



## Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis

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### Abstract

The biological benefits of certain carotenoids may be due to their potent antioxidant properties attributed to specific physico-chemical interactions with membranes. To test this hypothesis, we measured the effects of various carotenoids on rates of lipid peroxidation and correlated these findings with their membrane interactions, as determined by small angle X-ray diffraction approaches. The effects of the homochiral carotenoids (astaxanthin, zeaxanthin, lutein,  $\beta$ -carotene, lycopene) on lipid hydroperoxide (LOOH) generation were evaluated in membranes enriched with polyunsaturated fatty acids. Apolar carotenoids, such as lycopene and  $\beta$ -carotene, disordered the membrane bilayer and showed a potent pro-oxidant effect (>85% increase in LOOH levels) while astaxanthin preserved membrane structure and exhibited significant antioxidant activity (40% decrease in LOOH levels). These findings indicate distinct effects of carotenoids on lipid peroxidation due to membrane structure changes. These contrasting effects of carotenoids on lipid peroxidation may explain differences in their biological activity.

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**Keywords:** Astaxanthin;  $\beta$ -carotene; Antioxidant; Lipid peroxidation; Liposome; X-ray diffraction

### 1. Introduction

Carotenoids are a large group of naturally-occurring pigments that are found in plants, algae and various micro-organisms. To date, over 750 carotenoids have been identified in nature [1], but only 24 have been detected in human tissues [2]. Carotenoids are thought to have a protective effect against

degenerative conditions such as cancer, cardiovascular disease and cataracts [3,4]. The majority of epidemiologic studies on the incidence of various types of cancers and cardiovascular diseases indicate an inverse relationship with dietary carotenoids [5,6] and circulating carotenoid levels [7]. The mechanism(s) by which carotenoids exert their health benefits are not completely understood, but may be due in part to their antioxidant activities [8,9]. Although earlier research on the antioxidant activities of carotenoids was focused primarily on  $\beta$ -carotene, other carotenoids have been shown to have more potent antioxidant effects [10] which may explain contradictory clinical findings including evidence for increased cardiovascular risk with  $\beta$ -carotene [11–15].

The common chemical feature of carotenoids is the polyene chain, a long conjugated double bond system forming the backbone of the molecule. This chain may be terminated by cyclic end groups that contain oxygen-bearing substitutes (Fig. 1). The electron-rich conjugated system of the polyene

**Abbreviations:** ABIN, 2,2'-azobis-isobutyronitrile; AMVN, 2,2'-azobis (2,4'-dimethylvaleronitrile); C/P, cholesterol to phospholipid mole ratio; DLPC, 1,2-dilinoleoyl-3-*sn*-glycero phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-glycero phosphatidylcholine; DOPC, 1,2-dioleoyl-3-*sn*-glycero phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-glycero phosphatidylcholine; EPR, electron paramagnetic resonance; EYPC, egg-yolk phosphatidylcholine; LOOH, lipid peroxide; POPC, 1-palmitoyl 2-oleoyl-3-*sn*-glycero phosphatidylcholine

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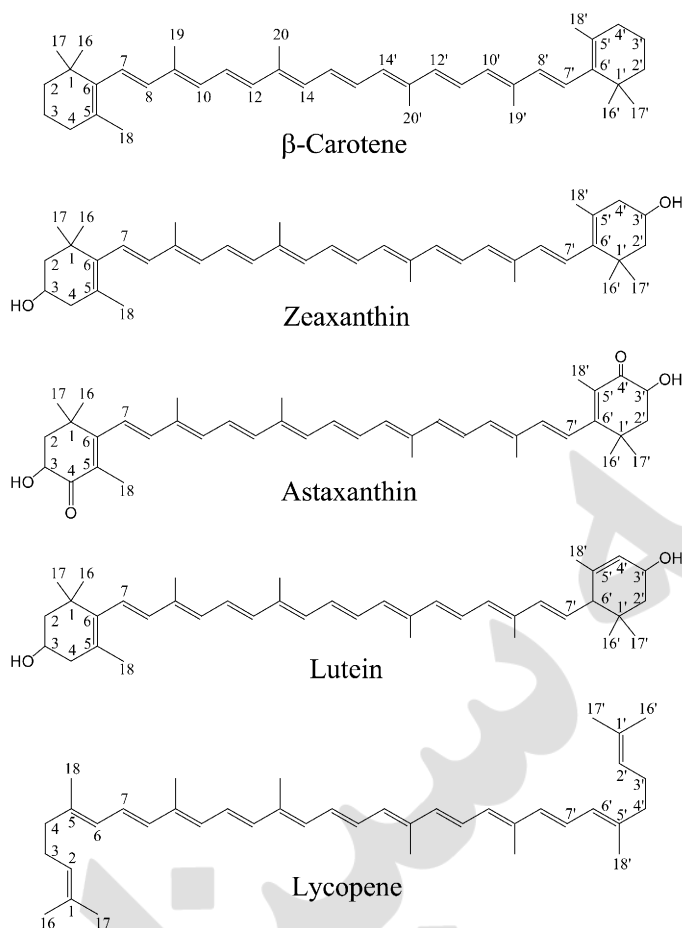


Fig. 1. Molecular structures of the carotenoids investigated in this study.

is responsible for the antioxidant activities of the carotenoids, both by quenching singlet oxygen [16,17], and scavenging radicals to terminate chain reactions [18–21].

Woodall et al. reported that the antioxidant capacities of various carotenoids in liposomes were very different from those in free solution and proposed that the antioxidant properties of carotenoids were determined by their inherent chemical reactivity as well as how they interact with the membrane bilayer [22]. This view has led to a number of studies designed to better understand the interactions between carotenoids and membranes using various techniques, including nuclear magnetic resonance [23–25], electron paramagnetic resonance (EPR) spin label [26–28], differential scanning calorimetry [29–31], fluorescence measurement [32–34] and X-ray diffraction approaches [35,36]. However, it should be noted that virtually all previous studies were carried out with model membranes lacking cholesterol. Cholesterol is a ubiquitous component of animal cell membranes, comprising up to 50 mol % of plasma membrane lipids [37]. Cholesterol is known to modulate the physical properties of membrane phospholipids [38]. Despite the evidence supporting a relationship between carotenoid membrane interactions and their antioxidant behavior, such a relationship has not been directly measured. In this study, we examined the membrane interactions of five homochiral carotenoids with various polarities (astaxanthin,

zeaxanthin, lutein,  $\beta$ -carotene, and lycopene). Membrane interactions were directly correlated with their effects on lipid peroxidation in membranes containing cholesterol. The results of this study demonstrate remarkable differences among these various carotenoids with respect to their effects on membrane lipid structure and rates of lipid peroxidation.

## 2. Materials and methods

### 2.1. Materials

1,2-Dilinoleoyl-3-*sn*-glycero phosphatidylcholine (DLPC) and 1-palmitoyl-2-oleoyl-3-*sn*-glycero phosphatidylcholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL) and stored in chloroform (25 mg/ml) at  $-80^{\circ}\text{C}$ . Cholesterol was also purchased from Avanti Polar Lipids and stored in chloroform (10 mg/ml) at  $-20^{\circ}\text{C}$  until use. Crystalline forms of  $\beta$ -carotene (all-trans), zeaxanthin (3*R*, 3'*R* all-trans), and astaxanthin (3*S*, 3'*S* all-trans) were obtained from Sigma-Aldrich (Saint Louis, MO), CarotNature, GmbH (Lupsingen, Switzerland), and Synchem, Inc. (Des Plaines, IL), respectively. Lutein (3*R*, 3'*R*, 6'*R* all-trans) and lycopene (all-trans) were purchased from Chromadex (Santa Ana, CA). The molecular structures of the carotenoids investigated in this study are shown in Fig. 1. Each carotenoid was solubilized in chloroform at a concentration of 50 mM and stored at  $-20^{\circ}\text{C}$  until use. The CHOD-iodide color reagent (stock) was prepared according to a procedure modified from el-Saadani et al. [39]. It consisted of 0.2 M  $\text{KH}_2\text{PO}_4$ , 0.12 M KI, 0.15 mM  $\text{NaN}_3$ , 10  $\mu\text{M}$  ammonium molybdate (tetrahydrate), and 0.1 g/l of benzalkonium chloride. The active reagent was freshly prepared prior to use by adding 0.2% Triton-X100, 24  $\mu\text{M}$  ethylenediaminetetraacetic acid (EDTA) and 20  $\mu\text{M}$  of butylated hydroxytoluene.

### 2.2. Preparation of multilamellar liposomes

For peroxidation analyses, liposomes were prepared with DLPC and cholesterol at a physiologically relevant cholesterol-to-phospholipid (or C/P) mole ratio of 0.2. Each of the five carotenoids was incorporated into the liposomes at a concentration of 10  $\mu\text{M}$ . For small angle X-ray diffraction analysis, POPC was used in preparation of membrane liposomes due to its lower susceptibility to oxidation as compared to DLPC and due to its similarity to DLPC in acyl chain length. Cholesterol was incorporated into POPC membranes at the same level as in DLPC membranes. Carotenoids were incorporated into POPC membranes at a carotenoid-to-phospholipid mole ratio of 0.07. Membrane samples were prepared as follows. Component lipids and carotenoids (in chloroform) were transferred to glass test tubes and dried under a steady stream of nitrogen gas while vortex mixing. Residual solvent was removed by drying for a minimum of 1 h under vacuum. Samples were resuspended in diffraction buffer (0.5 mM HEPES, 154 mM NaCl, pH 7.3) to yield a final phospholipid concentration of 2.5 and 1.0 mg/ml for POPC and DLPC, respectively. Multilamellar liposomes were prepared by vortexing vigorously for 3 min at ambient temperature [40]. We did not measure the efficiency of carotenoid incorporation into liposomes in this study; however, we assumed 100% incorporation based on previous reports that demonstrated carotenoid miscibility in DMPC [41] and unsaturated EYPC [41] in the range of 0 to 10 mol%. The concentrations used in this study were well below 10 mol %–6.2 mol% with respect to phospholipids and 5 mol% for phospholipids and cholesterol.

### 2.3. Lipid peroxidation analysis

All DLPC membrane samples were subjected to autooxidation by incubating at  $37^{\circ}\text{C}$  in an uncovered, shaking water bath. An aliquot (20  $\mu\text{l}$ ) of each sample was removed at the 48 h time point and combined with 1 ml of active CHOD-iodide color reagent. Test samples were immediately covered with foil and incubated at room temperature for over 3 h in the dark. Absorbances were then measured against a CHOD blank at 365 nm using a Beckman DU-640 spectrophotometer. This assay is based on the oxidation of iodide ( $\text{I}^-$ ) to triiodide anion ( $\text{I}_3^-$ ) by hydroperoxides. Concentrations of LOOH are directly proportional

to triiodide concentration and calculated by the use of the molar absorptivity of  $I_3^-$  measured at 365 nm ( $\epsilon = 2.46 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ).

#### 2.4. Preparation of samples for X-ray diffraction analysis

Membrane samples were oriented for X-ray diffraction analysis as previously described [42]. Aliquots containing 250  $\mu\text{g}$  of multilamellar vesicles (based on phospholipids) were transferred to custom-designed Lucite sedimentation cells, each containing an aluminum foil substrate upon which to collect a single membrane pellet. Samples were then loaded into a Sorvall AH-629 swinging bucket ultracentrifuge rotor (Dupont Corp., Wilmington, DE) and centrifuged at  $35,000 \times g$ ,  $5^\circ\text{C}$  for 1 h. Following membrane orientation, the supernatants were aspirated and the aluminum foil substrates, supporting the membrane pellets, were removed from the sedimentation cells and mounted onto curved glass slides. The samples were then placed in hermetically-sealed brass canisters in which temperature and relative humidity were controlled during X-ray diffraction experiments. Potassium sodium tartrate ( $\text{C}_4\text{H}_4\text{O}_6 \text{ NaK} \cdot 4\text{H}_2\text{O}$ ) and L(+) tartaric acid ( $\text{K}_2\text{C}_4\text{H}_4\text{O}_6 \cdot \frac{1}{2}\text{H}_2\text{O}$ ) were used to establish relative humidity levels of 87% and 74%, respectively, at  $20^\circ\text{C}$ .

#### 2.5. Small angle X-ray diffraction analysis

The oriented membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic X-ray beam produced by a Rigaku Rotaflex RU-200, high-brilliance microfocuss generator (Rigaku-MS, The Woodlands, TX). Analytical X-rays are generated by electron bombardment of a rotating copper anode and are filtered through a thin nickel foil to provide monochromatic  $\text{CuK}\alpha$  radiation ( $\text{K}\alpha_1$  and  $\text{K}\alpha_2$ , unresolved;  $\lambda = 1.54 \text{ \AA}$ ). Collimation of the X-ray beam was achieved using a single Franks mirror. Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Hecus X-ray Systems, Graz, Austria) using a sample-to-detector distance of 150 mm. Each diffraction peak is Lorentz- and background-corrected, as previously described [43]. The phases of the diffraction data are determined by swelling analysis [44], using the saturated solutions of the following salts (relative humidities in parentheses): tartaric acid (74%); potassium sodium tartrate (87%); and ammonium dihydrophosphate (93%). Fourier transformations of the collected X-ray diffraction data are generated using customized program modules written for Origin (OriginLab Co., Northampton, MA) and provides the time-averaged electron density distribution (distance,  $\text{\AA}$ , vs. electrons/ $\text{\AA}^3$ ). In addition to direct calibration, crystalline cholesterol monohydrate was used to verify the calibration of the detector.

This technique allows for precise measurement of the unit cell periodicity, or  $d$ -space, of the membrane lipid bilayer, which is the distance from the center of one lipid bilayer to the next, including surface hydration. The  $d$ -spaces for the membrane multilayer samples were calculated using Bragg's Law:

$$h\lambda = 2d\sin\theta \quad (1)$$

in which  $h$  is the diffraction order number,  $\lambda$  is the wavelength of the X-ray radiation ( $1.54 \text{ \AA}$ ),  $d$  is the membrane lipid bilayer unit cell periodicity including surface hydration, and  $\theta$  is the Bragg angle equal to one-half the angle between the incident beam and scattered beam.

### 3. Results

#### 3.1. Effects of carotenoids on membrane structure

A representative diffraction pattern of POPC model membranes containing cholesterol at a C/P mole ratio of 0.2 is shown in Fig. 2. Fig. 3 shows representative membrane electron density profiles that were generated from the small angle X-ray diffraction data. To understand the effects of the carotenoids on membrane structure, the electron density profiles were superimposed on the same scale. The two peaks of electron density on either side of the centrosymmetric

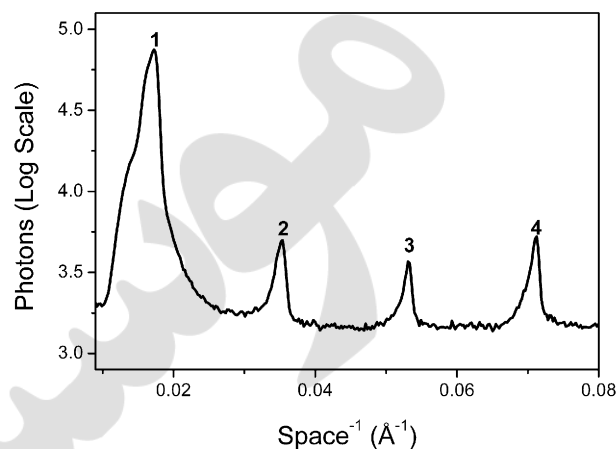


Fig. 2. Representative X-ray diffraction pattern of POPC membrane samples containing cholesterol at a C/P mole ratio of 0.2. Data were collected on a position-sensitive electronic detector at  $20^\circ\text{C}$  and 87% relative humidity. Four diffraction orders were obtained from these membranes, as indicated by the numbers above each peak.

profile correspond to phospholipid head groups, while the minimum of electron density at the center of the membrane is associated with terminal methyl segments of the phospholipid acyl chains.

With the exception of astaxanthin, the carotenoids altered the electron density associated with the membrane hydrocarbon core over a broad area,  $\pm 10 \text{ \AA}$  from the center of the membrane (Figs. 3 and 5). To assess the effects of the carotenoids on membrane structure, we directly subtracted the electron density profiles of the carotenoid-containing samples from the untreated samples (Fig. 5). The greatest disordering effect was observed with lycopene, followed by  $\beta$ -carotene, lutein, and zeaxanthin (Fig. 5). In addition to a decrease in electron density in the acyl chain regions, these carotenoids affected an increase in membrane width (Table 1).

We also measured the effects of these compounds on membrane structure at reduced hydrostatic pressure to order the membrane lipid. Decrease in relative humidity from 87% to 74% attenuated the disordering effects of the carotenoids and reduced changes in lipid width (Table 1). This effect was particularly noticeable with lycopene (Fig. 4). This observation supports the hypothesis that carotenoids exert their effects on membranes via a biophysical mechanism. If the changes were biochemical in nature, such as cleaved acyl chains, the imposed physical change would not have demonstrated this reversibility.

#### 3.2. Effects of carotenoids on lipid peroxidation

Each carotenoid was incorporated into DLPC/cholesterol composite membranes (C/P 0.2) at  $10 \mu\text{M}$  and the resultant membranes were subjected to autooxidation at  $37^\circ\text{C}$  for 48 h. The degree of lipid peroxidation was expressed in the percent increase or decrease in LOOH formation compared to controls containing no carotenoids. A strong positive correlation was observed between the degree of membrane peroxidation and

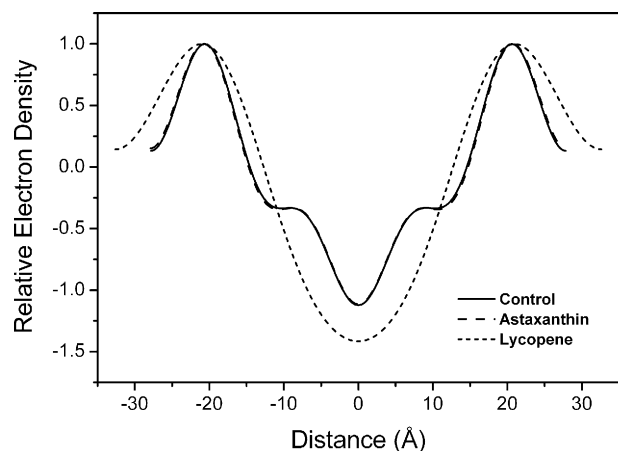


Fig. 3. Effects of polar versus apolar carotenoids on membrane structure. Superimposed one-dimensional electron density profiles (electrons/Å<sup>3</sup> vs. Å) of POPC membrane bilayers at a C/P mole ratio of 0.2. The data were collected at 87% relative humidity and 20 °C. Lycopene and astaxanthin were incorporated into membranes at a carotenoid/phospholipid mole ratio of 0.07. The two peaks of electron density on either side of the centrosymmetric profile correspond to phospholipid headgroups, while the minimum of electron density at the center of the membrane is associated with terminal methyl segments of the phospholipid acyl chains. Addition of astaxanthin did not appreciably affect membrane structure, whereas the other carotenoids altered the lipid packing characteristics in the hydrophobic region. The disordering effect on the membrane was especially evident for lycopene, as shown in this figure.

membrane disorder caused by the incorporated carotenoids. Astaxanthin showed a considerable reduction in lipid peroxidation of 41% (Fig. 5) while preserving membrane structure. By contrast, the other carotenoids disturbed the membrane bilayer and showed pro-oxidant activities. The strongest pro-oxidant activity was observed with lycopene

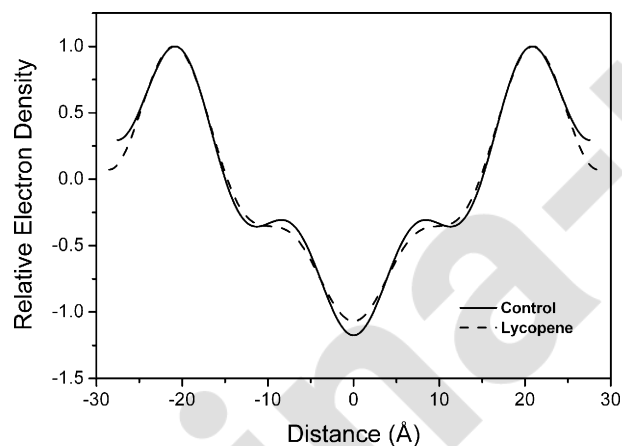


Fig. 4. Effect of hydrostatic pressure on membrane structure changes with lycopene. Superimposed one-dimensional electron density profiles (electrons/Å<sup>3</sup> vs. Å) of POPC membrane bilayers at 20 °C. The relative humidity was systematically decreased from 87% to 74% in hermetically-sealed sample chambers. Lycopene was incorporated into the membrane at a carotenoid/phospholipid mole ratio of 0.07. The two peaks of electron density on either side of the centrosymmetric profile correspond to phospholipid headgroups, while the minimum of electron density at the center of the membrane is associated with terminal methyl segments of the phospholipid acyl chains. The disordering effect of lycopene was greatly reduced compared to that of 87% relative humidity (Fig. 3).

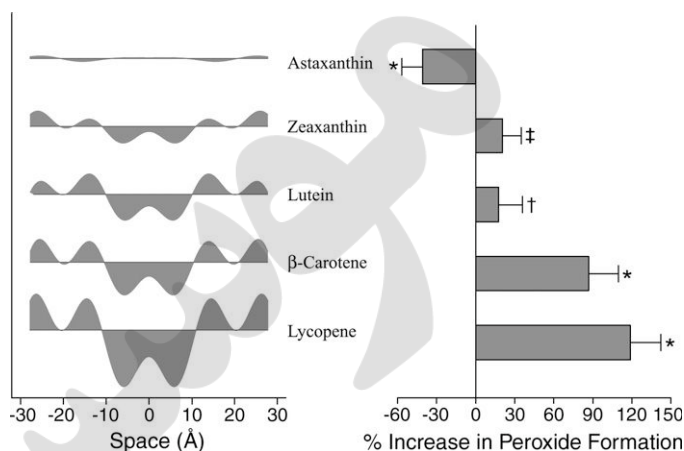


Fig. 5. Correlation between membrane structure changes and LOOH formation. Differences in relative electron density as a function of treatment with various carotenoids in POPC membranes containing a C/P mole ratio of 0.2. For the peroxidation study, various carotenoids (10 μM) were incorporated into DLPC membranes and underwent lipid peroxidation at 37 °C for 48 h. \**P*<0.001 vs. control; †*P*<0.01 vs. control; ‡*P*<0.05 vs. control; *n*=5–6.

(119%), followed by β-carotene (87%), zeaxanthin (21%) and lutein (18%) (Fig. 5).

#### 4. Discussion

The essential finding from this study is that carotenoids have contrasting effects on rates of peroxidation that relate to their membrane lipid interactions. The apolar carotenoids (β-carotene, lycopene) altered the packing of phospholipid acyl chains in a manner that correlated with potent pro-oxidant actions. By contrast, the addition of astaxanthin to the membrane did not modify the structure of constituent lipids. As a result, astaxanthin had opposite, antioxidant properties under identical conditions. Thus, the common chemical properties of carotenoids (i.e., the presence of a central polyene chain) may not be the only determinant of pro- or antioxidant behavior. To reach such conclusions, it is critical to ensure that differences in lipid peroxidation activities do not influence their intrinsic structure effects. For this reason, POPC was chosen due to its reduced susceptibility to lipid oxidation compared to DLPC, which is enriched with polyunsaturated fatty acids. Previous experiments in our laboratory have shown that lipid oxidation profoundly affects lipid order and membrane bilayer structure [45], and thereby could obfuscate membrane structural changes induced by incorporation of carotenoids. In addition, Woodall et al. [22] reported that all carotenoids (β-carotene, astaxanthin, zeaxanthin, and lycopene) tested in their study showed antioxidant activity, with astaxanthin and lycopene least effective, in organic solutions where membrane structure is not an issue. When carotenoids were incorporated into an ordered membrane system (liposome), they displayed different protective effects, indicating the membrane–carotenoids interaction is a critical factor that influences their antioxidant activity.

The differential effects of carotenoids on lipid peroxidation rates may be attributed, in part, to their orientation and location within the membrane. Both resonance Raman [46] and linear

dichroism [47] spectroscopy have been used to show that apolar carotenoids lie deep in the hydrophobic core, perpendicular to the long axis of the phospholipid acyl chains. Consequently, these rigid, rod-like molecules disrupted the intermolecular packing of the phospholipid molecules, leading to a decrease in molecular density, as observed in this study. Consistent with our findings, X-ray diffraction analyses by Suwalsky et al. demonstrated that lycopene induced structural perturbations of acyl chains in DPPC membranes when incorporated at a 2.5 mol % level, as evidenced by attenuation of the acyl-chain peak intensity in X-ray diffractograms [48]. EPR spin label and anisotropy studies have also been used to demonstrate the membrane disordering effects of  $\beta$ -carotene and lycopene, respectively [27,33].

Polar carotenoids, such as zeaxanthin and astaxanthin, appear to span the membrane with their polar end groups extending toward the polar regions of the membrane bilayer, spanning the membranes in a parallel fashion [49]. However, such orientation of carotenoids may be highly influenced by the lipid composition (e.g., acyl chain length, degree of saturation) of the membranes, leading to potential “mismatch” conditions between carotenoids and membranes. Gruszecki proposed that zeaxanthin acted like a “rivet” strengthening the membrane in this orientation [49]. This view has been supported for zeaxanthin (5 mol%) using DMPC, DPPC and egg-yolk phosphatidylcholine (EYPC) membranes by using X-ray diffraction [35], anisotropy [32], and  $^1\text{H-NMR}$  [23] studies, respectively; in contrast, Suwalsky [48] and Lazrack [50] reported no effect of zeaxanthin on DPPC and EYPC membranes at even higher incorporation levels (9 to 10 mol %) by X-ray diffraction analysis and measurement of the osmotic swelling of liposomes, respectively.

Several EPR studies have shown that polar carotenoids (zeaxanthin, violaxanthin, and lutein) decreased phosphatidylcholine membrane fluidity in the liquid crystalline phase and increased the order parameter of the membrane bilayer, particularly in the central, or hydrophobic core region [26,27,41,51]. The hydrophobicity of the membrane might play an important role in determining its susceptibility to oxidation because it restricts not only the membrane permeation of polar molecules [41,52–55] but also the depth of ion penetration into the bilayer [56–58]. By EPR technique using probes at various depths across the membrane, Wisniewska and Subczynski [28] estimated the shape of the hydrophobic barrier that is determined by the extent of water penetration into the membrane. In their study, the polar carotenoids – zeaxanthin, violaxanthin, and lutein – increased the hydrophobicity associated with the center of the bilayer at 10 mol%, but decreased hydrophobicity in the polar headgroup region. The degree of acyl chain length and lipid unsaturation also affected the hydrophobicity of membranes: longer acyl chains decreased the effect of polar carotenoids in the polar headgroup region but not in the hydrocarbon core. In unsaturated EYPC membranes, the hydrocarbon core hydrophobicity was elevated to a higher level than in saturated phosphatidylcholine membranes [28].

The primary consideration for a favorable interaction between the membrane and a particular carotenoid is its

molecular length. The length of the zeaxanthin molecule, defined as the distance between the hydroxyl group on opposing terminal rings, is known to be 32 Å [41]. Linear dichroism revealed that zeaxanthin adopts different tilt angles normal to the bilayer plane as a function of the membrane thickness, in order to best accommodate the stable transmembrane orientation; when comparing the thickness of the hydrophobic core of DMPC (25 Å) and EYPC (23 Å) membranes, as defined by the distance between the ester carbonyl groups of the opposite of phospholipids molecule, the angle of 24° was observed with DMPC [35] whereas the wider angle of 44° was observed with EYPC [23]. Compared to the studies discussed above, our membrane system was characterized by a larger membrane width, estimated to be 36 Å, due to the longer acyl chain of POPC and the presence of cholesterol, which is well known to increase membrane width [38]. This mismatch between the hosting membrane and zeaxanthin might explain the disordering effects of this carotenoid in the current study (Fig. 5). In addition to membrane width, the presence of unsaturated bonds in acyl chains is also known to affect the membrane-modulating ability of carotenoids. Subczynski et al. observed that the ordering effect of zeaxanthin was less pronounced in EYPC than DMPC membranes [59]. They proposed that the long axis of the rigid rod-like carotenoids lie parallel to the saturated acyl chains, thereby enhancing the extended conformation of lipid hydrocarbon chains. The ordering effect on lipid structure was less apparent in membranes containing unsaturated acyl chains, as in EYPC and POPC, due to the presence of cis double bonds.

Despite its structure similarity to zeaxanthin, astaxanthin (molecular length of 32 Å) did not significantly change the electron density of the membranes studied (Figs. 3 and 5). The only chemical difference between the two compounds is the presence of a ketone group at the C4 and C4' positions of the terminal rings of astaxanthin, which may act to further stabilize astaxanthin's membrane interactions with respect to the polar terminal groups (Fig. 1). This would allow the rest of the molecule to span the entire width of the membrane. This orientation proposed by Woodall et al. [22] enhances antioxidant activity by providing protection throughout the entire depth of membranes, interfering with the propagation of free radicals in the hydrophobic core, and quenching radicals generated at the surface of membranes. Goto et al. reported that once astaxanthin takes this favorable orientation within membranes, its polar terminal ring moiety is able to trap radicals both inside of the membrane and at the membrane surface via inter- and intramolecular hydrogen bonding [60]. Such structure–activity relationships for astaxanthin has been proposed to lead to its potent antioxidant activity in membranes [60].

Lutein, a polar dihydroxy constitutional isomer of zeaxanthin, caused a slightly greater degree of membrane perturbation than zeaxanthin (Fig. 5). Lutein have been reported to act quite differently in model membranes from zeaxanthin, due to the subtle modification at one terminal ring ( $\epsilon$ -ring). In lutein, one double bond in the  $\epsilon$ -ring is located between C4' and C5', differentiating it from the double bond between C5' and C6' in the  $\beta$  end ring of zeaxanthin (Fig. 1). This structural feature

endows the entire  $\epsilon$ -ring of lutein with relative rotational freedom around the C6'–C7' single bond. Due to this chemical property, lutein can adopt an orientation in which the hydroxyl group on the  $\epsilon$ -ring is either vertical or horizontal to the membrane plane [49]. The latter property allows lutein to assume a more apolar like orientation in the membrane, thus potentially shielding its OH groups from the water interface. This might explain why lutein produced a similar effect on membrane structure, compared to apolar carotenoid, as a function of relative humidity (Table 1). The 3'*R* allylic hydroxyl group of lutein also confers additional antioxidant capability as compared to zeaxanthin [61].

Among the five carotenoids studied, only astaxanthin essentially preserved the membrane structure, while at the same time demonstrating a strong antioxidant effect. Consistent with these findings, previous studies have indicated a link between rates of lipid peroxidation and membrane structure. It was observed that peroxidative damage to membranes may be modified by lipophilic drugs that have membrane ordering effects [62–64]. More recently, Berglund et al. [65] and Subczynski et al. [59] demonstrated that the addition of polar carotenoids to membranes increased membrane order and the permeability barrier to small molecules such as oxygen. Using the EPR spin label technique, Subczynski [66] demonstrated that an addition of 50 mol% cholesterol to DMPC and DOPC membranes decreased oxygen permeability of membranes by a factor of 5 and 2.5, respectively. They found that the resistance to oxygen transport is located in and near the polar headgroup region of membrane bilayers. These observations indicate that the ordering state of membranes might influence peroxidation by controlling oxygen diffusion into membranes. Since astaxanthin differs from zeaxanthin only by the presence of keto groups at its terminal rings, the antioxidant activity of astaxanthin in this study suggests highly specific mechanisms which need to be further investigated. Our study demonstrated that the effect of astaxanthin on membrane order may be one such mechanism. Future studies will explore the effects of carotenoids on fluidity, molecular motion, and lipid hydrophobicity and, consequently, on oxygen penetration and water diffusion into membranes.

Table 1  
Effect of carotenoids on structure of POPC model membranes as a function of hydration

Sample	<i>d</i> -Space (Å)	
	87% RH	74% RH
Control	57	55
Astaxanthin	57	55
$\beta$ -Carotene	61	57
Zeaxanthin	58	57
Lutein	61	56
Lycopene	66	57

Carotenoids were incorporated at a carotenoid/phospholipid mole ratio of 0.07 (or 6.25 mol%) into POPC membranes containing cholesterol at a C/P mole ratio of 0.2. Small angle X-ray analysis was conducted sequentially at two different relative humidities of 87% and 74% at 20 °C. Decreasing the relative humidity from 87% to 74% attenuated the effects of the carotenoids, including changes in lipid membrane width.

A number of studies exist on the role of carotenoids in the formation of LOOH in liposomes. Many reported the antioxidant activities of various carotenoids (including  $\beta$ -carotene, lycopene, zeaxanthin, astaxanthin, and canthaxanthin) [22,67,68], contrary to the current observations. In addition to the disordering effect of carotenoids on membranes discussed above, the peroxidation assay employed in the current study may have contributed to the different outcomes. Antioxidant assay methods employed in the studies cited above are based on the inhibition of free radicals induced by strong radical initiators, such as 2,2'-azobis-isobutyronitrile (ABIN) and 2,2'-azobis (2,4'-dimethylvaleronitrile) (AMVN), added exogenously to the system. It is noteworthy that the outcomes of the assays are affected by the type and amount of initiator used in the system. To avoid the use of strong peroxide radical initiators employed by most antioxidant capacity assays, we chose the method developed by el-Saadani [39], which occurs under normal atmospheric oxygen conditions without addition of exogenous initiators, making the present model study more valid physiologically.

This method also allowed us to observe the effects of carotenoids on the progress of peroxidation over a longer period of time (48 h) compared to the conventional methods where membranes are exposed to high concentrations of free radicals over a shorter time period (within a few hours). Reaction time is important in this type of study, since all carotenoids are known to react to oxidizing agents at different rates. Woodall et al. investigated reaction rate of various carotenoids in three different oxidation systems, using  $\text{Fe}^{2+}$ , AIBN, and AMVN as radical initiators [22]. In all cases, lycopene was the most reactive carotenoid studied, and therefore was nearly depleted within a few hours; lycopene was typically followed by  $\beta$ -carotene. By contrast, the diketocarotenoids, such as astaxanthin and canthaxanthin, were the least reactive, and therefore would remain for a longer period of time to scavenge free radicals. These results agree with those of Terao, in which carotenoid auto-oxidation was initiated by AMVN [69]. The differences in auto-oxidation rates might explain why only astaxanthin showed such strong antioxidant properties, whereas lycopene and  $\beta$ -carotene exhibited strong pro-oxidant properties in this study. This differentiation in antioxidant activities among the carotenoids caused by background auto-oxidation in the current study could have been missed with conventional antioxidant assays based on short peroxidation reaction times.

Biological membrane systems are much more complex than the membrane system used in this study, and therefore the physiological relevance of the outcome of this study is not clear. Within this limitation, however, our findings still provide additional insight into the apparently contradictory effects of chemically similar carotenoids on biological and clinical outcomes. In large and prospective randomized human trials, administration of  $\beta$ -carotene was shown to actually increase the incidence of cardiovascular disease in various groups, including healthy subjects [14,15], smokers [11,12], and patients with known or suspected coronary disease [13]. Others have shown that  $\beta$ -carotene – particularly at high concentration and high

oxygen tension – has auto-oxidative and pro-oxidant properties [19,70,71]. By contrast, astaxanthin has been shown to have cardioprotective benefits in various models [72–74]. Such beneficial actions of certain carotenoids may be attributed to antioxidant activity [8,9]. Thus, the differential effects of the carotenoids on lipid peroxidation may provide a clue to their apparent contradictory effects in various clinical trials.

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