

Antioxidant Activity of β -Carotene-Related Carotenoids in Solution

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The effect of the antioxidant activity of β -carotene and related carotenoids on the free radical-oxidation of methyl linoleate in solution was examined by measuring the production of methyl linoleate hydroperoxides. Canthaxanthin and astaxanthin which possess oxo groups at the 4 and 4' positions in the β -ionone ring retarded the hydroperoxide formation more efficiently than β -carotene and zeaxanthin which possess no oxo groups. The rates of autocatalytic oxidation of canthaxanthin and astaxanthin were also slower than those of β -carotene and zeaxanthin. These results suggest that canthaxanthin and astaxanthin are more effective antioxidants than β -carotene by stabilizing the trapped radicals.

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In recent years, epidemiological studies in humans (1-4) have suggested that β -carotene aids in cancer prevention. It was also implied that dietary β -carotene may exert an anticarcinogenic effect by a mechanism independent of its role as a vitamin A precursor (5). On the other hand, β -carotene is an effective singlet oxygen quencher (6), and we have found that β -carotene can prevent singlet oxygen-initiated oxidation of methyl linoleate in cooperation with α -tocopherol (7). Krinsky and Deneke (8,9) demonstrated that carotenoids including β -carotene are capable of inhibiting free radical-induced oxidation in liposomal lipids. Burton and Ingold (10) have shown that β -carotene belongs to a previously unknown class of biological antioxidants especially effective at low oxygen partial pressures such as those found in most tissues under physiological conditions. Therefore, the anticarcinogenic effect of β -carotene may be, at least partly, attributable to its antioxidant effect insofar as oxygen radicals are related to the process leading to human cancer (11).

However, little is known about the antioxidant activity of naturally occurring carotenoids other than β -carotene. We selected β -carotene (structure [1] in Fig. 1) and related carotenoids containing oxo groups and/or hydroxyl groups in the β -ionone rings as a common structural unit (that is, zeaxanthin [2], canthaxanthin [3], and astaxanthin [4] in Fig. 1) and examined their antioxidant effect upon the azo-initiated oxidation of methyl linoleate in solution. The results strongly suggest that the introduction of oxo groups at 4 and 4' positions enhances the antioxidant activity of carotenoids.

MATERIALS AND METHODS

Materials. β -Carotene was obtained from E. Merck, Darmstadt. Canthaxanthin, astaxanthin and zeaxanthin were

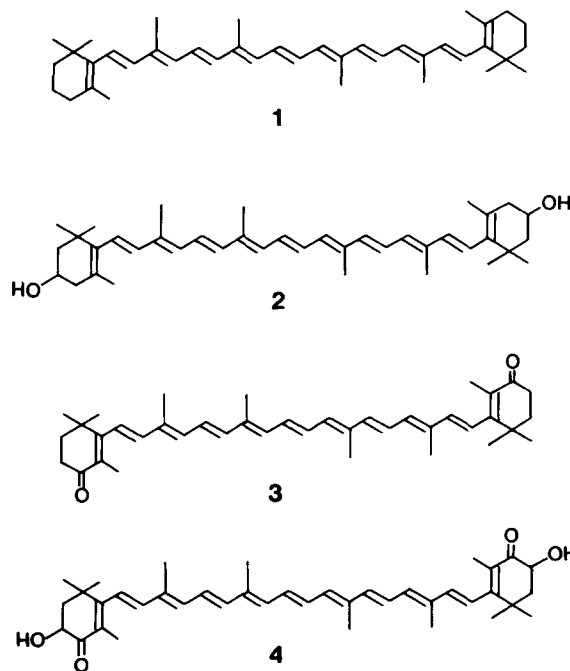


FIG. 1. Structures of carotenoids. (1) β -carotene, (2) zeaxanthin, (3) canthaxanthin, (4) astaxanthin.

generously provided by Hoffmann-La Roche. The product of Nacalai Tesque Inc., Kyoto, Japan, was dl- α -tocopherol. Obtained from Wako Pure Chemical Industries, Osaka, Japan, was 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). Methyl linoleate (99%), supplied by Nacalai Tesque, was further purified by column chromatography with Florisil (100/200 mesh) (12). Other reagents and solvents were of analytical grade and used without purification.

Procedures. An appropriate amount of carotenoid in tetrahydrofuran (5 μ mol/ml) was added to a mixture of hexane/isopropanol (1:1, v/v, 1.0 ml) containing methyl linoleate (100 μ mol). Oxidation was initiated by adding a hexane solution of AMVN (10 μ mol in 0.1 ml) and the mixture was incubated with continuous shaking under air in the dark at 37°C. At regular intervals, aliquots of the sample (10 μ l) were withdrawn and injected into the HPLC column. The HPLC conditions employed and the procedure for the determination of methyl linoleate hydroperoxides have been described in a previous paper (12). Carotenoids and α -tocopherol were also quantified by HPLC using a column of YMC-Pack ODS (6 \times 150 mm, 5 μ m particle size, Yamamura Kagaku, Japan). The column was eluted with a mixture of acetonitrile/isopropanol (3:1, v/v). The flow rate was maintained at 3.0 ml/min and the effluent was monitored at 470 nm for carotenoids and 290 nm for α -tocopherol.

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Abbreviations: AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile); HPLC, high performance liquid chromatography.

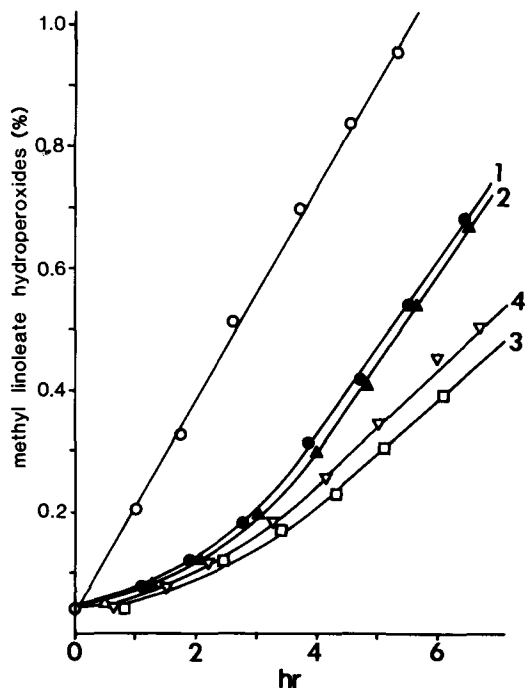


FIG. 2. Effect of carotenoids on the oxidation of methyl linoleate in solution. Reaction system consisted of methyl linoleate (77 mM), carotenoids (0.77 mM) and AMVN (7.7 mM) in a mixture of hexane/isopropanol/tetrahydrofuran (6:5:2, v/v/v, 1.3 ml). ●, β -Carotene; ▲, zeaxanthin; ▽, canthaxanthin; □, astaxanthin; ○, no addition.

RESULTS

Figure 2 shows the effect of the four carotenoids at 0.77 mM (1.0 mol % relative to methyl linoleate) on the rate of formation of methyl linoleate hydroperoxides. In the absence of carotenoids, methyl linoleate hydroperoxides accumulated linearly at the rate of $2.0 \mu\text{M}\cdot\text{min}^{-1}$. Each carotenoid suppressed the oxidation of methyl linoleate, although the reaction curve showed no distinct induction period. During the first 2 hr of oxidation, the rate was kept at less than $0.5 \mu\text{M}\cdot\text{min}^{-1}$ in the presence of each carotenoid, but thereafter canthaxanthin and astaxanthin retarded the hydroperoxide formation more efficiently than β -carotene and zeaxanthin.

When canthaxanthin or astaxanthin was added to the solution at 0.18 mM (0.2 mol % to methyl linoleate), little effect was observed, compared with α -tocopherol present at the same concentration (Fig. 3A). The concentrations of canthaxanthin and astaxanthin both decreased simultaneously with the formation of methyl linoleate hydroperoxides (Fig. 3B). On the other hand, an obvious induction period (approximately 4 hr) appeared in the presence of α -tocopherol, and the oxidation started only after the α -tocopherol was completely consumed.

In order to compare the reactivity of the carotenoids toward the radical chain reaction, the carotenoids were incubated in the presence of AMVN (8.8 mM) without methyl linoleate (Fig. 4). Evidently canthaxanthin and astaxanthin disappeared more slowly than β -carotene and zeaxanthin. The initial rates of the disappearance of the carotenoids were $2.7 \mu\text{M}\cdot\text{min}^{-1}$ (β -carotene), $2.5 \mu\text{M}\cdot\text{min}^{-1}$ (zeaxanthin), $1.3 \mu\text{M}\cdot\text{min}^{-1}$ (canthaxanthin), and $1.4 \mu\text{M}\cdot\text{min}^{-1}$ (astaxanthin), respectively. The rate of radical

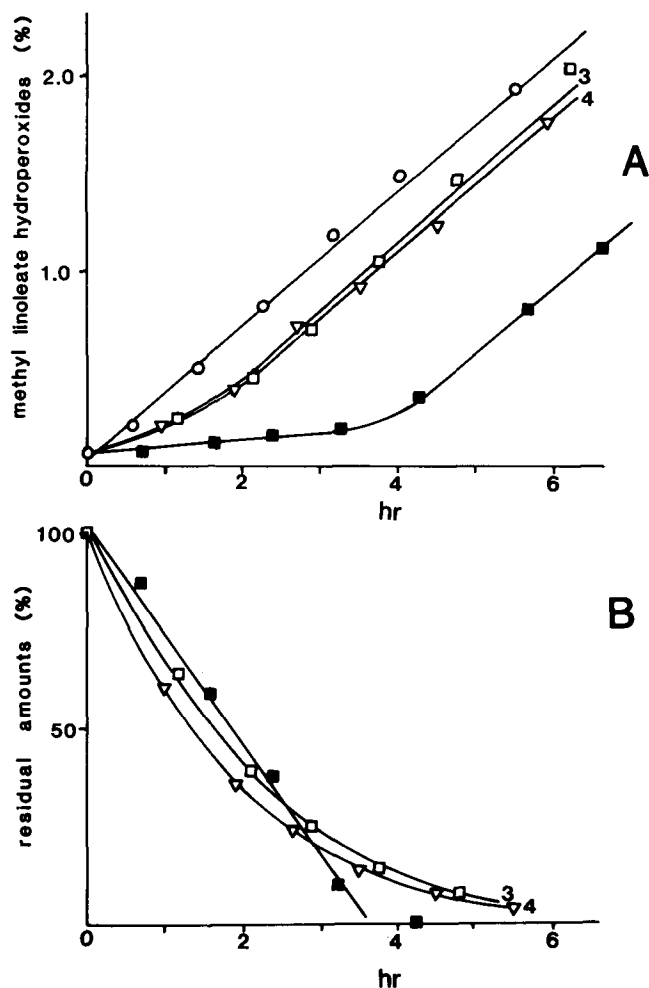


FIG. 3. Formation of methyl linoleate hydroperoxides (A) and loss of canthaxanthin, astaxanthin and α -tocopherol (B) during the oxidation of methyl linoleate. Reaction system consisted of methyl linoleate (88 mM), carotenoids (0.18 mM) or α -tocopherol (0.18 mM) and AMVN (8.8 mM) in a mixture of hexane/isopropanol/tetrahydrofurane (6:5:0.4, v/v/v, 1.14 ml). ▽, Canthaxanthin; □, astaxanthin; ■, α -tocopherol; ○, no addition.

production from AMVN was calculated to be $1.5 \mu\text{M}\cdot\text{min}^{-1}$ ($2 \times 180 \mu\text{M}/240 \text{ min}$) by the induction period in the presence of α -tocopherol shown in Figure 3A (13,14). Thus, the ratio of the rate of carotenoid-disappearance to that of radical production from AMVN (that is, effective chain length of carotenoid oxidation) was determined to be 1.8 (β -carotene), 1.7 (zeaxanthin), 0.9 (canthaxanthin), and 1.0 (astaxanthin), respectively. Accordingly, canthaxanthin and astaxanthin were found to be quite resistant to autocatalytic radical chain reaction.

DISCUSSION

We have used AMVN-induced lipid peroxidation in a solution to measure the antioxidant activity of carotenoids. The unimolecular decomposition of this initiator induces a free radical chain oxidation of methyl linoleate via lipid peroxy radicals as intermediates resulting in the exclusive formation of methyl linoleate hydroperoxides (15). The inhibition of the hydroperoxide formation by carotenoids has been attributed to their lipid peroxy radical-trapping ability (10).

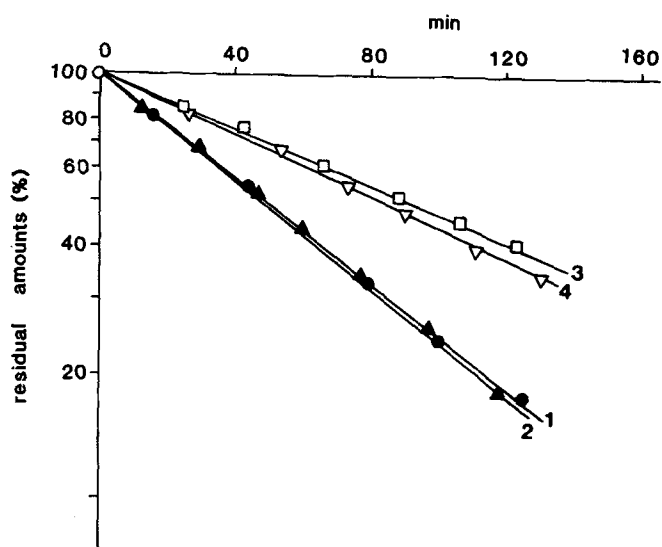


FIG. 4. Loss of carotenoids induced by AMVN. Reaction system consisted of carotenoids (0.18 mM) and AMVN (8.8 mM) in a mixture of hexane/isopropanol/tetrahydrofuran (6:5:0.4, v/v/v, 1.14 ml). ●, β -Carotene; ▲, zeaxanthin; ▽, canthaxanthin; □, astaxanthin.

Conventional chain-breaking antioxidants such as tocopherols trap peroxy radicals by donating a hydrogen atom. However, β -carotene seems to exert an antioxidant activity by a mechanism in which the chain-propagating peroxy radical is trapped by addition to the conjugated polyene system of β -carotene rather than the mechanism of hydrogen-donation (10). The resulting carbon-centered radical is resonance-stabilized because of the delocalization of the unpaired electron in the conjugated polyene system, leading to chain termination. This means that the reaction of β -carotene or related carotenoids with the peroxy radicals competes with the production of methyl linoleate hydroperoxides via a chain reaction. Actually the loss of carotenoids is accompanied by the formation of methyl linoleate hydroperoxides (Fig. 3). The lack of a distinct induction period during the carotenoid-inhibited oxidation, in contrast to α -tocopherol (16), can be explained by the idea that the rate of the antioxidant activity of the carotenoids is similar to the rate of chain propagation of methyl linoleate-hydroperoxidation.

The fact that the antioxidant activity of canthaxanthin and astaxanthin lasted longer than β -carotene and zeaxanthin (Fig. 2) indicates that the substitution of a hydrogen atom by an oxo group at the 4 (4')-position, but not by the corresponding substitute of a hydroxyl group at the 3 (3')-position, increases the efficiency of the peroxy radical-trapping ability of carotenoids containing the β -ionone ring system. It is most likely that the electron-withdrawing character of the oxygen atoms substantially reduces the unpaired electron density on the carbon skeleton resulting in the decrease of the reactivity of the carbon-centered radical toward molecular oxygen. Therefore, the presence of a conjugated carbonyl presumably enhances the stability of the trapped radical by decreasing its tendency for continued chain-propagation reaction. As shown in Figure 4, carotenoids serve as substrates for autocatalytic oxidation when incubated with a free radical initiator (10). The result that canthaxanthin and astaxanthin are more resistant to such chain reaction than

β -carotene is surely indicative of the enhancement of the stability of the chain-propagating radical.

The physiological concentration of carotenoids in human plasma is known to be much higher than that of short-lived primates and nonprimate mammals (17). It was also reported that the major carotenoid species from human plasma (18) has lycopene (0.2–0.5 $\mu\text{g/ml}$), α -carotene (0.1–0.2 $\mu\text{g/ml}$) and β -carotene (0.1–0.2 $\mu\text{g/ml}$). In addition, β -cryptoxanthin, lutein and zeaxanthin are detected in human plasma (19,20), and lutein and zeaxanthin are the dominant carotenoids in the whole human retina (21). On the other hand, canthaxanthin has been used as a therapeutic agent in certain photodermatoses, a tanning agent, and a color-additive in human foodstuffs. Canthaxanthin and other carotenoids are expected to accumulate in the blood plasma beyond the therapeutic level (3–4 $\mu\text{g/ml}$) when used clinically (22).

In conclusion, canthaxanthin, astaxanthin and probably the other carotenoids containing oxo groups at the 4 (4')-position in the β -ionone ring system can serve as more effective antioxidants than β -carotene in peroxy radical-dependent lipid peroxidation. These so-called xanthophylls may be of importance as biological antioxidants, although further studies at different conditions seem to be necessary to fully understand the inhibitory effect of carotenoid pigments on lipid peroxidation.

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REFERENCES

1. Willet, W.C., Polk, D.B., Underwood, B.A., Stamper, M.J., Pressel, S., Rosner, B., Taylor, J.O., Schneider, K., and Hames, C.G. (1984) *N. Engl. J. Med.* 310, 430–434.
2. Nomura, A.M.Y., Stemmermann, G.N., Heilbrun, L.K., Salkeld, R.M., and Vuilleumier, J.P. (1985) *Cancer Res.* 45, 2369–2372.
3. Menkes, M.S., Comstock, G.W., Vuilleumier, J.P., Helsing, K.J., Rider, A.A., and Brookmeyer, R. (1986) *N. Engl. J. Med.* 315, 1250–1254.
4. Gey, K.F., Brubacher, B., and Stahelin, H.B. (1987) *Am. J. Clin. Nutr.* 45, 1368–1377.
5. Peto, R., Doll, R., Buckley, J.D., and Sporn, M.B. (1981) *Nature* 290, 201–208.
6. Foote, C.S., and Denny, R.W. (1968) *J. Am. Chem. Soc.* 90, 6233–6235.
7. Terao, J., Yamauchi, R., Murakami, H., and Matsushita, S. (1980) *J. Food Process Preserv.* 4, 79–83.
8. Krinsky, N.I. (1979) *Pure Appl. Chem.* 51, 649–660.
9. Krinsky, N.I., and Deneke, S.M. (1982) *J. Natl. Cancer Inst.* 69, 205–210.
10. Burton, G.W., and Ingold, K.U. (1984) *Science* 224, 569–573.
11. Ames, B.N. (1983) *Science* 221, 1256–1264.
12. Terao, J., and Matsushita, S. (1986) *Lipids* 21, 255–260.
13. Barclay, L.R.C., and Ingold, K.U. (1981) *J. Am. Chem. Soc.* 103, 6478–6485.
14. Burton, G.W., and Ingold, K.U. (1981) *J. Am. Chem. Soc.* 103, 6472–6477.
15. Yamamoto, Y., Niki, E., and Kamiya, Y. (1982) *Bull. Chem. Soc. Jpn.* 55, 1548–1550.
16. Niki, E., Saito, T., Kawakami, A., and Kamiya, Y. (1984) *J. Biol. Chem.* 259, 4177–4182.
17. Cutler, R.G. (1984) *Proc. Natl. Acad. Sci. USA* 81, 7627–7631.
18. Miller, K.W., Lorr, N.A., and Yang, C.S. (1984) *Anal. Biochem.* 138, 340–345.
19. Nells, H.J.C.F., and De Leenheer, A.P. (1983) *Anal. Chem.* 55, 270–275.
20. Ohmacht, R., Toth, Gy., and Voigt, G. (1987) *J. Chromatogr.* 395, 609–612.
21. Handelman, J., Dratz, E.A., Reay, C.C., and Kujik, J.G.M. (1988) *Invest. Ophthalmol. Vis. Sci.* 29, 850–855.
22. Meyer, J.Ch., Grundmann, H.P., Seeger, B., and Schnyder, U.W. (1985) *Dermatologica* 171, 76–81.

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