggregation of lycopene in *tert*-butanol/water mixtures. The insert shows the rate of loss of 10 μM lycopene in a *tert*-butanol water mixture (80:20%, v/v) containing various concentrations of nitrate.

(60:40%, v/v) mixture of *tert*-butanol and water, as evidenced by partial loss of ground-state absorption, the appearance of two new bands at 340 and 570 nm, and absorption extending into the far red (Fig. 1) due to aggregation [30]. The measurements on lycopene were therefore performed in an (80:20%, v/v) mixture of *tert*-butanol and water. Lycopene still aggregates in this mixture but to a much smaller extent and only slowly (Fig. 1). The kinetic experiments were performed by bubbling the *tert*-butanol/water mixture, adding the lycopene stock solution while bubbling and measure the time traces within ca. 5–10 min of adding the lycopene thereby minimising the extent of aggregation during the experiments. Potassium nitrate was found to increase the rate and extent of aggregation (Fig. 1) and lower concentrations (0.01 M for the kinetics and 0.05 M for the spectral analysis) were therefore employed. The rates were found to decrease by less than 10% in (80:20%, v/v) mixtures of *tert*-butanol and water compared to (60:40%, v/v) mixtures.

Nitrogen dioxide is generated by reduction of nitrate by solvated (e_{sol}^-) and hydrated (e_{aq}^-) electrons [31] according to reaction 1 followed by rapid protonation and dissociation of the nitrate radical anion (NO_3^{2-}) at pH 5 via reaction 2:

$$e_{aq}^-/e_{sol}^- + NO_3^- \rightarrow NO_3^{2-} \quad (1)$$

$$NO_3^{2-} + H^+ \rightarrow [HNO_3^-] \rightarrow NO_2^{\bullet} + OH^- \quad (2)$$

Nitrous oxide converts electrons into hydroxyl radicals which are then rapidly scavenged by *tert*-butanol according to reactions 3 and 4:

$$e_{aq}^-/e_{sol}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \quad (3)$$

$$(CH_3)_3COH + {}^{\bullet}OH \rightarrow {}^{\bullet}CH_2(CH_3)_2COH + H_2O \quad (4)$$

The $^{\bullet}CH_2(CH_3)_2COH$ radicals exhibit negligible reactivity toward the carotenoids even on millisecond timescales. Thiyl radicals ($RS^{\bullet}$) are generated by the classical repair reaction [32,33] which involves hydrogen atom transfer from the thiol to the carbon-centred radical:

$$^{\bullet}CH_2(CH_3)_2COH + RSH \rightarrow (CH_3)_3COH + RS^{\bullet} \quad (5)$$

The thiyl-sulphonyl radical was generated by dissociative electron capture by methanesulphonyl chloride [34]:

$$e_{aq}^-/e_{sol}^- + CH_3SO_2Cl \rightarrow RSO_2^{\bullet} + Cl^- \quad (6)$$

The solvated and hydrated electrons are scavenged by tetrachloromethane to give the trichloromethyl radical ($CCl_3^{\bullet}$) which rapidly conjugates molecular oxygen to generate the corresponding halogenated peroxyl radical [35]:

$$e_{aq}^-/e_{sol}^- + CCl_4 \rightarrow CCl_3^{\bullet} + Cl^- \quad (7)$$

$$CCl_3^{\bullet} + O_2 \rightarrow CCl_3O_2^{\bullet} \quad (8)$$

The radiation chemical yields (G) of the various free radical species were previously determined as $G(NO_2^{\bullet}) = G(RSO_2^{\bullet}) = 0.09$ μmol J^{-1} and $G(RS^{\bullet}) = 0.55$ μmol J^{-1} [19] and used to determine the rate of decay of carotenoid radicals. Typical doses per pulse of <5 Gy (where 1 Gy = 1 J kg^{-1}) resulted in $<30\%$ conversion of carotenoid (10 μM) to products.

3. Results

3.1. Carotenoid scavenging of nitrogen dioxide

The oxidation of the carotenoids by nitrogen dioxide generated the transient spectra shown in Fig. 2. For all the carotenoids under study the spectra were characterised by an increase in absorption in the near infrared region (>700 nm) and a bleaching of the carotenoid ground-state absorption (<600 nm). The former has previously been assigned to the carotenoid radical cations [17,19,36]. The absorption maxima of the radical cations are 850 nm for canthaxanthin and astaxanthin, 910 nm for zeaxanthin, 880 nm for lutein, and 960 nm for lycopene. These values are similar to those generated by the $CCl_3OO^{\bullet}$ radical in a micellar system [17]. Although the absorption bands of the carotenoid radical cations are asymmetric showing a distinct shoulder in the region 700–900 nm (dependent on the carotenoid) the rate of growth and decay of the radical absorption is identical across the entire absorption band indicating that this band is only due to a single radical species. Consistent with the previous observations for β-carotene [19] the carotenoids all react with the $NO_2^{\bullet}$ radical via an electron transfer process (reaction 9) with-

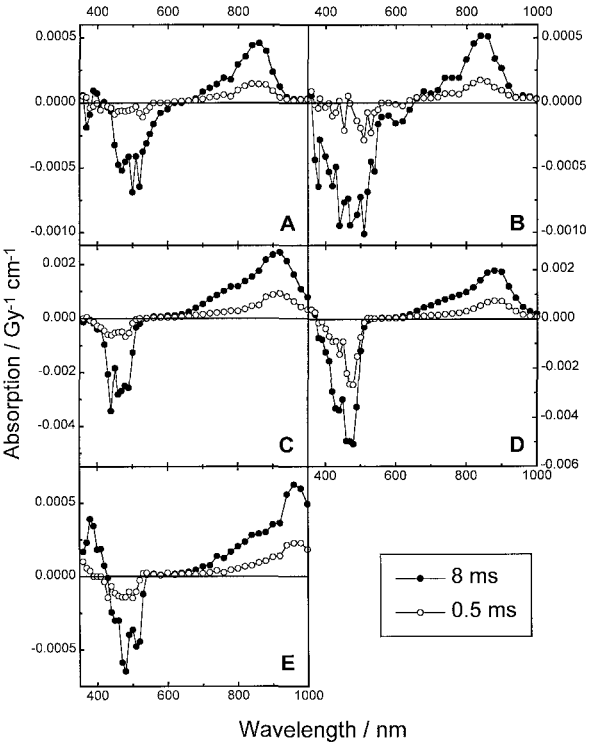

Fig. 2. Spectra obtained on the scavenging of $NO_2^{\bullet}$ radicals by various carotenoids. Spectra recorded at 500 μs and 8 ms after pulse radiolysis (1.5 Gy) of 10 μM (A) astaxanthin, (B) canthaxanthin, (C) zeaxanthin, (D) lutein and (E) lycopene. All experiments were carried out in N_2-saturated *tert*-butanol/water (60:40%, v/v) mixtures containing 0.1 M nitrate except lycopene which was 50 mM nitrate in *tert*-butanol/water (80:20%, v/v).

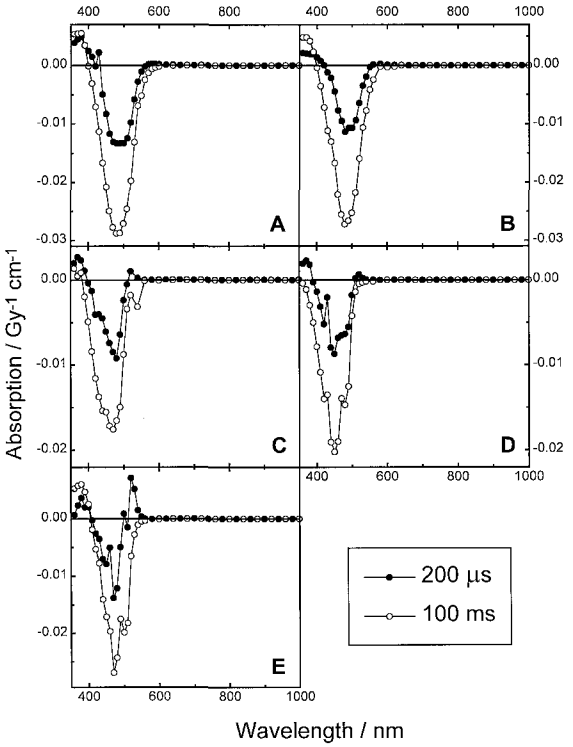

Fig. 3. Spectra obtained on the scavenging of $HO(CH_2)_2S^{\bullet}$ radicals by various carotenoids. Spectra recorded at 200 μs and 100 ms after pulse radiolysis (1.5 Gy) of 10 μM (A) astaxanthin, (B) canthaxanthin, (C) zeaxanthin, (D) lutein and (E) lycopene. All experiments were carried out in N_2O-saturated *tert*-butanol/water (60:40%, v/v) mixtures containing 20 mM β-mercaptoethanol except lycopene which was in *tert*-butanol/water (80:20%, v/v).

out any intermediate radical adduct $[NO_2\text{-Car}]^{\bullet}$ formation.

$$Car + NO_2^{\bullet} \rightarrow Car^{\bullet+} + NO_2^- \quad (9)$$

Concurrently with carotenoid radical cation formation is the loss of ground-state absorption of the carotenoid in the region 400–600 nm. The bleaching of the carotenoid ground-state absorption and formation of the radical cation were both exponential and first-order in carotenoid concentration. The rates of $NO_2^{\bullet}$ radical scavenging by each carotenoid were determined from the slopes of the individual linear plots (data not shown) of the observed rate of radical cation formation versus carotenoid concentration and are displayed in Table 1. The carotenoids exhibit a narrow range of reactivity (less than a factor of 2 between the fastest zeaxanthin and slowest canthaxanthin) toward the $NO_2^{\bullet}$ radical with $k_9 = (1.2\text{–}1.9) \times 10^7$ M^{-1} s^{-1}. Within the limits of error lycopene scavenges nitrogen dioxide as fast as zeaxanthin with astaxanthin exhibiting a slower reactivity on a par with that of canthaxanthin. Lutein has an intermediate reactivity falling between zeaxanthin and canthaxanthin.

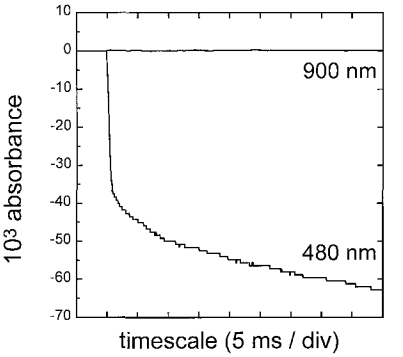

Fig. 4. Typical kinetic traces recorded at 480 and 900 nm for the scavenging of $HO(CH_2)_2S^{\bullet}$ radicals by astaxanthin under the experimental conditions described in Fig. 3. The biphasic bleaching of the ground-state absorption of astaxanthin at 480 nm is not accompanied by an absorption change at 900 nm where the radical cation is expected to absorb (see Fig. 3A).

The carotenoid radical cation decayed by pure second-order kinetics with a half-life which decreased with an increase in the initial concentration of radicals. This was attributed to the bimolecular decay of the radical cations via reaction 10.

$$2Car^{\bullet+} \rightarrow product(s) \quad (10)$$

The rate constants k_{10} were determined from the slopes of the linear plots of the reciprocal of the first half-life of the $Car^{\bullet+}$ radical cation versus initial radical concentration and are displayed in Table 1. The less polar radical cation of lycopene decays faster than the radical cations of the other more polar carotenoids in the rather polar solvent mixture. This is the opposite of what is observed in more lipophilic solvents like chloroform [27]. The rate of decay of the astaxanthin radical cation could not be determined due to the low solubility of astaxanthin in the toluene stock solution.

3.2. *Carotenoid scavenging of the 2-mercaptoethanol thiyl radical*

Reaction between $HOCH_2CH_2S^{\bullet}$ and the carotenoids leads to loss of ground-state absorption of the carotenoids but no transient absorption was observed at wavelengths longer than 600 nm (Fig. 3). A small positive transient absorption is observed between 500 and 600 nm (Fig. 3) indicating that a transient species absorbing in the same spectral region as the carotenoid is being formed. This positive transient absorption appears stronger in the case of lycopene. We ascribe this to aggregation taking place in the rather polar reaction me-

Table 1
Absolute rate constants for the formation and decay of carotenoid radicals (see text for experimental details)

Carotenoid	Absolute rate constants (M^{-1} s^{-1})						
	$NO_2^{\bullet}$		GS$^{\bullet}$		$HO(CH_2)_2S^{\bullet}$		$CH_3SO_2^{\bullet}$
	10^{-7} k_9	10^{-7} $2k_{10}$	10^{-8} k_{11}	10^{-8} $2k_{12}$	10^{-9} k_{11}	10^{-7} $2k_{12}$	10^{-8} k_{13}
Canthaxanthin	1.2±0.1	1.1±0.2	6.1±0.2	2.5±0.2	1.5±0.1	2.6±0.1	10.3±0.1
Astaxanthin	1.3±0.1	0.4±0.1	6.6±0.2	2.0±0.2	1.5±0.1	1.4±0.1	9.4±0.1
Lutein	1.6±0.1	0.8±0.2	2.4±0.2	1.6±0.2	1.0±0.1	5.0±0.1	9.2±0.1
Zeaxanthin	2.1±0.1	1.1±0.2	2.5±0.2	1.2±0.2	1.7±0.1	4.6±0.2	9.9±0.1
Lycopene	1.9±0.1	4.0±0.2	4.8±0.4	1.3±0.1	1.6±0.1	6.6±0.2	12.6±0.1

dium (see Section 2). Consequently, the resultant carotenoid radical species has a greater absorption than that of the lycopene ground-state absorption. The bleaching of the carotenoids is biphasic (Fig. 4) with a fast reaction first-order in carotenoid concentration and a slower second-order reaction. The fast step is due to reaction between carotenoid and $HOCH_2CH_2S^{\bullet}$. This reaction has been previously proposed to yield an adduct [19] via reaction 11.

$$Car + RS^{\bullet} \rightarrow [RS - Car]^{\bullet} \quad (11)$$

It is clear from the absence of the radical cation of the carotenoids in the spectra (Fig. 4) that the thiyl radical does not react with the carotenoids by electron transfer. The second slower step in the bleaching of the carotenoids is due to the bimolecular decay of the adduct formed in reaction 11:

$$2[RS{-}Car]^{\bullet} \rightarrow \text{product(s)} \quad (12)$$

The positive transient absorption observed below 400 nm (Fig. 3) is probably due to both the adduct and the product(s) formed in reaction 12, as it is formed at the same rate as the fast bleaching, but it does not decrease as the adduct decays because the degradation product(s) absorb(s) in the same spectral region as the radical adduct.

All the carotenoids react very rapidly with the highly reactive thiyl radical (Table 1) with a rate constant in the order of

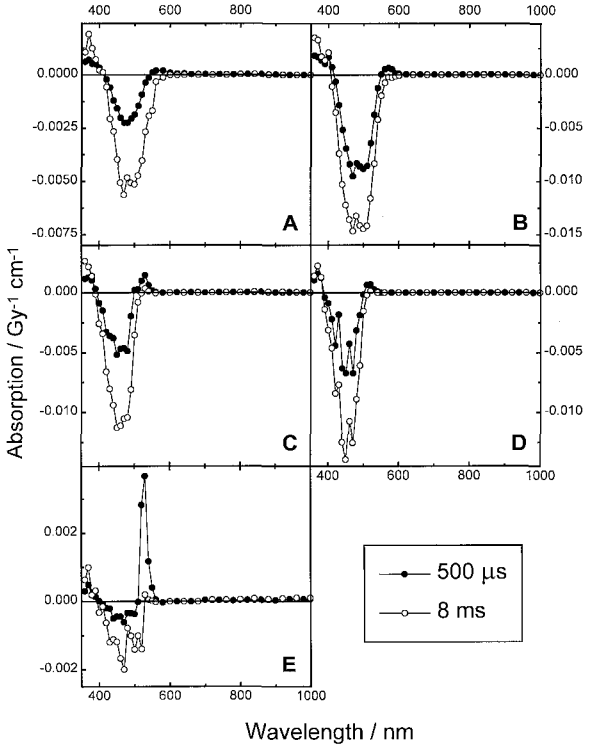

Fig. 5. Spectra obtained on the scavenging of GS$^{\bullet}$ radicals by various carotenoids. Spectra recorded at 500 µs and 8 ms after pulse radiolysis (1.5 Gy) of 10 µM (A) astaxanthin, (B) canthaxanthin, (C) zeaxanthin, (D) lutein and (E) lycopene. All experiments were carried out in N_2O-saturated *tert*-butanol/water (60:40%, v/v) mixtures containing 2 mM glutathione except lycopene which was in *tert*-butanol/water (80:20%, v/v). All the carotenoids under study did not give any absorption >600 nm.

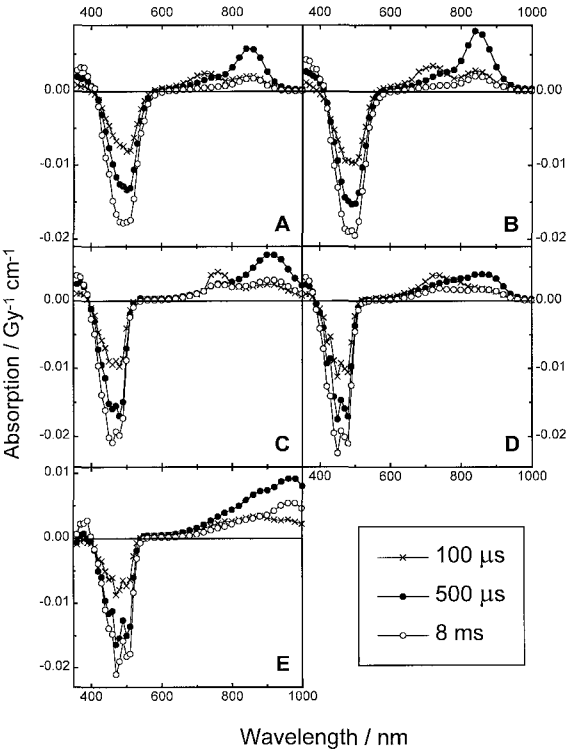

Fig. 6. Spectra obtained on the scavenging of the $CH_3SO_2^{\bullet}$ radical by various carotenoids recorded at 100 µs and 8 ms after pulse radiolysis (1.5 Gy) of 10 µM (A) astaxanthin, (B) canthaxanthin, (C) zeaxanthin, (D) lutein and (E) lycopene. All experiments were carried out in N_2-saturated *tert*-butanol/water (60:40%, v/v) mixtures containing 0.1 M methanesulfonyl chloride except lycopene which was in *tert*-butanol/water (80:20%, v/v).

ca. $k_{11} \sim 10^9$ M^{-1} s^{-1}. Astaxanthin, canthaxanthin, zeaxanthin and lycopene all react at the same rate, whereas lutein, having one less conjugated double bond, reacts somewhat slower. The less polar lycopene-thiyl radical adduct(s) decay faster than the other more polar carotenoid-thiyl radical adducts, as was observed for the lycopene radical cation (Table 1), and the more polar canthaxanthin and astaxanthin adducts decay with the slowest rate.

3.3. Carotenoid scavenging of glutathione thiyl radicals

GS$^{\bullet}$ radicals are scavenged by carotenoids in the same way as 2-mercaptoethanol-thiyl radicals are, i.e. by adduct formation via reaction 11. The bleaching is also biphasic and there is no transient absorption above 600 nm (Fig. 5). Again, positive transient absorption between 500 and 600 nm is observed. With the more polar and less reactive glutathione-thiyl radical the rate of scavenging is slower by a factor of 2–7 than with the 2-mercaptoethanol-thiyl radical (Table 1). The more polar carotenoids astaxanthin and canthaxanthin scavenge the glutathione-thiyl radical faster than the less polar carotenoids zeaxanthin and lutein. Although lycopene is the most hydrophobic carotenoid, the greater number of conjugated double bonds probably enough to elevate lycopene above zeaxanthin and lutein in the hierarchy of GS$^{\bullet}$ radical scavenging. The glutathione-carotenoid radical adduct(s) decay faster than the corresponding 2-mercaptoethanol-carotenoid radical adduct(s), and the astaxanthin and canthaxanthin adducts in this case decay fastest.

3.4. Carotenoid scavenging of thiyl-sulphonyl radicals

Scavenging of $CH_3SO_2^{\bullet}$ by carotenoids leads to formation of transient species absorbing in the near infrared (Fig. 6). The relative change in absorption with time varies with wavelength in the region 600–1000 nm indicating that more than one species is being formed. The time traces (Fig. 7A) show that one species absorbing at 700–800 nm (dependent on the carotenoid, Fig. 6) forms fast and decays rapidly (within 1 ms) whereas the other species absorbing at 850–970 nm (Fig. 6) is formed more slowly and decays more slowly than the other species. This latter species can be identified as the carotenoid radical cation by comparison of the transient spectra obtained by reaction with nitrogen dioxide (Fig. 2). The time trace at 700 nm, besides formation and decay of the rapidly formed species also, shows the contribution from slower formation and decay of the carotenoid radical cation because this latter species has a rather broad absorption band (Fig. 2) thereby making determination of the individual rates of formation and decay of the first formed intermediate difficult. However, the decay of the rapidly formed species correlates with the formation of the carotenoid radical cation (Fig. 7A) showing that a consecutive reaction takes place:

$$\text{Car} + CH_3SO_2^{\bullet} \rightarrow \text{Intermediate} \rightarrow \text{Car}^{\bullet+} + CH_3SO_2^{-} \quad (13)$$

Scavenging of $CH_3SO_2^{\bullet}$ leads to loss of carotenoid ground-state absorption in the spectral region 400–550 nm (Fig. 6). The bleaching is biphasic with a fast step first-order in carotenoid concentration and a slower second-order step (Fig. 7B), as was observed for the scavenging of thiyl radicals (Fig. 4). The rate of the fast bleaching is of the order of $k_{13} \sim 10^9$ M^{-1} s^{-1} with lycopene reacting slightly faster than the other four carotenoids (Table 1). The fast first-order bleaching correlates with the formation of the species absorbing at 700–800 nm (Fig. 7A). The slower second-order bleaching must be due to a species being formed that absorbs in the same spectral region as the parent carotenoid as was observed by scavenging of thiyl radicals. Previously, it has been suggested that this species is an adduct between the carotenoid and the $CH_3SO_2^{\bullet}$ radical [19].

$$\text{Car} + CH_3SO_2^{\bullet} \rightarrow [CH_3SO_2 - \text{Car}]^{\bullet} \quad (14)$$

The slower second-order step is then due to bimolecular decay of this adduct:

$$2[CH_3SO_2 - \text{Car}]^{\bullet} \rightarrow \text{product(s)} \quad (15)$$

The reaction between carotenoids and $CCl_3O_2^{\bullet}$ shows the same pattern of reaction, i.e. formation of an intermediate absorbing around 750 nm which decays to the radical cation. This initially formed intermediate was believed to be an adduct [17]. However, the reaction between carotenoids and $CCl_3O_2^{\bullet}$ also shows a biphasic bleaching indicating that an adduct is formed but that it absorbs in the same spectral region as the parent carotenoid and not around 750 nm. The intermediate in reaction 13 decaying to the carotenoid radical cation could be an ion-pair as has recently been suggested to be formed as an intermediate decaying to the carotenoid radical cation upon photobleaching of carotenoids in chloroform [27]. However, the characterisation of this intermediate remains elusive. It is interesting to note that nitrogen dioxide produces the carotenoid radical cation without apparently forming this intermediate. Another possibility for the nitrogen dioxide radical is that an adduct is formed but that it decays just as fast as it is formed thereby escaping detection.

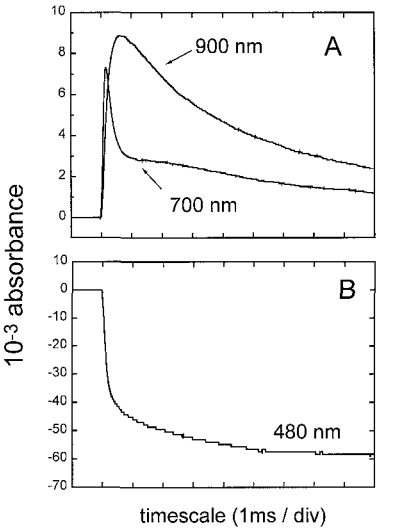

Fig. 7. Typical kinetic traces recorded at various wavelengths for the scavenging of $CH_3SO_2^{\bullet}$ radicals by astaxanthin under the experimental conditions described in Fig. 6. Panel A shows the rapid formation of the intermediate at 700 nm which subsequently decay to the radical cation at 900 nm. Panel B is the corresponding free radical bleaching of the astaxanthin ground-state absorption at 480 nm on the same timescale.

Comparing formation of the carotenoid radical cation formed by reaction with nitrogen dioxide and by reaction with $CH_3SO_2^{\bullet}$. to the extent of bleaching of the carotenoid shows that in the case of $CH_3SO_2^{\bullet}$, the bleaching relative to formation of radical cation is higher than in the case of $NO_2^{\bullet}$ confirming that $CH_3SO_2^{\bullet}$ reacts by more than one pathway. However, it was not possible reliably to determine the relative importance of the two pathways. The proportion of radical cation to radical adduct seems however, to be rather similar for all five carotenoids (Fig. 6).

4. Discussion

Previous studies have demonstrated that the initial interaction of a variety of free radicals with carotenoids results in electron transfer and $\text{Car}^{\bullet+}$ radical cation formation and/or radical addition, which gives rise to carotenyl adduct radicals [17–19]. In general, the rates of these reactions compare favourably with the corresponding reactions with the same oxidants and PUFAs or other major antioxidants such as the tocopherols, indicating that carotenoids possess the required reactivity to function as efficient antioxidants. Carotenoid structure may have a bearing on both the mechanism(s) and relative rates of free radical scavenging [14]. For example, the interaction of the stable radical cation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate), $ABTS^{\bullet+}$, demonstrated a ranking of carotenoids as antioxidants which followed the sequence lycopene > β-carotene = β-cryptoxanthin (-3-ol) > lutein/zeaxanthin (-3,3′-diol) > echinenone (-4-one) > canthaxanthin (-4,4′-dione) = astaxanthin (-3,3′-dihydroxy-4,4′-dione) [25]. Electrochemical studies on both natural and synthetic carotenoids confirm that carotenoids substituted with elec-

tron-donating groups are more easily oxidised than those with electron-accepting substituents [37,38]. Therefore, substitution of hydrogen by carbonyl groups at the 4 and 4′ positions reduces the unpaired electron density across the 11-double-bonded carbon skeleton resulting in a decreased propensity for electron donation and has been offered as an explanation for the lack of reactivity of the $ABTS^{\bullet+}$ with astaxanthin and canthaxanthin [14]. Similarly to β-carotene [19] the other carotenoids react exclusively with $NO_2^{\bullet}$ to generate the carotenoid radical cation. Although the sequence of reactivity closely resembles that for the $ABTS^{\bullet+}$ radical cation (see Table 1) the relative reactivities fall in a narrow range of $(1.2–2.1 \pm 0.1) \times 10^7$ M^{-1} s^{-1}. This study corroborates our previous findings with β-carotene that scavenging of $NO_2^{\bullet}$ radicals by carotenoids in general does not generate carotenyl adduct radicals although a recent study has suggested the contrary [24]. The requirement for electron transfer mechanisms in the case of the $NO_2^{\bullet}$ radical might explain the ready reactivity of this radical with the electron-dense structures of carotenes and its slower reactivity toward the ketoxanthophylls which exhibit greater electron delocalisation across the polyene chain. The lycopene radical cation decays faster than the other carotenoid radical cations (see Table 1). This is probably due to unfavourable solvation of the less polar lycopene radical cation compared to the more polar radical cations of the other carotenoids. The radical cations of the other carotenoids seem to decay at roughly the same rate.

Astaxanthin and canthaxanthin are more effective antioxidants than β-carotene or zeaxanthin in retarding hydroperoxide formation on azo-initiated lipid peroxidation in homogeneous methyl linoleate/AMVN systems [39]. It was proposed that substitution of the hydrogens with carbonyl groups at the 4 and 4′ positions increases the overall peroxyl radical trapping efficiency (astaxanthin ≈ canthaxanthin ≫ β-carotene ≈ zeaxanthin) by virtue of the fact that the electron-withdrawing character of the carbonyl oxygen atoms substantially reduces the unpaired electron density throughout the carbon skeleton, thereby decreasing the reactivity of the carbon-centred radical toward oxygen. The ranking of the antioxidant activities of astaxanthin > canthaxanthin > β-carotene > zeaxanthin in a homogeneous methyl linoleate/AIBN system is consistent with the idea that the ketocarotenoids are more effective antioxidants against lipid peroxidation in vitro [39,40]. Carotenoid radical adducts have been identified on the scavenging of AMVN-derived alkyl peroxyl radicals by β-carotene [23]. Both the thiyl radicals of glutathione and 2-mercaptoethanol exhibit contrasting mechanisms and sequence of reactivity to the $NO_2^{\bullet}$ during scavenging by the carotenoids. Both thiyl radicals react exclusively with the carotenoids via radical addition to generate carotenyl adduct radicals. In the case of the $GS^{\bullet}$ radical the ketocarotenoids astaxanthin and canthaxanthin exhibit a higher reactivity than the unsubstituted carotenoids suggesting a greater propensity to participate in radical addition pathways. The high reactivity of the $HO(CH_2)_2S^{\bullet}$ radical ($k_{11} \sim 10^9$ M^{-1} s^{-1}) toward the carotenoids made it difficult to identify trends in reactivity but no discernable differences were observed between the ketocarotenoids and the rest of the unsubstituted carotenoids. The lycopene thiyl radical adduct decays faster than the other carotenyl radical adducts. Zeaxanthin and lutein radical cations decay somewhat slower, whereas the most polar carotenoid radical adducts derived from canthaxanthin and astaxanthin decay at the slowest rate. The decay of the glutathione-carotenoid adduct is faster than the decay of the corresponding carotenoid-2-mercaptoethanol adduct (Table 1), and the order of rate of decay is the opposite of what is observed with this latter adduct.

The carotenoids also scavenge the thiyl-sulphonyl radical at a high rate approaching diffusion controlled. The four polar carotenoids astaxanthin, canthaxanthin, lutein and zeaxanthin all scavenge this radical at the same rate of $k_{13} = 1 \times 10^9$ M^{-1} s^{-1}. Lycopene, on the other hand, scavenges the sulphonyl-thiyl radical at a higher rate than the other four carotenoids.

In conclusion, there are no dramatic differences in the rate of carotenoid scavenging of individual radicals with rate constants varying by no greater than a factor of 2.5. The mechanism and rate of scavenging is more dependent on the nature of the radical species but much less dependent on the carotenoid structure. The effect of radical cation versus radical adduct formation on the antioxidant properties of carotenoids remains to be determined. A recent review concluded that there is little evidence as yet to suggest that the carotenoids exert their purported health-protective effects in vivo through their antioxidant properties [14]. Although this study provides a mechanistic basis for free radical scavenging by carotenes and xanthophylls, extrapolations to possible antioxidant effects in vivo must be tempered.

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