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Original Contribution

CAROTENOIDS AS SCAVENGERS OF FREE RADICALS IN A FENTON REACTION: ANTIOXIDANTS OR PRO-OXIDANTS?

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Abstract—The spin trapping EPR technique was used to study the influence of carotenoids (β-carotene, 8′-apo-β-caroten-8′-al, canthaxanthin, and ethyl 8′-apo-β-caroten-8′-oate) on the yield of free radicals in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$) in the organic solvents, DMSO, and methanol. DMPO and PBN were used as spin trapping agents. It was demonstrated that carotenoids could increase or decrease the total yield of free radicals depending on the oxidation potential of the carotenoids and the nature of the radicals. A reaction mechanism is suggested which includes the reduction of Fe^{3+} to Fe^{2+} by carotenoids. The effectiveness of this carotenoid-driven Fenton reaction increases with a decrease of the scavenging rates for free radicals and with decreasing oxidation potentials of carotenoids. © 2001 Elsevier Science Inc.

Keywords—Carotenoids, Free radicals, Antioxidant activity, Pro-oxidant activity, Fenton reaction, Spin trapping

INTRODUCTION

Numerous reported examples for the protective role of carotenoids against a number of diseases appeared in the past several years (see, for example, reviews [1,2]). It is usually suggested that the activity of carotenoids as nutrients and drugs is related to their ability to scavenge active free radicals and toxic forms of oxygen in living systems. One of the most widely discussed roles of the carotenoids is the interaction with free radicals that initiate harmful reactions such as lipid peroxidation. It is well known that the lipid peroxidation is a chain process with the participation of short-lived carbon-centered as well as oxygen-centered free radicals [3]. For lipid peroxidation, carotenoids exhibit an antioxidant character, as evidenced by the reaction of carotenoids with free peroxyl radicals as an additional chain-breaking process [1,2].

However, there are also some studies devoted to the appearance of pro-oxidant properties of β-carotene [4–6]. The term "pro-oxidant activity" involves the ability of β-carotene to increase the total radical yield in the system. It was shown by Burton and Ingold [4] that β-carotene might act in the process of lipid peroxidation as a pro-oxidant at high oxygen pressure and high carotenoid concentration. It was suggested that β-carotene reacts with peroxyl radicals to give a carbon-centered carotenyl radical which, similar to lipid free radical, in the presence of oxygen produces β-carotene peroxyl radical, so that chain propagation may occur.

A special case is the metal-induced lipid peroxidation. In this case metal ions (Fe^{2+} or Cu^{2+}) react with hydroperoxides, via a Fenton-type reaction, to initiate free radical chain processes [7,8]. There are several studies which indicate that β-carotene offers little protection against metal-induced lipid oxidation [9,10]. Also, it was found that this process is stimulated by glucose [11] and α-tocopherol [12], and this effect is associated with enhanced reduction of metal ions.

Although the role of β-carotene in metal-induced lipid peroxidation was not investigated in detail, it is known that carotenoids can react with ferric ions, and the formation of carotenoid radical cations in this reaction has been observed [13]. Recently we reported the mechanism of the scavenging reaction of a set of carotenoids towards ${}^{\bullet}OOH$ radical generated by the Fenton process in DMSO as a solvent [14]. A correlation of the scavenging rate towards ${}^{\bullet}OOH$ radicals with oxidation potentials of caro-

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tenoids was found. In that study we applied a combination of optical and spin trapping EPR techniques. Spin trapping EPR can provide direct evidence for the presence of free radicals in the reaction system and in principle could permit the separate observation of the reaction of carotenoids with different radicals [15,16].

Taking into account the above and the significance of the investigation of the antioxidant properties of carotenoids to medicine, we report the influence of β-carotene, 8′-apo-caroten-8′-al, canthaxanthin, and ethyl 8′-apo-β-caroten-8′-oate on the behavior of the different paramagnetic species generated in the Fenton system. It is expected that the reaction of carotenoids with Fe^{3+} will depend on the redox properties of carotenoids and the concentrations of reagents. As a result, the process of Fe^{2+} regeneration via the interaction of carotenoids with Fe^{3+} may decrease the antioxidant effect of carotenoids, and, depending on the nature of carotenoids and free radicals, will even result in an increase in the total yield of free radicals in the system (pro-oxidant effect).

MATERIALS AND METHODS

Chemicals

β-Carotene (I) was supplied by Sigma (St. Louis, MO, USA), 8′-apo-β-caroten-8′-al (II) by Roche Vitamins and Fine Chemicals, canthaxanthin (III) by Fluka, and ethyl 8′-apo-β-caroten-8′-oate (IV) was a gift from Hoffmann-LaRoche, Basel (see Scheme 1). Purity of the carotenoids was checked by ^1H NMR (360 MHz, $CDCl_3$) and TLC analyses. They were stored in a freezer at −14°C in a desiccator containing drierite.

The solvents CH_2Cl_2 (Aldrich, anhydrous) and dimethyl sulfoxide (DMSO, 99.5%) (Aldrich, A.C.S.) were used as received.

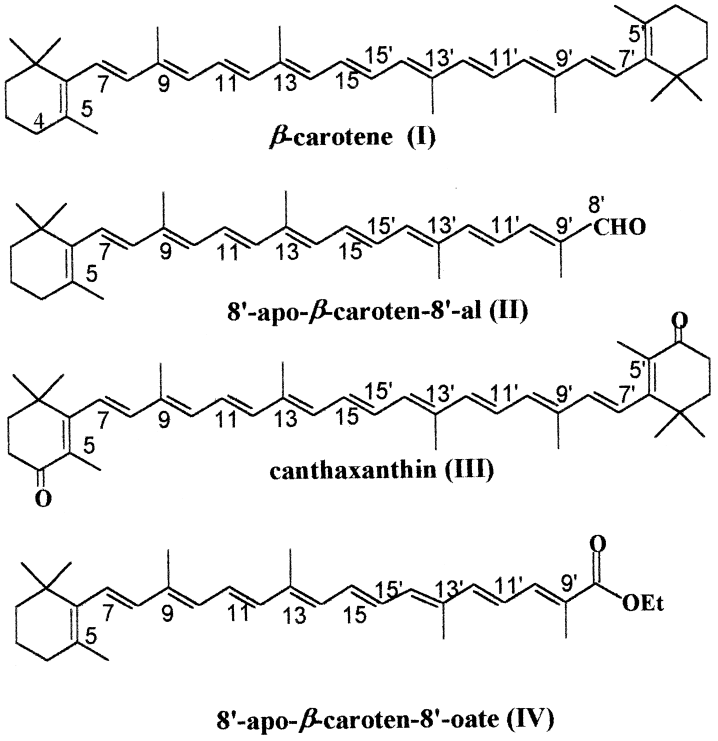
Fenton reagent: hydrogen peroxide (H_2O_2) (30%) (Fisher, A.C.S.), $FeCl_2$ (Aldrich).

Two spin traps were used in the present work: purified 5,5-dimethyl-pyrroline-N-oxide (DMPO), a gift from the National Institute of Environmental Health Science (NIEHS), and N-*tert*-butyl-α*a*-phenylnitrone (PBN) (Aldrich, 98%).

Instrumentation

EPR measurements were carried out with a X-band (9.5 GHz) Varian E-12 EPR spectrometer, equipped with a rectangular cavity. The magnetic field was measured with a Bruker EPR 035M gaussmeter, and the microwave frequency was measured with a Model HP 5245L frequency counter.

UV-visible absorption spectra were recorded with a Shimadzu UV-1610 spectrometer.



Scheme 1.

Fenton reaction

The solutions of all reagents were freshly prepared and deaerated by Ar bubbling. The concentration of carotenoids for optical analysis was of the order of 10 μM, and 1 mM for ESR measurements. The concentration was determined by UV absorption spectrum. Carotenoids were dissolved in CH_2Cl_2 and then added to DMSO solutions of spin traps (1–5 mM). The amount of CH_2Cl_2 in the solvent was kept at 20%. The concentration of H_2O_2 varied from 0.17 to 500 mM. The reaction was started by adding a fixed amount of $FeCl_2$ dissolved in CH_2Cl_2. Then, the solution was transferred to the fixed stop-flow EPR tube by a Hamilton syringe, and the EPR spectrum was recorded 1 min after mixing. In the optical experiments, decay kinetics were recorded at 450 nm a few seconds after mixing.

Spin adduct analysis

The changes in radical concentration were measured by the spin adduct yield. DMPO and PBN were used as spin traps. As was shown in a previous study [14] the spin adducts spectra detected in the Fenton reaction in DMSO showed three different types of spin adducts depending on the concentration of H_2O_2, namely, the $^{\bullet}OH$, $^{\bullet}OOH$, and $^{\bullet}CH_3$ radicals. The identification of all spin adducts were made by using The Spin Trap Data-Base, http://epr.niehs.nih.gov, [17]: PBN/OH ($\mathbf{a}_N$ = 15.00 G, $\mathbf{a}_H$ = 2.80 G, in ACN); PBN/OOH ($\mathbf{a}_N$ = 14.29 G, $\mathbf{a}_H$ = 2.38 G, in benzene); PBN/CH_3 ($\mathbf{a}_N$ = 14.2–15.0 G, $\mathbf{a}_H$ = 3.3–3.6 G, in benzene); DMPO/OH ($\mathbf{a}_N$ = 13.60 G, $\mathbf{a}_H$ = 13.60 G, in ACN); DMPO/OOH ($\mathbf{a}_N$ = 12.9 G, $\mathbf{a}_H$ = 10.3 G, in DMSO); DMPO/CH_3 ($\mathbf{a}_N$ = 14.7 G, $\mathbf{a}_H$ = 20.9 G, in DMSO).

At a high concentration of H_2O_2 only the spin adduct with $^{\bullet}OOH$ radical was observed.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \quad (1)$$

$$^{\bullet}OH + DMSO \rightarrow {}^{\bullet}CH_3 + OS(OH)Me \quad (2)$$

$$^{\bullet}CH_3 + H_2O_2 \rightarrow CH_4 + {}^{\bullet}OOH \quad (3)$$

The reaction with DMPO at the intermediate H_2O_2 concentration (10 mM) shows two types of spin adducts in the EPR spectrum, with $^{\bullet}CH_3$ and $^{\bullet}OH$ radicals. Because the $^{\bullet}OH$ radical reacts with the solvent at diffusion controlled rate, DMPO/OH adduct could not be produced directly under these conditions. As was shown in the previous study, the DMPO/OH adduct results from the DMPO/OOH adduct via reaction with Fe^{2+} [14]. Finally, at low H_2O_2 concentration (1 mM) only the adduct with $^{\bullet}CH_3$ radical was detected. The same measurements in methanol show the presence of $^{\bullet}CH_2OH$ radicals at low H_2O_2 concentration.

RESULTS AND DISCUSSION

It is well known that OH radicals produced in the Fenton reaction can react rapidly with the solvent resulting in secondary free radicals [18–21]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$$
$$^{\bullet}OH + R\text{-}H \rightarrow {}^{\bullet}R + H_2O \quad (4)$$

In the presence of a spin trap (ST) and carotenoids (Car) these secondary active free radicals can decay by three ways:

$$^{\bullet}R + {}^{\bullet}R \rightarrow \text{products} \quad (5)$$

$$^{\bullet}R + ST \rightarrow \text{spin-adduct} \quad (6)$$

$$^{\bullet}R + Car \rightarrow \text{products} \quad (7)$$

Three possible mechanisms are usually suggested for reaction 7: (i) the addition of R to the chromophore chain resulting in radical-adducts; (ii) hydrogen abstraction from the allylic C-4 position of the carotenoid by a free radical; and (iii) electron transfer between carotenoid and $^{\bullet}R$ [1,22]. For the $^{\bullet}OOH$ radical, a correlation of the scavenging rate and oxidation potential of carotenoids was found [14]. The discussion of the antioxidant activity of carotenoids is based on the suggestion that the carotenyl radicals or the radical adducts of carotenoid with free radicals are not reactive due to delocalization of the unpaired electron throughout the long unsaturated chain.

The presence of carotenoid in this reaction system shows, however, not only a decrease of the free radical concentration, but the reduction of Fe^{3+} to Fe^{2+} by carotenoids. Figure 1 demonstrates this for the reaction of IV with $FeCl_3$ in CH_2Cl_2. As was shown earlier, the absorption peak at 855 nm is due to carotenoid radical cation [13].

$$Car + Fe^{3+} \rightarrow Car^{\bullet+} + Fe^{2+} \quad (8)$$

When H_2O_2 is present in excess, this reaction can lead to a redox cycle of the Fenton process, that is carotenoid-driven. In this case the radical yield will depend on the comparative rates of reactions 7 and 8.

Let us discuss what experimental conditions are the most suitable for the appearance of the pro-oxidant effects of carotenoids. Because carotenoid neutral radicals are inactive, reaction 7 is a chain-breaking process and reaction 8 can be viewed as a chain propagation process.

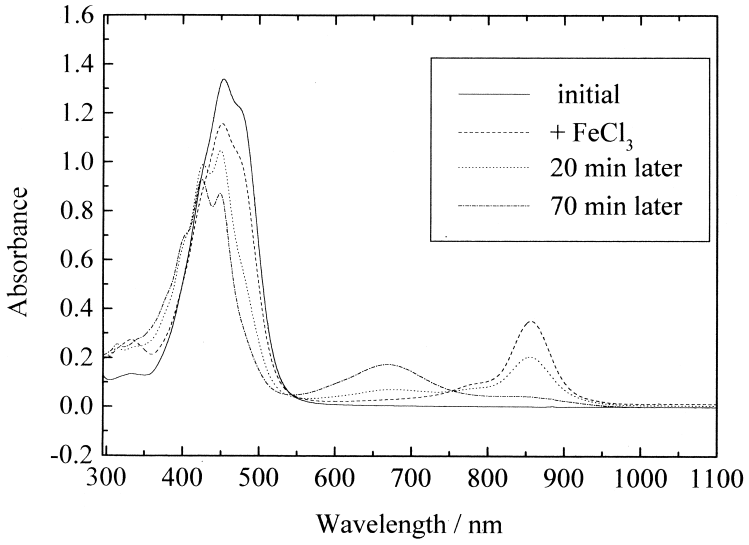


Fig. 1. Optical absorption spectra of carotenoid IV in CH_2Cl_2 before and during reaction with $FeCl_3$ at different time delay after starting.

So, depending on the competition between reactions 7 and 8, the presence of the carotenoid will result in a decrease or an increase in the total radical yield in the system. From this point of view, the pro-oxidant effect will increase with decreasing oxidation potential of the carotenoid. In our investigation, β-carotene has the lowest oxidation potential. On the other hand, the effectiveness of reaction 7 will decrease with a decrease in the free radical concentration and the scavenging rate constant. In principle, any effective decay channel for free radicals (except the reaction with carotenoids) will enhance the pro-oxidant effect of carotenoids. In the system under study such a channel is the reaction with spin trap, for example, and in living cells it is the reaction with lipids. As was shown in the previous paper [14], the spin adduct yield (A) decreases due to the competition reaction with carotenoid:

$$\frac{A}{A_0} = \frac{k_{ST}[ST]}{k_{ST}[ST] + k_{Car}[Car]}$$

Here, k_{Car} and k_{ST} are reaction rate constants of free radical with carotenoid (Car) and spin trap (ST), and A_0 is spin adduct yield at zero carotenoid concentration. Now, let us consider the possibility of a pro-oxidant effect of carotenoids at different ratios of k_{Car} and k_{ST}.

1. In the case of $k_{Car} << k_{ST}$ and [Car] $\approx$ [ST] $\approx$ [Fe], A/A_0 approaches to 1. In this case almost all carotenoid will react with Fe^{3+}, and, in the presence of excess H_2O_2, it will result in an additional portion of spin adduct, $A_{add} \approx$ [Car]. So, the total yield of spin adduct can increase up to $2A_0$.
2. An increase of k_{Car} will lead to the decrease of both A/A_0 and the remaining carotenoid, which can react with Fe^{3+} resulting in an additional concentration of radicals. For $k_{Car}/k_{ST} = 1$, $A/A_0 = 1/2$, so no pro-oxidant effect can be expected.
3. If $k_{Car} > k_{ST}$ and [Car] $\approx$ [ST], A/A_0 approaches to zero, so only the antioxidant effect is possible.

In the previous study we measured the k_{Car}/k_{ST} ratios for the reaction with $^{\bullet}OOH$ radical. They are equal to 0.64, 3.22, 1.96, and 12.4 $M^{-1}s^{-1}$ for carotenoids I–IV and DMPO as spin trap [14]. Thus, in the reaction with peroxyl radical only the antioxidant effect can be expected for all carotenoids, except β-carotene. A small pro-oxidant effect can appear for this carotenoid.

In the present work, in order to estimate the rate constant of the reaction of carotenoids with C-centered radicals ($^{\bullet}CH_3$ and $^{\bullet}CH_2OH$ radicals), the decay kinetics of carotenoid III were observed at low H_2O_2 concentration (see experimental part) by UV-absorption spectroscopy. As was described in the experimental part, only the spin adduct with $^{\bullet}CH_3$ radical was detected in DMSO and $^{\bullet}CH_2OH$ radical in methanol at low H_2O_2 concentration. Figure 2 shows the initial decay kinetics of the carotenoid III. Computer fitting of the full kinetics gives good agreement with single-exponential approximation, $I = I_0\exp(-t/\tau)$, where τ^{-1} is the decay rate of carotenoid absorption, $\tau(CH_3) = 7.94 \pm 0.02$ s, and $\tau(CH_2OH) = 40.84 \pm 0.24$ s. To obtain the value of k_{Car}, from τ, the analysis of decay kinetics of carotenoid has been made using equations 1, 5, and 7. Since under our experimental conditions, $[H_2O_2] <<$ [Fe], no chain processes occur. The following expression can be derived:

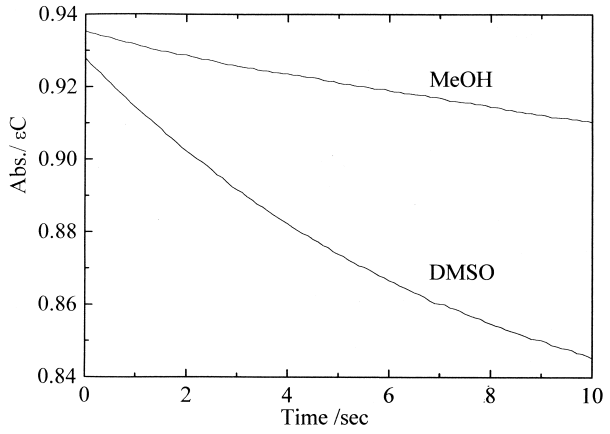


Fig. 2. Change in absorption of carotenoid III measured at 450 nm during the Fenton reaction in DMSO and CH_3OH at low H_2O_2 concentration (0.17 mM, Fe^{2+} = 1 mM).

$$C(t) = C_0\exp(-k_{Car}\sqrt{k_1[H_2O_2][Fe]_0/k_5 \cdot t})$$

Using the literature data of rate constants k_1 = 76 $M^{-1}s^{-1}$ [23], $k_5(^{\bullet}CH_3)$ = 9 · 10^8 $M^{-1}s^{-1}$, $k_5(^{\bullet}CH_2OH)$ = 1.5 · 10^9 $M^{-1}s^{-1}$ [24,25], and the concentrations of $FeCl_2$ (1 mM) and H_2O_2 (0.17 mM), the rate constants k_{Car} were calculated for reaction of III with $^{\bullet}CH_3$ radical: $k_{Car}(^{\bullet}CH_3)$ = 1.5 · 10^6 $M^{-1}s^{-1}$ and with $^{\bullet}CH_2OH$ radical: $k_{Car}(CH_2OH)$ = 2.5 · 10^5 $M^{-1}s^{-1}$. According to the kinetic DataBase [25], the trapping rate constants (k_{ST}) for spin traps PBN and DMPO towards C-centered radicals are on the order of 10^7–10^8 $M^{-1}s^{-1}$. So, $k_{Car}/k_{ST} << 1$, and for C-centered radicals a pro-oxidant effect of carotenoids can be expected.

To study the influence of carotenoids as anti- and pro-oxidants, the spin adduct yield was measured at different H_2O_2 concentrations in the presence and absence of carotenoids. As was expected, the maximum pro-oxidant effect was detected at the intermediate H_2O_2 concentration (10 mM) for reaction of carotenoid with $^{\bullet}CH_3$ radical (Fig. 3). Figure 3 shows the increase of DMPO/CH_3 spin adduct yield [spin adduct (2)] in the presence of carotenoid II, apo-carotenal, when [DMPO] > [Car].

At the same time, a decrease of the DMPO/OH adduct signal was observed [spin adduct (1) in Fig. 3]. As was mentioned in the experimental section, the DMPO/OH adduct results from the DMPO/OOH adduct via reaction with Fe^{2+}. Thus, Fig. 3 demonstrates both the decrease of the yield of OOH radicals (antioxidant effect) and the increase of the yield of $^{\bullet}CH_3$ radicals (pro-oxidant effect).

In order to obtain evidence for the importance of reaction 8 in this effect, we measured the spin adduct yield in the same reaction at low H_2O_2 concentration.

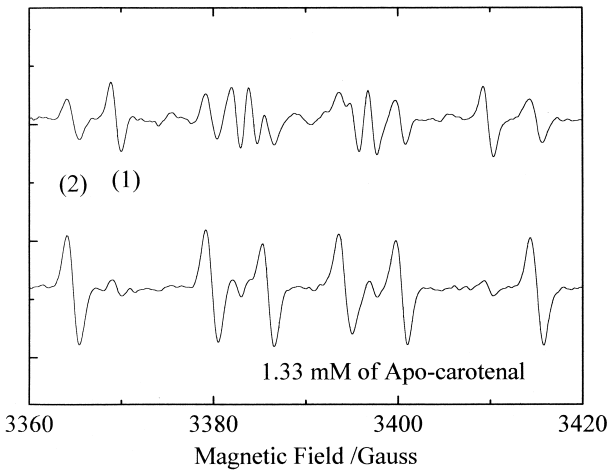


Fig. 3. ESR spectra of DMPO spin adducts recorded during the Fenton reaction in DMSO in the absence (top) and presence (bottom) of II (concentration of H_2O_2 = 10 mM, Fe^{2+} = 1 mM, and DMPO = 5 mM). (1) DMPO/OH adduct ($\mathbf{a}_N$=13.20 G, $\mathbf{a}_H$=14.50 G); (2) DMPO/CH_3 ($\mathbf{a}_N$=14.8 G, $\mathbf{a}_H$=21.1 G).

Under these conditions all H_2O_2 reacts with Fe^{2+}, so the chain propagation cannot occur and no increase of the spin adduct with the $^{\bullet}CH_3$ radical was observed at a low H_2O_2 concentration (see Fig. 4).

The effect of the redox properties of carotenoids on the appearance of a pro-oxidant effect can be demonstrated by using the results of our previous study [14]. For this purpose the chosen carotenoids had different oxidation potentials and different scavenging rates towards free radicals [14]. At high concentration of H_2O_2 when only $^{\bullet}OOH$ spin adducts were detected, a comparison of the spin adduct yields was made for carotenoids

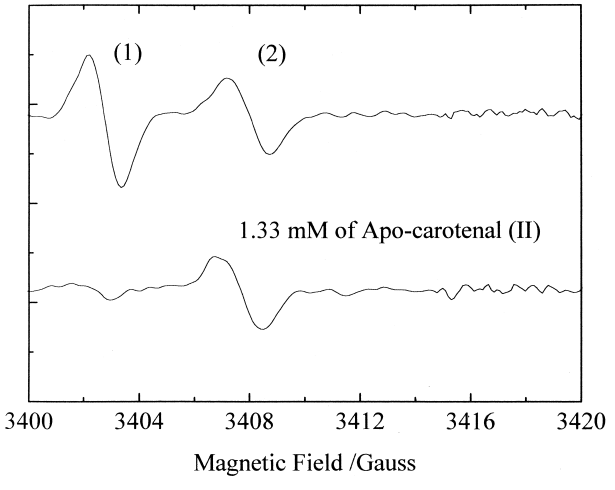


Fig. 4. ESR spectra (fragments) of DMPO/OH (1) and DMPO/CH_3 (2) spin adducts recorded during the Fenton reaction in DMSO in the absence (top) and presence (bottom) of II (concentration of H_2O_2 = 2 mM, Fe^{2+} = 2 mM, and DMPO = 5 mM).

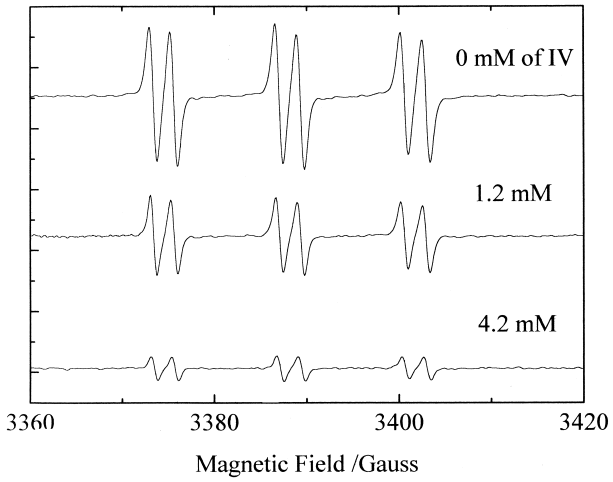


Fig. 5. ESR spectra of PBN/OOH spin-adduct ($\mathbf{a}$ = 14.2 G, $\mathbf{a}_H$ = 2.4 G) recorded during the Fenton reaction in DMSO (concentration of H_2O_2 = 500 mM, Fe^{2+} = 1 mM and PBN = 5 mM) at different concentration of IV.

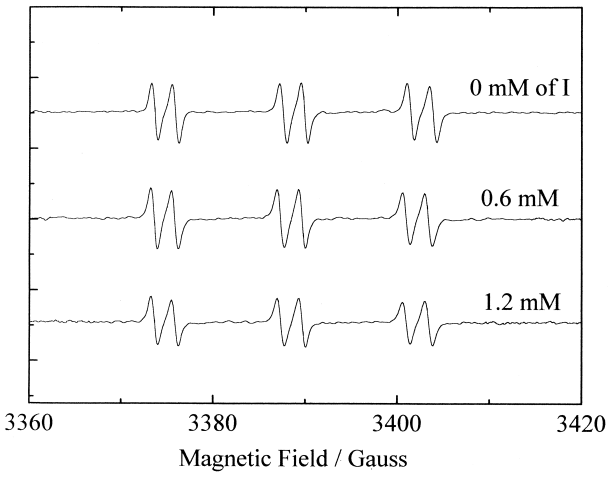
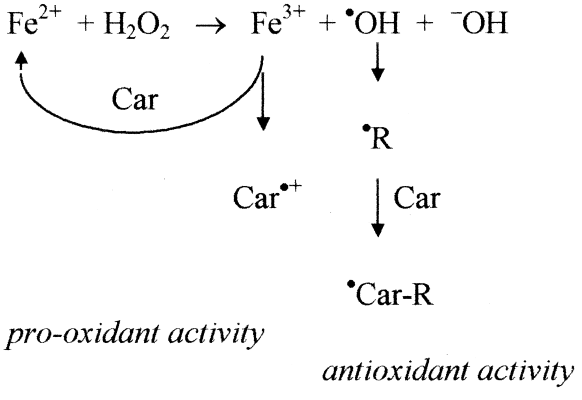


Fig. 6. ESR spectra of PBN/OOH spin adduct recorded during the Fenton reaction in DMSO (concentration of H_2O_2 = 500 mM, Fe^{2+} = 1 mM, and PBN = 3 mM) at different concentration of I.

I–IV with known half-wave potentials [26]: I ($E^{ox}_{1/2}$ = 0.54 V vs. SCE), II ($E^{ox}_{1/2}$ = 0.72 V), III ($E^{ox}_{1/2}$ = 0.69 V), and IV ($E^{ox}_{1/2}$ = 0.74 V). The monotonous decrease of the spin adduct yield with the increase of carotenoid concentration was observed for II–IV, but I showed some unusual behavior. As an example, Figs. 5 and 6 demonstrate the dependencies of PBN spin adduct yields for systems I and IV at different carotenoids concentrations. Carotenoid IV shows only the antioxidant activity (decrease of spin adduct yield) in the accord with our estimations ($k_{Car}/k_{ST} > 1$), but β-carotene ($k_{Car}/k_{ST} < 1$) shows no decrease of spin adduct yield at low carotenoid concentration. Because β-carotene has the lowest oxidation potential [27], its ability to reduce Fe^{3+} should appear most clearly. This could explain why β-carotene shows the unusual concentration dependence.

Thus, the following mechanism of the participation of carotenoids in the modified Fenton process can be suggested:



As was shown, the pro-oxidant effect grows with decreasing oxidation potential of the carotenoid, with increasing H_2O_2 concentration, and with decreasing in the scavenging rate of the carotenoid. Thus, all results confirm the suggested scheme. Analogous to the carotenoid-driven Fenton reaction described in the present paper, several examples of reversible reduction of iron are known for other reducing agents [28]. The prime example is reaction of ascorbate (AH_2) with Fe^{3+}-EDTA (ethylenediaminetetraacetate) chelate complex:

$$Fe^{3+} + AH_2 \rightarrow Fe^{2+} + AH^{\bullet} + H^{+} \qquad (9)$$

Another example is the superoxide driven-Fenton reaction:

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (10)$$

In all cases, iron functions as a catalyst governing the recycling mechanism.

CONCLUSION

The main interest in the reactions of carotenoids with free radicals is whether carotenoids can give effective protection against diseases caused by oxidative stress. It was shown by many authors that carotenoids are readily oxidized by a variety of oxidants. However, as was demonstrated in the present paper, the decay of carotenoids detected by optical methods does not necessarily mean the decrease of free radicals yield. The combination of ST EPR and optical methods can provide more detailed information about the reactivity of carotenoids with free radicals. The presented results show that caro-

tenoids can serve as effective scavengers of reactive free radicals. However, when carotenoids are involved in a reaction cycle with the participation of iron, an increase of total radical yield can be expected. Thus, it was demonstrated that carotenoids could affect metal ion-mediated processes. In the presence of Fe^{2+} the pro-oxidant effect will increase with decreasing carotenoid oxidation potential and its scavenging activity. Similar effects were earlier described for ascorbate, α-tocopherol and other reducing agents.

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