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The retinal carotenoids zeaxanthin and lutein scavenge superoxide and hydroxyl radicals: A chemiluminescence and ESR study

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Purpose: Carotenoids are present in many biological systems, often decreasing the formation of products of oxidative damage to biological molecules. In the macula their concentration is so high that it has been believed that the yellow color filters out damaging blue light. Recent reports that dietary lutein reduces the risk of cataract in the eye lens suggested that the antioxidant action of carotenoids, which has been inferred from decreased oxidative damage, warranted further direct investigation.

Methods: Superoxide and hydroxyl radical scavenging by lutein and zeaxanthin (retinal carotenoids), β-carotene, lycopene, lutein esters (from marigolds), and a commercial mixture of soy carotenoids were compared to scavenging by ascorbate and ascorbyl palmitate. Radical scavenging was measured with a chemiluminescent assay (luminol) and by electron spin resonance, ESR. Inhibitory concentrations, IC_{50}, were determined with the luminescent assay.

Results: All of the carotenoids scavenged both superoxide (in ESR 30-50% at 16.7 μM) and hydroxyl radicals (in ESR 50-70% at 16.7 μM, in a luminescent assay 90-99%).

Conclusions: While crocin may be unable to scavenge superoxide, some of the other carotenoids do so quite effectively. The mixtures of 15,15'-*cis* and all-*trans*-carotenoids studied by ESR and luminescent assay scavenge both superoxide and hydroxyl radicals. Lycopene and β-carotene both scavenge superoxide more effectively than the xanthophylls of the retina, zeaxanthin and lutein. All of the carotenoids examined scavenged the hydroxyl radicals more effectively than superoxide radicals. The predominant carotenoid in the fovea of the retina, zeaxanthin, scavenged hydroxyl radicals more effectively than the other retinal carotenoid, lutein. Possible mechanisms of radical scavenging by the carotenoids are discussed.

Carotenoids scavenge free radicals in both in vitro and in vivo systems [1-3] and quench singlet oxygen, $^1O_2$ ($^1\Delta_g$) rapidly [2,4]. These abilities are employed in many biological systems to prevent damaging oxidation. Further, carotenoids may be responsible for the decreased cancer incidence associated with the consumption of certain fruits and vegetables [1,2]. Based on an unrepresentative carotenoid, crocin, it has been assumed that carotenoids do not directly scavenge free radicals [5], except for the destruction of singlet oxygen [6] and peroxyl radicals, which is reported to only occur at low oxygen tensions [7]. Although the products of free radical oxidation are reduced in the presence of carotenoids [8], no direct chemical investigations have been reported of the scavenging of free radicals for both hydroxyl and superoxide radicals. Although two other reports of carotenoid radical scavenging of superoxide monitored by electron spin resonance have appeared [9,10], the source of the superoxide was activated leucocytes, which are also known to produce hypochlorite in the respiratory burst [11-13]. These reports did not investigate the scavenging of hydroxyl radical by carotenoids. Furthermore, the carotenoids studied, such as astaxanthin [10] and a zwitterionic C_{30} carotenoid phospholipid synthetic detergent [9], are not commonly consumed in the usual North American diet.

The luminescent assay [14-17] is another very sensitive and convenient method to determine the potency of the antioxidant activity of these carotenoids, as well as other antioxidants.

Different carotenoids play specialized physiological roles [4]. Vitamin A, required for vision, comes from dietary β-carotene. Zeaxanthin and lutein (isomeric dihydroxycarotenoids) are the major constituents of the retinal macular region [18]. The macula contains the highest density of cone photoreceptors in the retina and it is responsible for central (not peripheral) vision [19]. The macula appears as a bright yellow spot in the center of the typical primate retina, and deteriorates in some elderly humans (a condition called age-related macular degeneration, AMD or ARMD), reducing or eliminating central vision [20]. Supplementation with β-carotene, antioxidant vitamins C and E, zinc, and copper has been reported to decrease the progression of advanced AMD, suggesting a role for antioxidants in reducing risk of AMD [21].

The xanthophylls zeaxanthin and lutein are present throughout the neural retina of humans (from prenates through adult), although the relative amounts vary [18]. Studies using HPLC analysis have shown that the fovea, the center of the

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macula, has the greatest zeaxanthin concentration, out to a radial distance of 2.5 mm, beyond which lutein is found in greater abundance [18]. Bone and Landrum reported the total amount of carotenoids per unit of area decreased from 13 ng/mm^2 in the center of the fovea to 0.05 ng/mm^2 at a radial distance of 8.7 to 12.2 mm [18].

The function of carotenoids in the macula has not yet been determined, although it is generally believed that pigments may selectively absorb blue light [20,22], thereby protecting the retina against the formation of free radicals from the high energy photons of blue light.

Recent dietary studies have suggested that dietary carotenoids such as lutein and β-carotene can reduce the risk of cataract in humans [23-27]. It seems likely that this effect is related to their antioxidant effect, because antioxidants such as vitamins C and E have been shown to reduce the risk of cataract in humans [28-31,31,32] and animal models [33-35]. Because of the potential importance of antioxidants in decreasing free radical damage to light-exposed ocular tissues such as lens and retina, we decided to further investigate the possibility that the carotenoids exerted their protective effect by scavenging free radicals as well as filtering out damaging blue light.

Although carotenoids are generally ascribed to have poor superoxide scavenging ability (while reacting much more readily with hydroxyl, alkoxyl, and peroxyl radicals [7]), the primary literature is sparse [5]. The general statement that carotenoids do not scavenge superoxide is based on studies of crocin [5], a carotenoid with only seven conjugated double bonds, which is certainly not a representative sample of all carotenoids. Figure 1 shows the structures of the carotenoids discussed in this manuscript.

Lycopene has 13 double bonds, 11 of which are conjugated, and no rings. The other carotenoids shown (zeaxanthin, lutein, and β-carotene) each have six-membered rings at the ends, called β-ionone rings. Lycopene, β-carotene, and zeaxanthin each have 11 conjugated double bonds whereas lutein has only 10. Lutein and zeaxanthin also differ from β-carotene in the presence of alcohol groups in the β-ionone rings. The stereochemistry of the alcohol on the ε-ionone ring of lutein, and the one β-ionone ring of zeaxanthin is the final difference between lutein and zeaxanthin. Crocin is very different from the other carotenoids shown. It has only 7 conjugated carbon-carbon double bonds, capped through ester linkages to the disaccharide, gentobiose, at both ends.

Here we report the radical scavenging abilities of dietary carotenoids and some common antioxidants (for comparison purposes) based on a luminescent assay for free radicals and on electron spin resonance (ESR) spectroscopy spin-trapping.

METHODS

Materials and equipment for luminescent assay: The luminescent assay [14-17,28,29,36] used the following components in a total assay volume of 0.7 ml: for the control, dimethyl sulfoxide (DMSO; 0.4 ml), phosphate buffered saline (0.11 ml), deionized water (0.07 ml), luminol/albumin (0.02 ml), and 3% hydrogen peroxide (0.88 M, 0.1 ml), which was added immediately before mixing the contents and placing the cuvette in the Lumac Biocounter #2010 luminometer (Celsis, Chicago, Il). For the carotenoid to be tested, the assay was performed in sextuplicate for each control or each concentration of carotenoid; the assay contained the carotenoid dissolved in DMSO (0.07 ml) and DMSO (0.33 ml) instead of 0.4 ml of DMSO. The highest carotenoid concentration in the cuvette was 0.1 mM. Luminol/albumin was prepared by dissolving 10 mg/ml each of bovine albumin and luminol (5-amino-2,3-dihydro-1,4-phenylazinedione) in phosphate-buffered saline, stirring overnight and warming to 40 °C before filtering through a 2 μm filter. The maximal emission wavelength (MEW) of luminol (emitting species is 3-aminophthalate) is 425 nm.

The IC_{50} values were measured using five serial tenfold dilutions of the carotenoid solutions in the luminescent assay and regression analysis of the antioxidant activity versus $\log_{10}$ of the concentration of the carotenoid in Microsoft Excel. The IC_{50} values were determined using the Excel goal seek program to determine the $\log_{10}$ values of the concentration at which the antioxidant activity was 50% of the maximum antioxidant activity. The actual concentrations for IC_{50} values were obtained by conversion of the $\log_{10}$ values to actual concentrations, and the errors on the IC_{50} values were determined similarly by conversion of the $\log_{10}$ values for the 95% confidence limits for the regression analysis. Thus, the determination of the IC_{50} value using the luminescent assay was based on triplicate experiments, each of which used a minimum of thirty-six separate assays including the controls with no carotenoid added.

Materials for ESR studies: Ascorbic acid (100%), ascorbyl palmitate (99.3%), β-carotene (1% CWS), lutein (5% TG/P), lycopene (5% TG/P), and zeaxanthin (5% TG) were gifts from DSM (Mississauga, Ontario, Canada). Lutein esters and soy carotenoids were gifts from Cognis (LaGrange, IL). DMSO was purchased from EMScience (Gibbstown, NJ) and was dried over molecular sieves. Phosphate buffered saline (PBS), xanthine oxidase (X1875), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), and hypoxanthine were purchased from Sigma (St. Louis, MO).

Equipment for ESR studies: Electron spin resonance spectra were run of DMPO-spin-trapped free radicals in the reaction mixture containing xanthine oxidase in 57% DMSO or aqueous media. All spectra were run on a Bruker B-MN 120/125 with the following parameters: Center field: 348.439 mT (3484.39 G); Sweep width: 8.0 mT (80 G); Scan time: 10.49 s; Number of scans: 10; Microwave frequency: 9.774 GHz; Microwave power: 20 mW; Modulation amplitude: 0.70 G; Receiver Gain: $5.2x10^5$ (except $2x10^5$ for the ascorbate with hydroxyl radical).

The reaction mixtures were as follows: DMPO+superoxide control cell contained 0.02 ml of xanthine oxidase (0.1 dilution from 25 units per 0.8 ml), 0.28 ml of 0.5 mM hypoxanthine (in milliQ water), 3 μl DMPO, 0.3 ml PBS or milliQ water in some cases, and 1.2 ml DMSO for a total mixture volume of 2.1 ml; DMPO+superoxide with sample cell contained the same as the control cell, except the 1.2 ml

DMSO was replaced with 0.9 ml DMSO and 0.3 ml of 1 mM carotenoid in DMSO or 0.5 mM lutein esters or soy carotenoids in DMSO or 10 mM ascorbyl palmitate in DMSO. For the ascorbate sample, the 0.3 ml of milliQ water was replaced with 0.3 ml of 1 mM ascorbate in milliQ water. The final concentration of each sample carotenoid in the cell was 0.14 mM; DMPO+hydroxyl radical control cell contained 0.1 ml of 30% hydrogen peroxide, 3 µl DMPO, 0.8 ml milliQ water, and 1.2 ml DMSO for a total mixture volume of 2.1 ml; and DMPO+hydroxyl radical with sample cell contained the same as the control cell, except the 1.2 ml DMSO was replaced with 0.9 ml DMSO and 0.3 ml of 1 mM carotenoid in DMSO or 0.5 mM lutein esters or soy carotenoids in DMSO or 10 mM ascorbyl palmitate in DMSO. For the ascorbate sample, the 0.8 ml of milliQ water was replaced with 0.77 ml milliQ water and 0.03 ml of 10 mM ascorbate in milliQ water. The final concentration of each sample carotenoid in the cell was 0.14 mM.

ESR spectra collection: The flow cell was placed in the ESR chamber and the solutions from either the control or sample vials were pumped into the flow cell immediately after xanthine oxidase (for superoxide) or hydrogen peroxide (for hydroxyl radical) addition. The first scans were taken approximately one min after the addition of enzyme or peroxide, when the solution reached the chamber. Additional scans were taken at five min after enzyme addition.

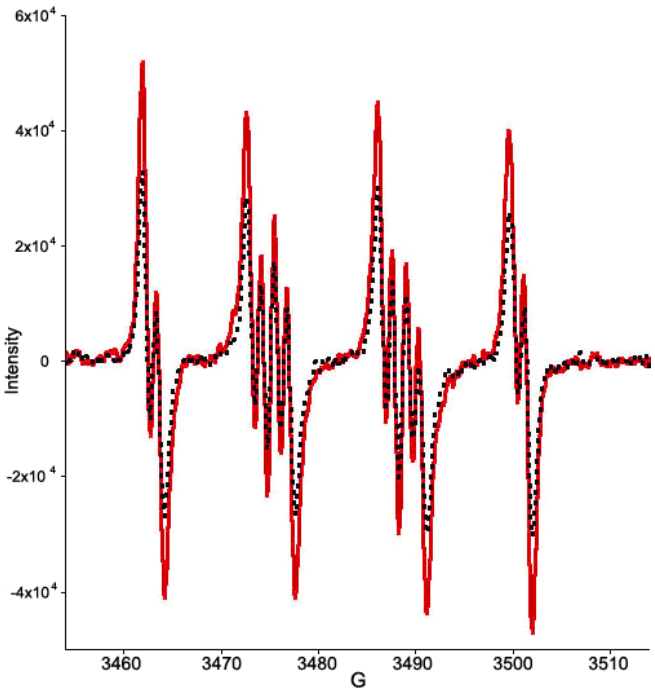

Figure 2. ESR spectra of the DMPO-O_2^- control with (black dashed line) and without (red solid line) $1.4x10^{14}$ M zeaxanthin. Data were collected at 1 min after addition of xanthine oxidase (when the solution reached ESR chamber flow cell). ESR parameters were as described in the methods section.

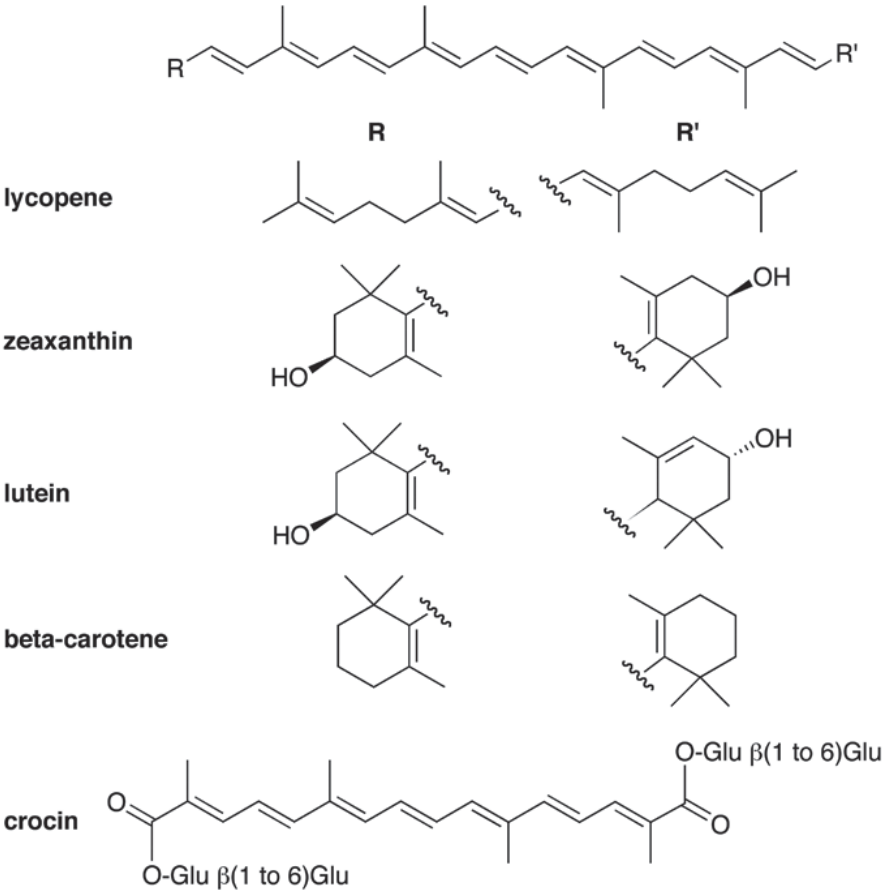

Figure 1. Structures of the carotenoids lycopene, zeaxanthin, lutein, β-carotene, and crocin. The disaccharides esterified in crocin are gentobiose units.

Ultraviolet-visible spectra were measured on a Beckman 650 DU spectrophotometer in the range of 200-600 nm.

RESULTS

In this study, the carotenoids most associated with ocular and vision protection, [18,19,23] zeaxanthin and lutein, were examined, determining their effect on superoxide and hydroxyl radicals. Other carotenoids and biological antioxidants were also examined for comparison purposes.

Superoxide scavenging ability of carotenoids by ESR: Using electron spin resonance, we monitored the superoxide formed by xanthine oxidase and hypoxanthine and trapped by 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) with and without a sample (carotenoid or well-characterized antioxidant). The magnitude of the peak heights of the ESR spectra of the spin-trapped radical was decreased in the presence of the added compound to be tested, indicating that the carotenoid or test substance was scavenging the radicals.

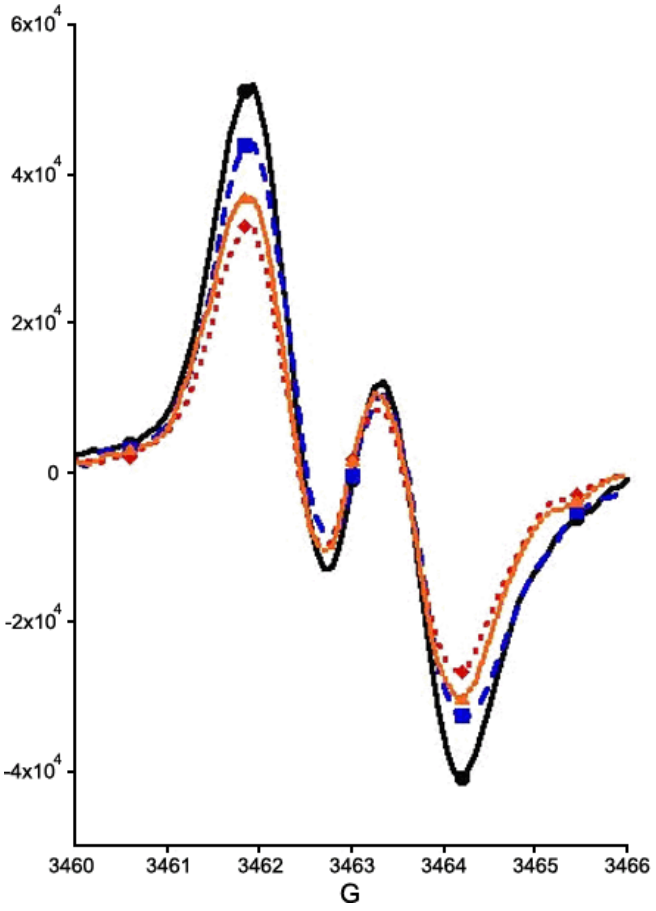

Figure 3. A section of ESR spectra of DMPO-OB2PB-P with and without $1.4x10^{14}$ M zeaxanthin. Data were collected at 1 min (when solution reached the ESR chamber flow cell; black line, control and red line with zeaxanthin) and at 5 min (blue line control and orange line with zeaxanthin) after the addition of xanthine oxidase. This axis expansion shows that given time, less DMPO-O_2^- is present in the control, but more DMPO-O_2^- is produced in the presence of zeaxanthin. ESR parameters were as described in the methods section.

Figure 2 depicts the ESR spectrum of the DMPO-O_2^- control and the spectrum of DMPO-O_2^- in the presence of $1.4x10^{-4}$ M zeaxanthin. The intensity of the DMPO-O_2^- signal was decreased by approximately one-third, thus zeaxanthin either suppressed the formation of or destroyed DMPO-O_2^-. The other carotenoids examined all showed a similar effect, so the change in DMPO-O_2^- intensity for each sample from control at one and five min is detailed in Table 1.

Figure 3 depicts the ESR spectrum of the DMPO-O_2^- with and without $1.4x10^{-4}$ M zeaxanthin at one and five min after xanthine oxidase addition. The axis is expanded to allow the differences in intensity and line width to be observed. While the DMPO-O_2^- intensity decreased after five min in the control solution, it increased in all of the carotenoid-containing solutions over the same time frame.

Hydroxyl radical scavenging ability of carotenoids by ESR: Using electron spin resonance, we monitored the hydroxide formed by hydrogen peroxide (and trace metal ions) and trapped by DMPO with and without a sample (carotenoid or well-characterized antioxidant).

Figure 4 depicts the ESR spectra of the DMPO-OH with and without $1.4x10^{-4}$ M zeaxanthin. The intensity of the DMPO-OH signal was decreased by approximately three-quarters, thus zeaxanthin either suppressed the formation of or destroyed DMPO-OH. The other carotenoids examined all showed a similar effect, so the change in DMPO-OH intensity for each sample from control at one and five min is detailed in Table 1.

The peak height of the ESR spectrum, which quantifies the DMPO-OH intensity, decreased most substantially for samples to which were added zeaxanthin, followed by lycopene, β-carotene, and lutein. The DMPO-OH signals are very noisy, putting the averaged peak height intensities at 3472.5 G (Gauss units), within each other's standard deviations.

Figure 5 depicts the ESR spectrum of the DMPO-OH with and without $1.4x10^{-4}$ M lutein at one and five min after hydrogen peroxide addition. The peak height, which quantifies the DMPO-OH spin-trapped radical concentration, increased

TABLE 1.

	% DMPO-O2- destroyed* (±1 standard deviation)		% DMPO-OH destroyed** (±1 standard deviation)	
Sample	At 1 min	At 5 min	At 1 min	At 5 min
Control	0 ±1.9	5.5±2.0	0 ±3.5	-21.6±2.7
Lycopene	48.0±1.4	30.3±2.1	64.8±17.1	60.5±11.4
β-carotene	46.4±2.3	36.2±1.2	65.1±9.3	50.7±8.9
Zeaxanthin	38.3±2.7	28.3±1.7	66.0±4.0	59.9±11.4
Lutein	32.2±2.3	27.2±1.7	54.3±10.7	50.8±8.9
Crocin	0#		6.4-26.4##	
Ascorbate	97.6±48	98.2±15	87.8±13.8	93.7±11.6
Ascorbyl palmitate	98.4±33	97.0±21	35.9±4.8	17.5±1.8
Lutein esters	41.3±1.4	24.6±1.3	50.0±9.8	39.7±14.0
Soy carotenoids	88.2±5.2	94.2±6.5	44.2±7.8	41.8±7.6

Radical scavenging ability of the carotenoids and ascorbate. The asterisk indicates that the percent DMPO-O_2^- destroyed calculations were the average intensity of three points at 3472 G. The double asterisk indicates that the calculations were the average intensity of three points at 3499 G. The sharp (hash mark) indicates that the value was not from an ESR study, but estimated from [5]. The double sharp (hash mark) indicates that the value was not from an ESR study, but reported by Bors, et al [5].

slightly after five min in the control solution and in all of the carotenoid-containing solutions over the same time frame.

An interesting phenomenon with ascorbate was also observed in both the superoxide and the hydroxyl radical ESR analyses. Ascorbate showed an unusual ESR spectrum upon scavenging the superoxide: a doublet with a hyperfine splitting constant of about 1.9 G, consistent with the O-centered ascorbate radical reported by Bernofsky and Bandara [37]. This doublet was also observed with a much lower intensity in the ascorbyl palmitate sample upon scavenging the superoxide.

Luminescent assay of antioxidant activity using hydrogen peroxide: The percent reduction of hydrogen peroxide counts per min by carotenoids is illustrated in Figure 6 with the standard deviations shown in the error bars. The addition of various carotenoids (0.07 ml of 1 mM carotenoid, unless otherwise specified, in DMSO, replacing 0.07 ml of the 0.4 ml of DMSO) to the assay decreased the luminescence. The carotenoid concentrations at which 50% of the peroxide is destroyed (IC_{50} values) are plotted in Figure 6, with hydroxyl radical scavenging from ESR experiments for comparison.

Ultraviolet-visible spectra of carotenoids: Foote et al. [6] reported the change in the absorbance spectra of β-carotene upon singlet oxygen quenching, corresponding to the *cis* to *trans* isomerization of the central double bond. In all-*trans*-β-carotene, the 464 nm absorbance dwarfs the 345 nm (extinction coefficients of 114,000 and 7,500 l/mol.cm, respectively) but the 15,15'-*cis* form absorbs much more at 345 nm (49,400 l/mol.cm) and the longer wavelength absorbance band shifts to 456 nm and the extinction coefficient drops (85,000 l/mol.cm) [6].

The all-*trans*-β-carotene has an $\varepsilon_{345}/\varepsilon_{464}$=0.07 while in the 15,15'-*cis*-β-carotene the $\varepsilon_{345}/\varepsilon_{464}$=0.58 [6]. Figure 7 depicts the UV-vis spectra of β-carotene, lycopene, lutein, zeaxanthin, soy carotenoids, and lutein esters. None of the carotenoid samples in Figure 7 match the ratios of the all-*trans*-β-carotene or the 15,15'-*cis*-β-carotene, and therefore, the samples examined are likely mixtures of the 15,15'-*cis* and all-*trans*-carotenoid forms.

Ascorbate, well-known for its antioxidant activity, showed an unusual ESR spectrum; a doublet, with a hyperfine splitting constant of about 1.9 G appeared. The same doublet, although much less intense, appears to be barely present in the ascorbyl palmitate spectrum in Figure 8. Perhaps this very small doublet is due to an ascorbate impurity in the ascorbyl palmitate sample. Bernofsky and Bandara [37] observed this doublet in solutions of ascorbate and PbO_2 using the spin trap DMPO and assigned it to an O-centered ascorbate radical. A C-centered ascorbate radical was also trapped by DMPO following the oxidation of ascorbate and DMPO with PbO_2 [37]. This C-centered ascorbyl radical appears as a 6-line spectrum with A_N=15.9 G and A_H=22.4 G, but this species was not observed in the presence of xanthine oxidase and hypoxanthine.

DISCUSSION

Superoxide scavenging ability of carotenoids by ESR: The magnitude of the peak heights quantified the intensity of DMPO-O_2^-. The carotenoid-induced decrease in magnitude of the spin-trapped radical became less over the first five min in the control cell, meaning that less superoxide is trapped as time increases. The opposite results appeared in the presence of the carotenoids where the DMPO-O_2^- intensity increased

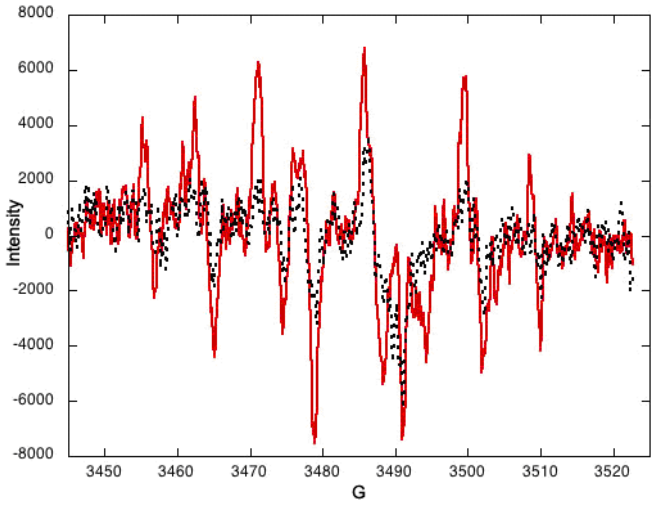

Figure 4. The ESR spectra of the DMPO-OH control with (dashed black line) and without (red solid line) 1.4x10^{-4} M zeaxanthin. Data were collected at 1 min after addition of hydrogen peroxide (when the solution reached ESR chamber flow cell). ESR parameters were as described in the methods section.

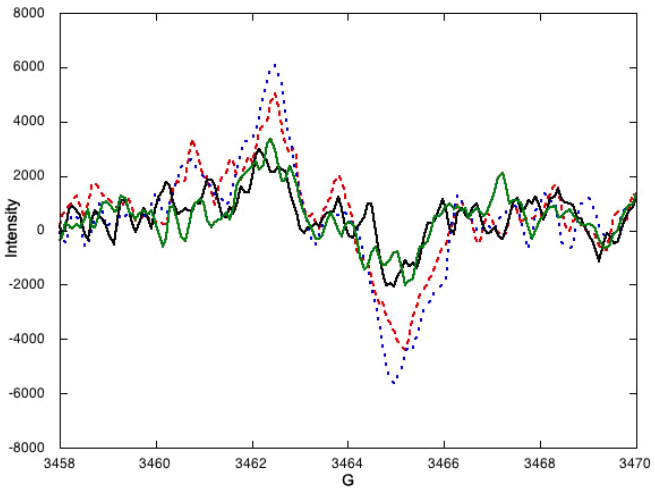

Figure 5. ESR spectra of DMPO-OH with and without 1.4x10^{-4} M lutein. Data were collected at 1 min (when solution reached the ESR chamber flow cell) and at 5 min after the addition of hydrogen peroxide. Lines shown are ESR spectra of controls at 1 min (red dots) and 5 min (blue dots), and with lutein after 1 min (black solid line) and 5 min (green solid line). After five min, more DMPO-OH is present in the control, and in the presence of lutein.

over the first five min, meaning that more superoxide is trapped as time increases *despite* the presence of carotenoids. We relate the increased trapping of superoxide over time to a decreased ability of the carotenoids to scavenge superoxide radicals [20]. This decreased ability of superoxide scavenging with time suggests that superoxide scavenging occurs via a chemical reaction that does not regenerate the carotenoid. This type of radical scavenging is consistent with the fact that carotenoids lack the type of moiety that allows regeneration after H-atom abstraction (such as the phenolic hydrogen in alpha-tocopherol).

The two retinal carotenoids, zeaxanthin and lutein, suppressed the formation of 35-40% of the DMPO-O_2^-; however, lycopene and β-carotene were both better superoxide scavengers than zeaxanthin and lutein. Since there is no difference in the number of allylic hydrogen atoms in β-carotene and zeaxanthin (see Figure 1), it is likely that the difference in superoxide scavenging abilities is due to some other feature.

One would have expected the doubly allylic hydrogen atom in lutein to be the most easily abstracted, enhancing rather than diminishing its superoxide scavenging. As lutein is the poorest superoxide scavenger of this group, the dominant mechanism is unlikely H atom abstraction. Thus, the carotenoids may scavenge superoxide by adding to a double bond. Given the extent of conjugation of these carotenoids, superoxide addition to any of the double bonds would produce highly resonance-stabilized C-centered radicals (with the exception of the nonconjugated double bonds at the ends of lycopene and lutein). The most stable of the C-centered radical products would result from superoxide addition to one of the ends of the conjugated systems.

Crocin has no allylic H atoms to abstract and superoxide addition to one end of the conjugated system can produce at most seven resonance contributors. Crocin is unique among these carotenoids because of the presence of ester functionalities. Superoxide is nucleophilic but the esters in crocin are not very electrophilic. Since crocin is not similar in structure to the carotenoids of the eye, lutein and zeaxanthin, it is imperative that the general assumption that carotenoids cannot scavenge superoxide [5] be set aside.

Hydroxyl radical scavenging ability of carotenoids by ESR: All four carotenoids substantially scavenge hydroxyl radicals or prevent the formation of DMPO-OH. The hydroxyl radical scavenging abilities of the four carotenoids are all within 15%, or one standard deviation of the relatively noisy signals. Over the first five min the DMPO-OH intensity did not remain constant in the control cell; the peak height, measuring the intensity at 3499 G, increased by 22%. The increase in the intensity of hydroxyl radical signal over the first five min is likely due to the continued production of hydroxyl radicals from hydrogen peroxide. In contrast, the intensity of DMPO-OH did not increase as much in the presence of the carotenoids over the first five min, suggesting that the carotenoids continued to scavenge the newly produced hydroxyl radicals. The increases in DMPO-OH intensity (at 3499 G) for the other carotenoids are within one standard deviation of their original values for all but β-carotene (with a 15% increase).

The mechanism of hydroxyl radical scavenging could occur via bond formation between the hydroxyl radical and one of the double bonds in the carotenoid. Obviously the nonconjugated double bonds are the most susceptible to radical

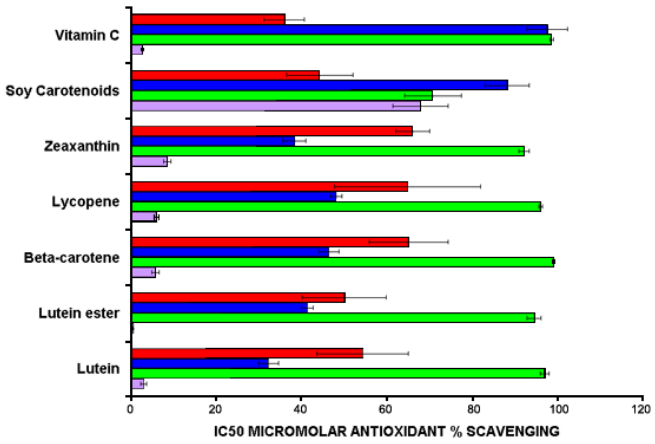

Figure 6. Comparison of antioxidant activity of carotenoids determined by chemiluminescent method and ESR antioxidant activity for hydroxyl and superoxide radicals. The purple bar represents the inhibitory concentration (IC_{50}), the green bar represents antioxidant activity measured from the luminescent assay, the blue bar represents superoxide scavenging measured by ESR, and the red bar represents hydroxyl radical scavenging measured by ESR. The IC_{50} values were determined by interpolation of antioxidant activities for carotenoid solutions diluted in tenfold serial dilutions, plotted in semi-log plots. The IC_{50} (concentration at 50% antioxidant activity) was obtained by conversion of the log concentration.

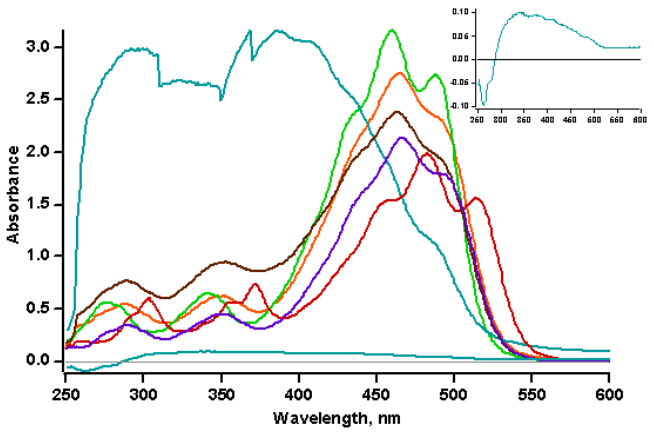

Figure 7. UV-VIS spectra of select carotenoids. All samples were dissolved in DMSO: 500 μM (blue line) and 8.4 μM (turquoise line) lutein esters; 16.7 μM (orange line) β-carotene; 16.7 μM (green line) lutein; 16.7 μM (red line) lycopene; 16.7 μM (purple line) zeaxanthin; 25 μM (brown line) soy carotenoids. The inset shows the UV-VIS spectrum of lutein esters (mostly palmitate from marigolds) at 8.4 μM.

addition, suggesting that lycopene should be the best scavenger with a total of 13 double bonds, 2 of which are separated from the conjugated chain. Zeaxanthin and β-carotene both contain 11 conjugated double bonds and no additional double bonds separated from the conjugated chain, whereas lutein contains 10 conjugated double bonds, and one non-conjugated double bond. All three (lycopene, β-carotene, and zeaxanthin), have similar hydroxyl radical scavenging abilities and contain 11 conjugated double bonds. Lutein, containing only 10 conjugated double bonds, scavenges hydroxyl radicals less effectively than the others.

The two retinal carotenoids, zeaxanthin and lutein, and lycopene and β-carotene all scavenge hydroxyl radicals. While zeaxanthin is the most powerful hydroxyl radical scavenger, above β-carotene, lycopene, and lutein (in that order) the differences between them are within the standard deviations of the relatively noisy signals.

The best scavenger of these four, zeaxanthin, predominates in the center of the fovea (responsible for central vision). Perhaps the center of the fovea has sufficient hydroxyl radical production that this powerful scavenger (of all the available carotenoids) is directed in vivo to the retina. Consistent with this possibility, we have previously reported that rat retina contains a high concentration of superoxide anion, which increases with age [15]. The source of such reactive oxygen species in the rat is probably the mitochondria of the retinal photoreceptor cells. Mitochondria are known to produce superoxide as a result of leakage of electrons from the electron transport chain by way of coenzyme CoQ_{10}, and damage to mitochondria which can occur with age can result in increased production of reactive oxygen species [38]. In addition to the well-known protective absorption of blue light by the carotenoids in the macula [18], their radical scavenging activities would provide important protection to the fovea.

Luminescent assay of antioxidant activity using hydrogen peroxide: In addition to the electron spin trapping assay, our luminescent assay [14] is a very sensitive and convenient method to determine the potency of the antioxidant activity of these carotenoids, as well as other antioxidants. More expensive reagents and equipment that can be used in other antioxidant assays are not necessary for this assay. When there are so many different antioxidant assays available to use to detect antioxidant activity of compounds, such as the commonly used FRAP [39] or ORAC [40] assay, what advantage does a luminescent assay of antioxidant activity [14] have? One advantage is that the luminescent assay reagents are easily available and simple to prepare. Other assays use a more sophisticated radical source and generate radicals slowly at a constant rate. The colored product of these radicals reacting with the reporter molecule over a relatively long period of time (15 min to an hour) is measured spectrophotometrically. For these assays the measurement of the antioxidant activity is by the decrease of the colored product. In contrast to this, the luminescent measurements of antioxidant activity are very rapid, counting in ten s the photons resulting from free radicals reacting with luminol. The assay uses easily available and inexpensive hydrogen peroxide as a source of hydroxyl radicals, or xanthine oxidase and hypoxanthine as a source of superoxide [14]. The antioxidant activity is calculated from the percent decrease in counts in the presence of added antioxidant. The specificity of the reaction for hydroxyl radical or superoxide can be tested by the controls to which catalase or superoxide dismutase, respectively, are added. In addition to these advantages, it is possible, using serial dilutions of added antioxidant, to accurately calculate a value for the inhibitory concentration for 50% inhibition (IC_{50}), an excellent estimate of the potency of the antioxidant in scavenging of the free radical selected for testing, either hydroxyl radical or superoxide.

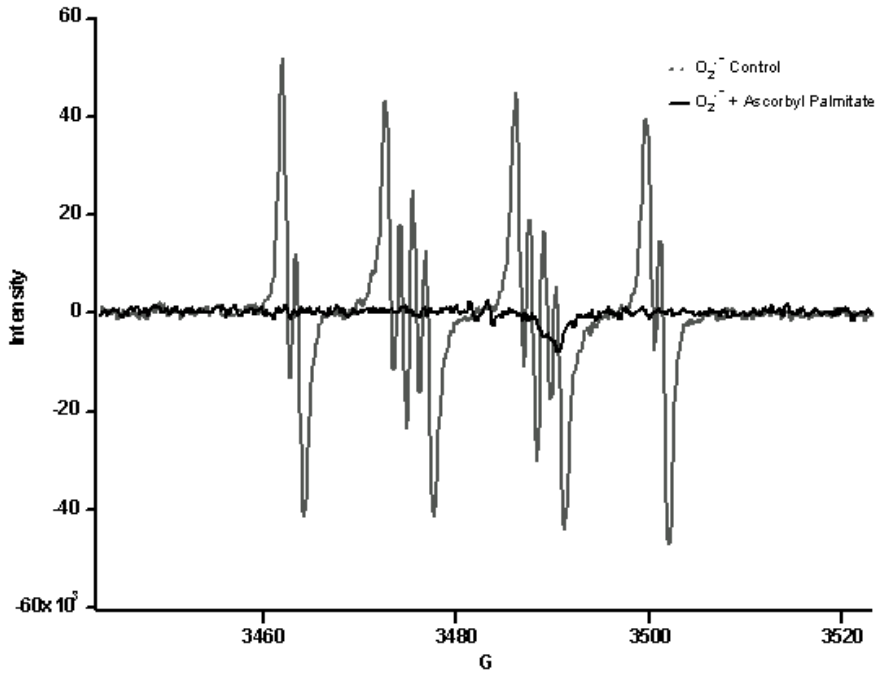

Figure 8. ESR spectra of DMPO-O_2^- with and without ascorbyl palmitate. The control cell contained 0.28 ml of 0.5 mM hypoxanthine (in water), 3 µl DMPO, 0.3 ml phosphate buffered saline (PBS), 0.3 ml milliQ water, and 1.2 ml DMSO (total mixture volume of 2.1 ml). The contents of the superoxide and ascorbyl palmitate cell were identical to the control cell, except for the presence of $1.4x10^{-4}$ M ascorbyl palmitate. To each vial, 0.02 ml of xanthine oxidase (0.1 dilution from 25 units per 0.8 ml) was added immediately prior to pumping into the flow cell in the ESR chamber and the spectra were collected approximately 1 min later.

While crocin may be unable to scavenge superoxide [5], some of the other carotenoids do so quite effectively. The mixtures of 15,15'-*cis* and all-*trans*-carotenoids studied by ESR and luminescent assay scavenge both superoxide and hydroxyl radicals. Lycopene and β-carotene both scavenge superoxide more effectively than the xanthophylls of the retina, zeaxanthin and lutein. The fully saturated fatty acid diesterified luteins scavenged superoxide more effectively than lutein, suggesting that perhaps the electrophilic ester groups may play a role in reacting with the somewhat nucleophilic superoxide.

All of the carotenoids examined scavenged the hydroxyl radicals more effectively than superoxide radicals. The predominant carotenoid in the fovea of the retina, zeaxanthin, scavenged hydroxyl radicals more effectively than the other retinal carotenoid, lutein, suggesting that hydroxyl radical scavenging may be more important in the fovea than elsewhere in the retina, perhaps due to increased hydroxyl radical production.

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