14 Dietary Carotenoids and their Metabolites as Potentially Useful Chemoprotective Agents against Cancer

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INTRODUCTION

One class of food components that has been widely studied for their protective effect against cancer is carotenoids. Carotenoids are among the most widespread of the naturally occurring groups of pigments and are found in all families of the plant and animal kingdoms. To date, as many as

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700 carotenoids have been isolated from various sources and their chemical structures have been characterized. During the past decade, the authors have isolated, identified, and quantified carotenoids from fruits and vegetables commonly consumed in the United States (Khachik *et al.*, 1986; Khachik and Beecher, 1988; Khachik *et al.*, 1989, 1992a,e). These studies have revealed that as many as 40 to 50 carotenoids may be available from the diet and absorbed, metabolized, or utilized by the human body (Khachik *et al.*, 1991). However, among these, only 13 *all-E-* and 12 *Z*-carotenoids are found routinely in human serum and milk (Khachik *et al.*, 1992b,c,d, 1995b, 1997c). In addition, there are 1 *Z*- and 8 *all-E*-carotenoid metabolites resulting from two major dietary carotenoids, lutein and lycopene, which have also been characterized by Khachik *et al.* (1992b,c,d, 1995b, 1997c). The correlation between dietary carotenoids and carotenoids found routinely in the extracts from human serum/plasma has revealed that only selected groups of carotenoids make their way into the human bloodstream. Some of these carotenoids are absorbed intact and others, such as lutein, zeaxanthin, and lycopene, are converted to several metabolites. Some dietary carotenoids are also present in human tissues, i.e., liver, lung, breast, and cervix (Khachik *et al.*, 1998a).

Evidence for the nutritional significance of carotenoids in the prevention of diseases such as cancer, cardiovascular, and macular degeneration (an age-related degenerative eye disease) has been obtained from various interdisciplinary studies, which may be classified as (a) epidemiological studies, (b) carotenoid distribution in fruits, vegetables, human serum, and milk, (c) carotenoids in human organs and tissues, (d) *in vitro* studies of chemopreventive properties, and (e) *in vivo* studies with rodents. Ever since the 1970s, it has been suggested that one mechanism by which carotenoids exert their biological activity in disease prevention was by functioning as an antioxidant. In 1992, for the first time, the authors reported on the isolation and characterization of several oxidation products of carotenoids in human plasma (Khachik *et al.*, 1992c). More recently the *in vivo* oxidation of specific carotenoids, has been demonstrated, e.g., lutein, zeaxanthin, and possibly lycopene in humans (Khachik *et al.*, 1995a, 1997d; Paetau *et al.*, 1998).

This chapter identifies several new optical isomers of carotenoid metabolities in human plasma and provides additional evidence for previously proposed metabolic oxidation–reduction reactions of dietary carotenoids in humans. This chapter also presents studies that indicate that carotenoids, in addition to their antioxidant mechanism of action, can exert their biological activity in disease prevention by other mechanisms. These are (a) gap junctional intercellular communications (GJC) (King *et*

al., 1997), (b) anti-inflammatory and antitumor promoting properties and (c) induction of detoxication (phase 2) enzymes.

EXPERIMENTAL PROCEDURES

Instrumentation

For gradient high-performance liquid chromatography (HPLC) analysis, a binary solvent delivery system interfaced into a rapid-scanning ultraviolet (UV)/visible photodiode array detector was employed.

Chromatographic Conditions

For separation of the benzoate derivatives of carotenoids and their metabolites, which were prepared either from extracts of human plasma or by synthesis, a Sumichiral OA-2000 (5 μm, 25 cm × 4 mm) column (Phenomenex, Torrance, CA) was employed. The column was protected with a Brownlee Cyano guard cartridge (5 μm, 3 cm × 4.6 mm). A combination of an isocratic and a two-step gradient at the flow rate of 0.70 ml/min affected the separations in about 100 min. Details of the isocratic and the gradient conditions are summarized in Table I.

Reference Samples of Carotenoids

Carotenoid standards were either synthesized or isolated from natural sources according to published procedures (Khachik *et al.*, 1986, 1992c, 1995b, 1997c, 1998b). (3*R*,6′*R*)-3-Hydroxy-β,ϵ-3′-one was prepared from

Table I Chromatographic Conditions for Separation of a Mixture of Benzoate Derivatives of Carotenoids and Their Metabolites Prepared from an Extract of Human Plasma

Combination of isocratic and two-step gradient			
Mobile phase	Pump A: Hexane		
	Pump B: Hexane (79%), dichloromethane (20%), methanol (1%)		
Time 0–10 min	Time 65 min		Time 75 min
90% (A)	60% (A)		10% (A)
Linear gradient Over 55 min →		Linear gradient Over 10 min →	
10% (B)	40% (B)		90% (B)

lutein by partial synthesis and (3*R*,6′*R*)-3-hydroxy-β,ε-caroten-3′-one was prepared by total synthesis (Khachik *et al.*, 1992c). Lactucaxanthin was isolated from an extract of Romaine lettuce (*Lactuca sativa*).

Preparation of Carotenoid Benzoates

Hydroxycarotenoids were dissolved in 2.0 ml of pyridine and cooled to 0°C under an atmosphere of nitrogen. A few drops of benzoyl chloride were added and the mixture was stirred for 30 min at 0°C and for 1 hr at room temperature. The product was partitioned between hexane and water. The hexane layer was washed with water several times, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in hexane (80%) and dichloromethane (20%) for HPLC analysis.

Preparation of Benzoate Derivatives of Carotenoids from an Extract of Human Plasma

Human plasma (American Red Cross, Baltimore, MD) was extracted according to published procedures (Khachik *et al.*, 1992c, 1997c). The extract was dried over sodium sulfate, dissolved in pyridine, and treated with benzoyl chloride; the product was worked up similar to the previous procedure.

Intercellular Communication Assays

Cell Culture Conditions

The human immortalized keratinocyte cell line HaCaT (Ryle *et al.*, 1989; Fitzgerald *et al.*, 1994) was used to examine carotenoid effects in human cells. All cultures were incubated at 37°C, in 5% carbon dioxide and 95% humidity. HaCaT cells were cultured in low calcium (0.1 mmol/liter) serum-free keratinocyte medium (GIBCO-BRL, Grand Island, NY) supplemented with epidermal growth factor (5 μg/ml), bovine pituitary extract (35 μg/ml), and insulin (5 μg/ml) in a monolayer culture until subconfluent. They were then harvested by trypsin/EDTA, resuspended in low calcium medium, and placed on Millicell-CM collagen-coated culture plate inserts (Millipore, Bedford, MA). Filters were incubated submerged in low calcium medium for approximately 7 days, at which point high calcium medium (1.15 mmol/liter), supplemented as described earlier, was placed below the filters while the surface of the filters were exposed to the atmosphere for 7 days. During this time a multilayered differentiating organotypic culture was produced. Cells were treated with carotenoids or retinoic acid dis-

solved in THF or acetone, respectively, using the precautions and procedures discussed previously (Cooney *et al.*, 1993) and added to culture medium concurrent with exposure to the air interface. Cells were refed and retreated every 2 days.

Protein Electrophoresis and Western Blotting

HaCaT cells were harvested and connexin 43 (Cx43) solubilized as described previously (Rogers *et al.*, 1990). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), and equal amounts of protein were electrophoresed on 10% SDS polyacrylamide gels. Proteins were electroblotted onto an Immobilon PVDF membrane (Millipore) and subsequently incubated with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 15 residues of the predicted sequence of rat cardiac Cx43 (Beyer *et al.*, 1987). This sequence is 100% homologous in mouse, rat, and human. Bound antibody was visualized using a chemiluminescent detection system (Tropix, Bedford, MA) and recorded on X-ray film.

Phase 2 Enzyme Induction Assay

Purified carotenoids were dissolved in tetrahydrofuran (THF) and their quinone reductase (QR) inducer potency was determined by a coupled tetrazolium dye assay performed on digitonin extracts of Hepa 1c1c7 cells grown in microliter plates. The procedures have been described previously by Prochaska and Santamaria (1988) and Prochaska *et al.* (1992) and modified by Fahey *et al.* (1997). The protein content of digitonin extracts was determined using a bicinchoninic acid assay (Smith *et al.*, 1985). Carotenoids were tested over a concentration range of 0.78 to 100 μM and the final concentration of THF was 0.5% by volume. Activity is reported as follows. Concentration for doubling (CD) inducer activity is the amount of compound required to double the QR activity of a microtiter plate well, initially seeded with 10,000 Hepa 1c1c7 (murine hepatoma) cells and containing 0.15 ml of α-MEM culture medium amended with 10% fetal calf serum, streptomycin, and penicillin.

SEPARATION OF OPTICAL STEREOISOMERS OF CAROTENOIDS AND THEIR METABOLITES IN HUMAN PLASMA BY HPLC

Dietary hydroxycarotenoids and their metabolites, identified in human serum, contain two or three stereogenic centers in their molecules and would therefore be expected to exist as a number of optically active iso-

mers. Although the authors have reported on the separation and identification of carotenoids and their metabolites from extracts of human serum and milk previously (Khachik *et al.*, 1992c, 1997c), the absolute configuration of these compounds has not been determined to date. Understanding the stereochemistry of carotenoids in serum can provide valuable information regarding the pathways leading to the formation of carotenoid metabolites in humans. Metabolites found in serum result from three major dietary carotenoids: lutein, zeaxanthin, and lycopene (Fig. 1). The chemical structures of carotenoid metabolites are shown in Fig. 2. Among these, only the absolute configurations of 3′-epilutein and the two dehydration products of dietary lutein, (3*R*,6′*R*)-3′,4′-didehydro-β,γ-carotene-3-ol and (3*R*,6′*R*)-2′3′-didehydro-β,ε-carotene-3-ol, are known (Khachik *et al.*, 1995b). In the case of the two lycopene metabolites, 2,6-cyclolycopene-1,5-diols A and B, only the relative but not the absolute configuration of these compounds at C-2, C-5, and C-6 is known at present (Khachik *et al.*, 1998b). The rest of the carotenoid metabolites shown in Fig. 2 are monoketo- and diketocarotenoids that have also been isolated from hen's egg yolk by Matsuno *et al.* (1986). These investigators employed a Sumipax OA-2000 chiral HPLC column to separate the 3 stereoisomers of ε, ε-caroten-3,3′-dione and all 10 stereoisomers of lactucaxanthin [(3*S*,6*S*,3′*S*,6′*S*-ε, ε-carotene-3,3′-diol] (Fig. 1), a dihydroxycarotenoid with four stereogenic centers (Ikuno *et al.*, 1985). This was accomplished by derivatization of this dihydroxycarotenoid with benzoyl chloride and separation of the resulting dibenzoates by chiral chromatography. A chiral HPLC column was employed to separate the benzoate derivatives of lutein, zeaxanthin, lactucaxanthin, and the metabolites of carotenoids typically found in human serum. The objective was to determine whether it was possible to simultaneously separate the benzoate derivatives of carotenoids as well as the optical isomers of their metabolites by chiral HPLC in such a complex mixture by derivatizing all the mono- and dihydroxycarotenoids in an extract from human plasma with benzoyl chloride. Because of the lack of a hydroxyl group in ε, ε-caroten-3,3′-dione and the tertiary nature of the hydroxyl groups in 2,6-cyclolycopene-1,5-diols A and B (Fig. 2), these metabolites could not be derivatized with benzoyl chloride. Therefore, these compounds were mixed directly with a synthetic mixture of the benzoate derivatives of all the hydroxycarotenoids in human serum for HPLC studies. Employing a combination of isocratic and two-step gradient HPLC (Table I), the various optical isomers of carotenoids and their metabolites in the just-described synthetic mixture were resolved adequately as shown in Fig. 3. The HPLC profile of an extract from human plasma derivatized with benzoyl chloride is shown in Fig. 4. The benzoate derivatives of monohydroxycarotenoids,

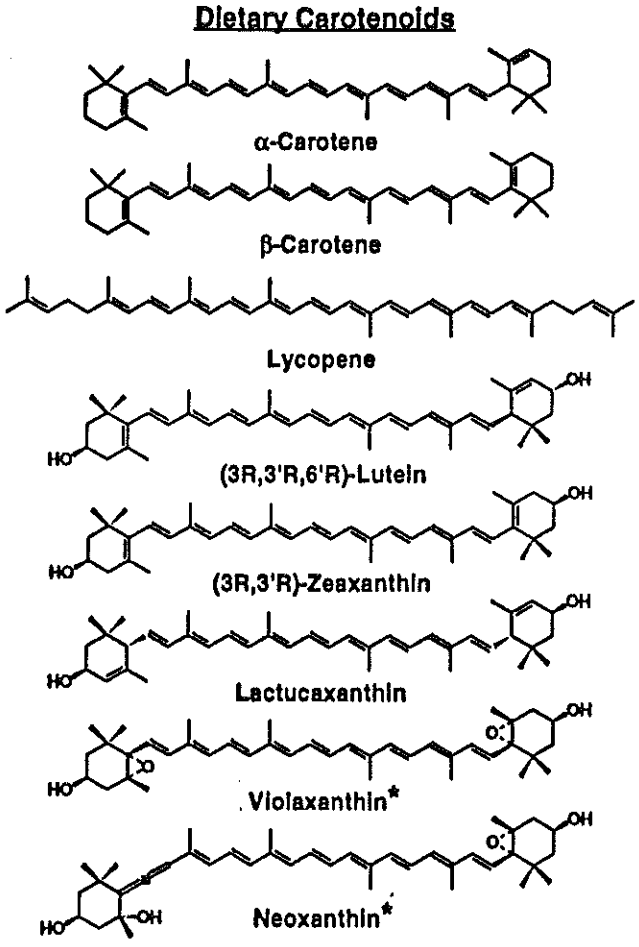

Figure 1 Chemical structures of some of the dietary carotenoids and two noncarotenoids, curcumin and sulforaphane, evaluated as inducers of phase 2 enzymes. Asterisks indicate carotenoids not absorbed into human serum/plasma.

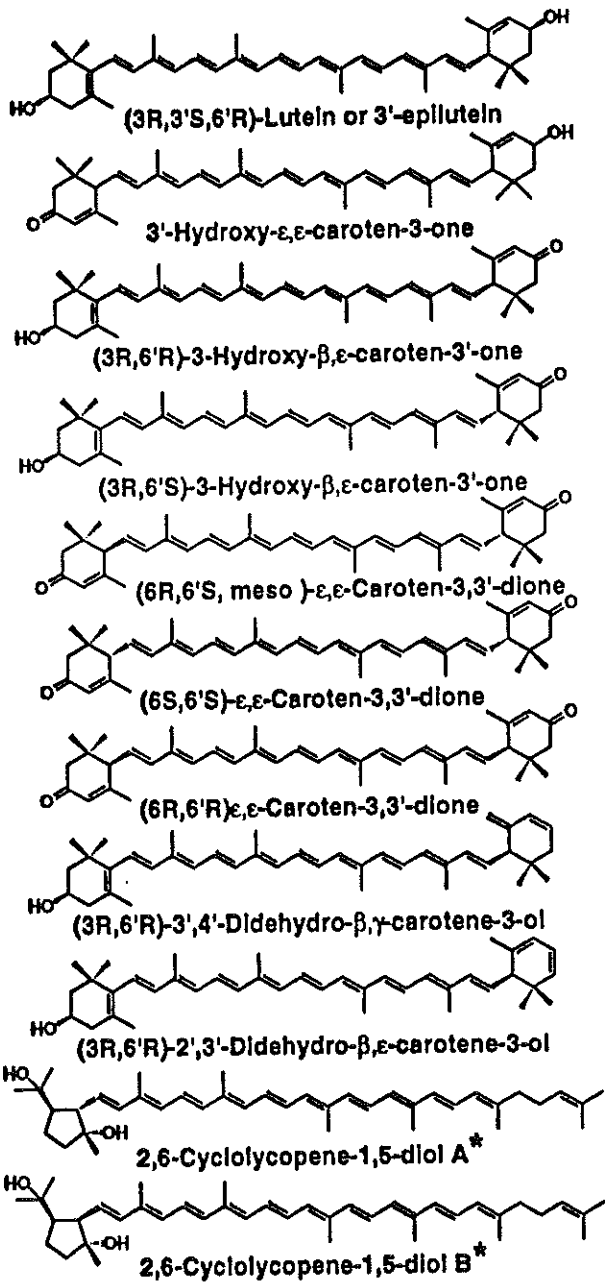

Figure 2 Chemical structures of carotenoid metabolites and their optical stereoisomers identified in an extract from human plasma. Absolute configurations of the various optical isomers of 3'-hydroxy-ε,ε-caroten-3-one are not known at present. Asterisks indicate that only the relative but not the absolute configuration of these compounds at C-2, C-5, and C-6 is known.

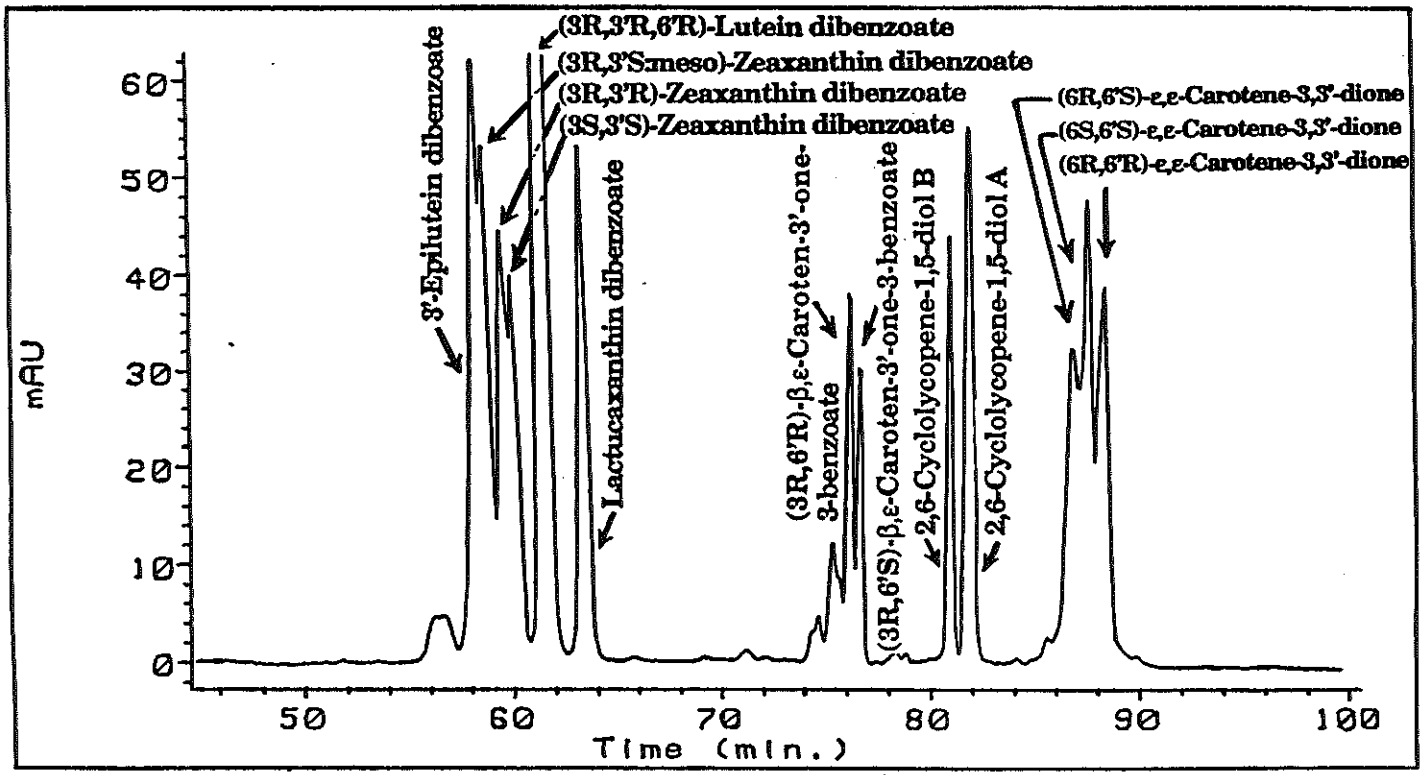

Figure 3 Chiral HPLC separation of optical isomers of a mixture of synthetic benzoate derivatives of certain carotenoids and their metabolites. For clarity of presentation, only the latter part of the HPLC profile is shown. Conditions are as described in the text.

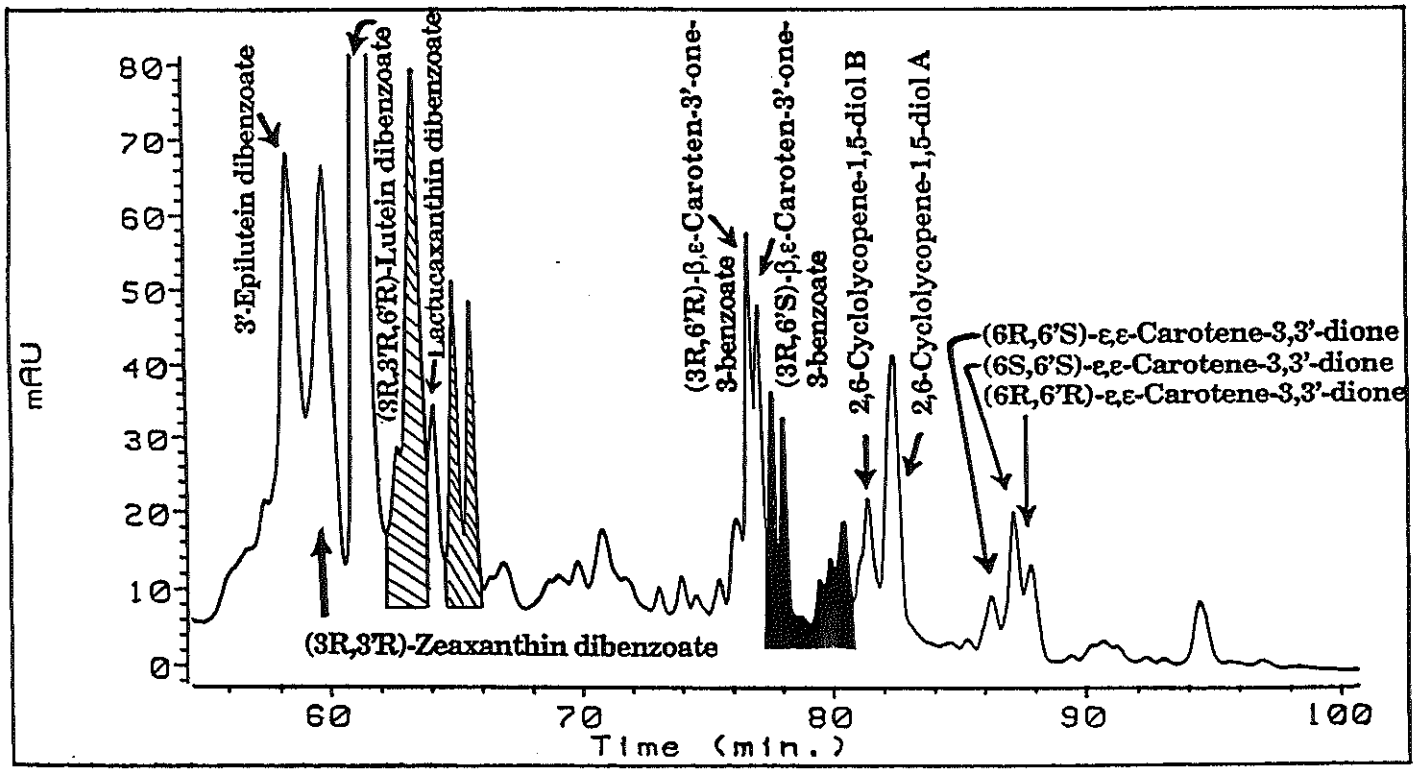

Figure 4 Chiral HPLC separation of the optical isomers of benzoate derivatives of carotenoids and their metabolites prepared from an extract of human plasma. For clarity of presentation, only the latter part of the HPLC profile is shown. Conditions are as described in the text. Shaded peaks are the *Z*-isomers of lutein and zeaxanthin dibenzoates and dark peaks are the optical isomers of ε,ε-caroten-3-one-3′-benzoate.

such as α- and β-cryptoxanthin, as well as the dehydration products of lutein, which are normally present in human plasma, were also prepared and examined by HPLC. However, due to early retention times (25–30 min), the separation of these compounds is not shown in the chromatograms in Figs. 3 and 4. Although 3′-hydroxy- ϵ, ϵ-caroten-3-one benzoate was not prepared by synthesis, the presence of the various optical isomers of this compound with three stereogenic centers was tentatively established by HPLC (dark peaks highlighted in Fig. 4). Individual carotenoid benzoates were separated by preparative HPLC and were identified by comparison of their circular dichroism spectra with those reported in the literature (Ikuno *et al.*, 1985; Matsuno *et al.*, 1986). Details of the structural elucidation will be described in a later publication. As shown in Fig. 4, there appears to be some HPLC peak overlap between 3′-epilutein dibenzoate and zeaxanthin dibenzoate. Therefore, lutein, zeaxanthin, and 3′-epilutein were isolated by preparative HPLC from an extract of human plasma (Khachik *et al.*, 1992c, 1992d) and then derivatized with benzoyl chloride separately. HPLC results indicated that in addition to dietary (3*R*,3′*R*,6′*R*)-lutein and its metabolite (3*R*,3′*S*,6′*R*)-lutein (3′-epilutein), none of the other optically active isomers of lutein were present in human plasma. However, in the case of dietary (3*R*,3′*R*)-zeaxanthin, approximately 2–3% of an optically inactive isomer of this compound, namely (3*R*,3′*S*,*meso*)-zeaxanthin, could be detected in plasma.

DIETARY CAROTENOIDS AND THEIR METABOLITES AS CANCER CHEMOPREVENTIVE AGENTS

There is very little disagreement within the scientific community regarding the protective effect of high consumption of fruits and vegetables and the lower risk for human cancers. However, the identity of the active components in such a diet and the mechanisms by which this protective effect may be provided are the subjects of intense research and much debate. Carotenoids are one such class of compounds, which are present at high concentrations in fruits and vegetables associated with risk reduction in many epidemiologic studies (Mayne, 1996). Although the number of dietary carotenoids is in excess of 40, it has been shown that 13 *all-E* and 12-*Z* dietary ones are actually absorbed into the bloodstream. These dietary carotenoids are metabolized to 8 *all-E* and 1-*Z* metabolites and, as a result, a total of 34 carotenoids can be detected in human serum and milk (Khachik *et al.*, 1997c). Despite the presence of all these dietary carotenoids and their metabolites in the serum, β-carotene has received much of the attention due to its provitamin A activity. The nutritional significance of

β-carotene and carotenoids in general in the prevention of cancer has been based solely on their provitamin A activity and, to some extent, on their antioxidant property. In 1991, Bertram and colleagues reported on the cancer preventive property of carotenoids by a different mechanism involving the upregulation of the expression of gap junctional communication proteins. However, to date, other mechanisms of chemoprotection by carotenoids, particularly their metabolites, have not been fully investigated. Possible mechanisms by which chemopreventive agents may work against events that are involved in the development of cancer have been reviewed by Kelloff *et al.* (1994).

The authors have examined the chemopreventive properties of major dietary carotenoids and their metabolites in light of some of these mechanisms and have established that these compounds may serve as potentially useful chemoprotective agents against cancer. The chemical structures of some of the dietary carotenoids investigated are shown in Fig. 1. Figure 1 also shows the structures of two noncarotenoids, curcumin and sulforaphane, which due to their unique and well-established role as chemoprotective agents were selected for comparative studies with carotenoids. α-Carotene, β-carotene, lycopene, lutein, and zeaxanthin are among the major dietary carotenoids found in human serum or plasma whereas only trace levels of lactucaxanthin can be detected. This is probably related to the dietary source of this compound, which is limited to Romaine lettuce (*L. sativa*). Although violaxanthin and neoxanthin are two major carotenoid epoxides found in fruits and green vegetables (Khachik *et al.*, 1986), these compounds have not been detected in human serum or plasma (Khachik *et al.*, 1992c, 1997c).

Studies with the carotenoids shown in Fig. 1 and some of their metabolites (Fig. 2) have revealed that these compounds may impart their biological activity by several mechanisms. These are (a) antioxidant function, (b) gap junctional intercellular communication, (c) anti-inflammatory and antitumor promoting properties, and (d) induction of detoxication (phase 2) enzymes.

Antioxidant Function

One hypothesis regarding the role of carotenoids as cancer chemopreventive agents is based on their antioxidant capability to quench singlet oxygen and other oxidizing species and to inhibit lipid peroxidation, thus preventing further promotion and replication in the neoplastic cell. If a free radical mechanism is involved in the initiation and promotion of carcinogenesis, carotenoids such as lutein, zeaxanthin, and lycopene may participate in quenching peroxides and protecting cells from oxidative damage.

This would be expected to result in the formation of a number of oxidative metabolites of these carotenoids. In 1992, for the first time, the authors reported on the isolation and characterization of oxidation products of carotenoids in human plasma (Khachik *et al.*, 1992c) and conducted several human supplementation studies to demonstrate the *in vivo* oxidation of certain dietary carotenoids such as lutein and zeaxanthin to their metabolites (Khachik *et al.*, 1995a, 1997d). In a more recent publication, the structures of two oxidative metabolites of lycopene, 2,6-cyclolycopene-1,5-diols A and B (Fig. 2) in human serum and milk, were established (Khachik *et al.*, 1997c, 1998b) and a mechanism for the formation of these metabolites was proposed (Khachik *et al.*, 1997d). Further evidence for possible *in vivo* oxidation of lycopene to these metabolites was obtained from a study with purified lycopene supplements involving healthy human volunteers (Paetau *et al.*, 1998). Based on these studies, the authors have proposed the most likely pathways leading to the formation of the oxidative metabolites of carotenoids in humans (Khachik *et al.*, 1995a, 1997d). These metabolic pathways are summarized in Fig. 5. With the exception of the metabolites of lycopene (2,6-cyclolycopene-1,5-diols A and B) (Khachik *et al.*, 1997d) and the two dehydration products of lutein (Khachik *et al.*, 1995b) reported previously, the carotenoid metabolites, shown in Fig. 5, can be formed by three types of reactions. These are (1) allylic oxidation of the ε-end group of lutein to give an α,β-unsaturated ketocarotenoid, (2) reduction of the resulting ketocarotenoid with epimerization at C-3, and (3) double bond isomerization in the β-end group of dietary zeaxanthin or other intermediate carotenoid metabolites to give an ε-end group. While the oxidation reactions remove the stereogenic centers in the carotenoid metabolites, the reduction and double bond isomerization reactions create new stereogenic centers. As described earlier, all the optical isomers of monoketo- and diketocarotenoids appear to be present in the extracts of human plasma. These results are in complete agreement with earlier proposed metabolic pathways of dietary lutein and zeaxanthin in humans. Another carotenoid that may serve as an antioxidant is lactucaxanthin (Fig. 1). Due to the presence of two allylic hydroxyl groups in its molecule, lactucaxanthin would be expected to oxidize readily to mono- and diketocarotenoids; metabolic pathways of this compound are not shown in Fig. 5. Because of its limited dietary source (Romaine lettuce), only a trace amount of lactucaxanthin has been detected in human serum (Khachik *et al.*, 1997c). It is imperative to point out that although only 2–3% of (3*R*,3′*S*,*meso*)-zeaxanthin has been found inhuman plasma, the presence of this compound completes the cycle of metabolic reactions and interconversion of dietary lutein and zeaxanthin in humans.

This chapter presents additional evidence for the *in vivo* oxidation of

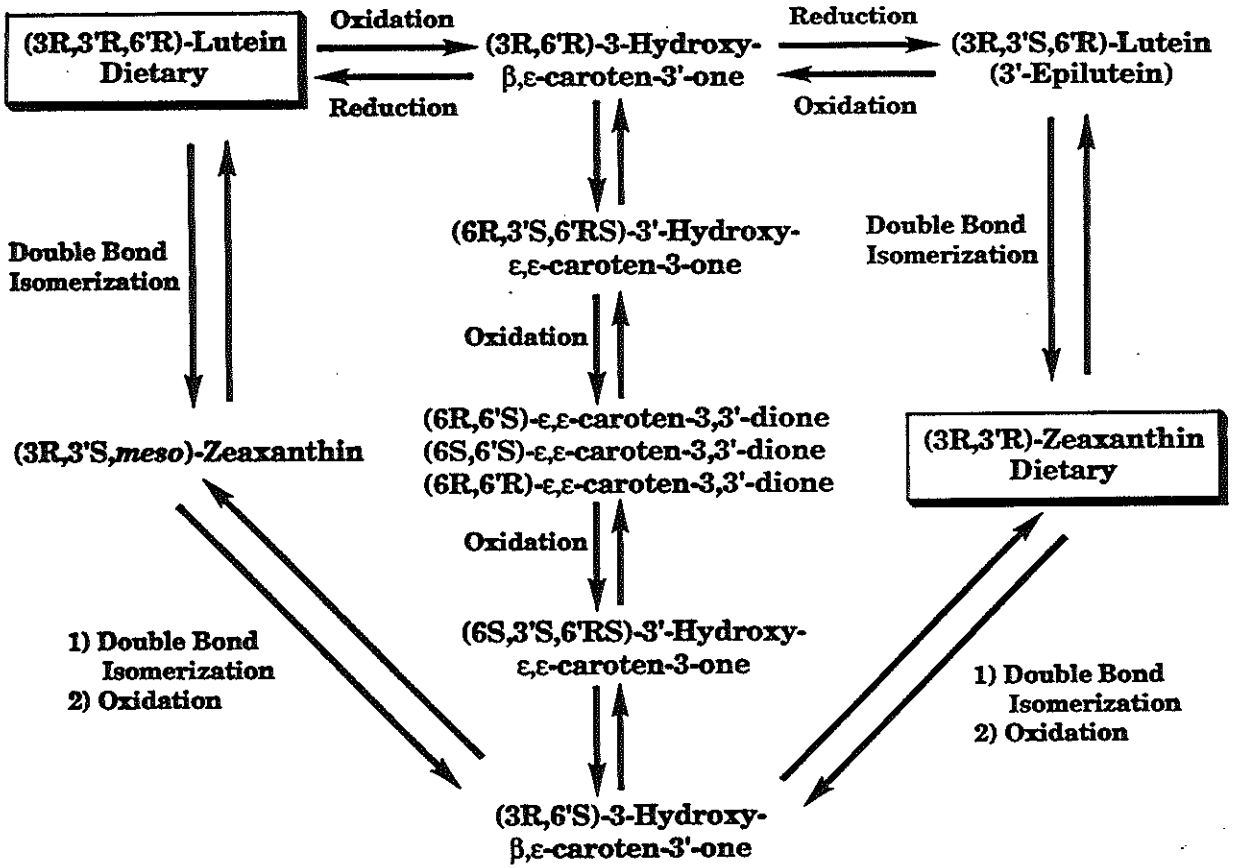

Figure 5 Metabolic pathways of dietary lutein and zeaxanthin in humans.

carotenoids by further elucidating the stereochemistry of some of their oxidative metabolites. However, the pharmacokinetics and pharmacodynamics of the oxidative metabolites of carotenoids, as well as the nature of the enzymes that may be involved in these metabolic reactions, are not known at present and they clearly need to be investigated.

Gap Junctional Intercellular Communications

Clincal studies have confirmed the experimental evidence that retinoids can act as cancer preventive agents (Hong *et al.*, 1990); however, the strong epidemiological association between dietary carotenoids and a decreased risk of cancer has not been substantiated in clinical trials of β-carotene (Mayne, 1996). The authors have been investigating the mechanistic basis for cancer prevention by these agents using a mouse assay for neoplastic transformation. In these studies, it has been demonstrated that certain retinoids (Merriman and Bertram, 1979) and carotenoids, including β-carotene (Bertram *et al.*, 1991), can inhibit the carcinogen-induced neoplastic transformation of 10T½ cells and that this inhibition correlates with increased gap junctional intercellular communication (Hossain *et al.*, 1989; Zhang *et al.*, 1991). In both cases the observed increase in GJC is mediated through an increase in the gap junctional protein connexin 43 (Cx43) at the mRNA and protein level (Rogers *et al.*, 1990; Zhang *et al.*, 1992). This increase in GJC is believed to be of functional significance to their actions as chemopreventive agents, as many separate studies have demonstrated an association between GJC and growth control (Neveu and Bertram, 1997).

An important question arises of how do carotenoids elevate expression of this gene? Because β-carotene is converted readily in mammals to retinoids, many of its effects have been considered to be mediated through its breakdown, either spontaneously or enzymatically to retinoids. However, the demonstration that several non-provitamin A carotenoids, including the acyclic lycopene, also mediate responses similar to retinoic acid (Zhang *et al.*, 1992) has led to a reevaluation of this concept. While conversion of many carotenoids to compounds known to have retinoid-like properties is certainly feasible on a chemical basis, it is more difficult to imagine such a conversion in the case of the straight-chain carotenoid lycopene (Fig. 1), which is also active in mouse cells (Bertram *et al.*, 1991). As described later, a possible explanation for this surprising activity of lycopene is its conversion, after ingestion to a novel five-membered ring cyclic metabolite known as 2,6-cyclolycopene-1,5-diol (Fig. 2) as a consequence of oxidation and subsequent rearrangement (Khachik *et al.*, 1997d).

As indicated earlier, lutein is another abundant dietary carotenoid that undergoes a series of oxidation–reduction and double bond isomerization

reactions after ingestion. Found in green leaves, lutein was reported previously to possess only a moderate ability to inhibit transformation and increase Cx43 expression in 10T½ cells in comparison with β-carotene (Bertram *et al.*, 1991), indicating that its chemopreventive potential may be low. However, lutein has been shown to convert partially to zeaxanthin in the course of its metabolism (Khachik *et al.*, 1995a). The authors have shown that zeaxanthin has increased activity in elevating Cx43 expression in comparison with lutein. Most studies demonstrating biological effects of carotenoids relevant to carcinogenesis have been conducted in experimental animals or animal cell cultures. In order to more closely address the question of their effects in humans, studies have been conducted in human keratinocytes in organotypic culture. Under these conditions, cells differentiate to form a multilayered tissue with many of the characteristics of intact human skin. For reasons of reproducibility, availability, and ease of use, these new studies did not utilize primary keratinocytes derived from newborn foreskins, instead the authors used the immortalized human keratinocyte cell line HaCaT. Studies by others had shown this line to closely resemble normal human keratinocytes in its differentiation pattern in organotypic culture (Ryle *et al.*, 1989). Preliminary studies using immunofluorescent techniques had also demonstrated expression of connexin 43 in suprabasal cells (unpublished data); moreover, levels of Cx43 in HaCaT cells as detected by Western blotting were consistently higher than those detected in cell cultures of normal human keratinocytes (unpublished results). This may simply reflect a more homogeneous population of cells or that HaCaT cells are better adapted to cell culture.

The following section shows that products derived from the oxidation of dietary carotenoids possess enhanced biological activity in comparison to their parent dietary compounds.

Carotenoids and Their Effects on Human Keratinocytes

HaCaT cells respond to retinoic acid and β-carotene by increasing Cx43 expression. As shown in Fig 6A, retinoic acid caused a major increase in Cx43. HaCaT cultures were treated for 7 days, harvested, and analyzed by Western blotting using an antibody specific for Cx43. Digital image analysis showed this to be 2.2-fold at 10^{-7} *M* and 4.1-fold at 10^{-6} *M* (lanes 2 and 3, respectively). β-Carotene was less potent: no effect was seen at 10^{-7} *M*, whereas 10^{-6} and 10^{-5} *M* caused 1.6- and 3.1-fold increases, respectively (lanes 6 and 7). It was shown previously that retinoic acid treatment of intact human skin causes increased expression of Cx43 (Guo *et al.*, 1992), thus this response of HaCaT cells mirrors the *in vivo* situation. By extension, these data suggest that β-carotene can also produce this response.

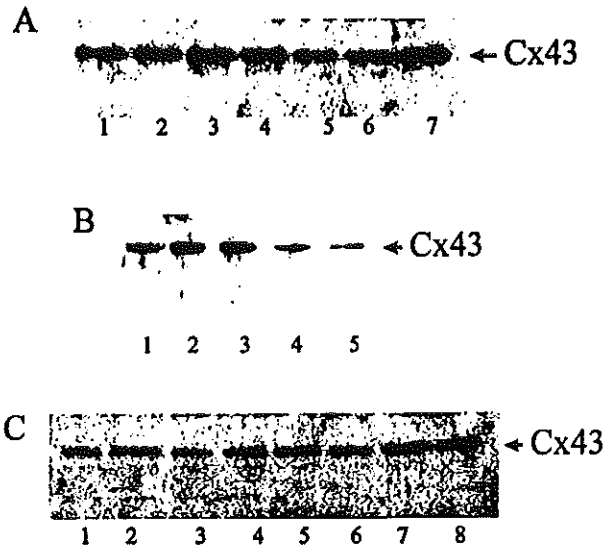

Figure 6 Induction of Cx43 by retinoic acid, carotenoids, and their metabolites in organotypic HaCaT cell cultures. Confluent HaCaT cells were grown in organotypic culture as described previously (King *et al.*, 1997). Cells were treated with retinoic acid or carotenoids for 7 days. Cells were then harvested and lysed, and the extracts were solubilized as described in the methods section. Gel electrophoresis, Western transfer, and detection of Cx43 were performed as described previously (Rogers *et al.*, 1990). Equal amounts of protein (25 μg) were loaded in each lane. (A) Lane 1, solvent control 0.5% THF; lanes 2–4, retinoic acid 10^{-7}, 10^{-6}, and 10^{-5} *M*, respectively; lanes 5–7, β-carotene 10^{-7}, 10^{-6}, and 10^{-5} *M*, respectively; (B) Lane 1, THF control; lanes 2 and 3, zeaxanthin 10^{-6} and 10^{-5} *M*, respectively; lanes 4 and 5, lutein 10^{-6} and 10^{-5} *M*, respectively. (C) Lane 1, control (no treatment; lane 2, THF 0.5% lanes 3–5, lycopene 10^{-7}, 10^{-6}, and 10^{-5} *M*, respectively; lanes 6–8, 2,6-cyclolycopene-1,5-diol 10^{-7}, 10^{-6}, and 10^{-5} *M*, respectively. Modified from King *et al.* (1997).

Lycopene, lutein, and their oxidation products induce Cx43 in human cells. To investigate if lycopene and lutein modulated Cx43 expression in human keratinocytes, cultures were treated as described earlier and were then subjected to Western blotting. As shown in Fig. 6B, although lutein did not elevate Cx43 expression above that seen in solvent controls (lanes 4 and 5), zeaxanthin 10^{-6} *M* (lane 2) caused a 5.3-fold increase in expression in comparison with lutein at the same concentration (lane 4). These results are in accord with previous studies in murine cells in which lutein was only marginally active (Bertram *et al.*, 1991; Zhang *et al.*, 1991) and suggest strongly that its conversion to zeaxanthin increases its biological effects greatly.

The actions of lycopene are shown in Fig. 6C. Treatment with lycopene itself resulted in increased expression (2-fold) of Cx43 at 10^{-6}–10^{-5} *M* (lanes 4 and 5) but not at the lowest concentration. However, the metabolite of lycopene, 2,6-cyclolycopene-1,5-diol, caused a marginal increase in expression at 10^{-7} *M* and a 2.7 and 3.1-fold increase at 10^{-6} and 10^{-5} *M* concentrations, respectively (lanes 6–8). These results demonstrate that the oxidation of lycopene to 2,6-cyclolycopene-1,5-diol results in a more active compound. It has not been determined what proportion, if any, of added lycopene becomes converted to 2,6-cyclolycopene-1,5-diol under conditions of cell culture. If substantial oxidation of lycopene occurs, the apparent activity of lycopene may be a consequence of this conversion.

These data strongly suggest that zeaxanthin and 2,6-cyclolycopene-1,5-diol are more potent than their dietary counterparts, lutein and lycopene, in their ability to upregulate connexin 43. However, the caveat must be added that the stability of these metabolites in cells and in culture medium has yet to be investigated. Should they be significantly more stable and/or attain higher cellular concentrations than lutein or lycopene, respetively, this factor must also be considered as contributing to the observed effects.

Anti-inflammatory and Antitumor Promoting Properties

Due to the detailed and time-consuming experiments involved, the authors' anti-inflammatory and antitumor promoting studies to date have only focused on two major dietary carotenoids: lycopene and lutein. However, in order to elucidate structure/activity relationships, the anti-inflammatory and antitumor properties of other carotenoids and their metabolites need to be investigated.

Anti-inflammatory Property: Inhibitory Effect of Lycopene and Lutein on TPA-Induced Inflammation

12-O-Tetradecanoyphorbol-13-acetate (TPA) induces many biochemical, molecular, and morphological changes in mouse skin. Many of these changes appear to be associated with skin tumor promotion. For example, topical application of TPA to the skin of mice induces skin inflammation rapidly and results in an increase in epidermal ornithine decarboxylase activity, epidermal DNA synthesis, the number of epidermal cell layers, production of hydrogen peroxide, and c-Fos and c-Jun oncogene expression. Chemicals that inhibit these TPA-induced biochemical, molecular, or morphological changes usually inhibit TPA-induced tumor promotion in mouse skin. These biochemical, molecular, and morphological changes may be use-

ful as biomarkers for the early detection of inhibitors of skin tumor promotion (Huang, *et al.*, 1992, 1994,1997).

The authors investigated the effect of purified lycopene and lutein on topical application of TPA-induced inflammation and tumor promotion in mouse skin. The possibility that lycopene or lutein could inhibit TPA-dependent inflammation was evaluated by studying the effect of lycopene and lutein on TPA-induced edema of mouse ears. Topical application of 0.07 or 0.36 mg of lycopene, together with 0.5 nmol TPA to ears of mice, inhibited TPA-induced edema of mouse ears by 72 or 100%, respectively. Topical application of 0.07 or 0.36 mg of lutein with 0.5 nmol TPA to ears of mice inhibited TPA-induced edema of mouse ears by 20 or 47%, respectively. Application of 0.07 or 0.36 mg of curcumin, a known potent anti-inflammatory agent (Huang *et al.*, 1988), with 0.5 nmol TPA, inhibited TPA-induced edema of mouse ears by 81 or 100%, respectively. These results indicate that the anti-inflammatory activity of lycopene is equal to that of curcumin, followed by lutein, which exhibits somewhat lesser anti-inflammatory property.

Antitumor Promoting Property: Inhibitory Effect of Lycopene and Lutein on TPA-Induced Tumor Promotion in Mouse Skin

In another experiment, the protective effect of lycopene and lutein on a two-stage mouse skin tumorigenesis model was investigated. The number of skin tumors in mice was measured every 2 weeks for a duration of 14 weeks; results after 6 and 14 weeks are summarized in Table II. The skin tumors were initiated in CD-1 mice with a single dose of 200 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA) and were promoted with 5 nmol TPA twice a week for a duration of 14 weeks, which resulted in the development of an average of 11.3 skin tumors per mouse. Topical application of 1.2 or 2.5 μmol of lycopene with 5 nmol TPA twice a week for 14 weeks to DMBA-treated mice inhibited the number of skin tumors per mouse by 23 or 74%, respectively (Table III). This resulted in a reduction of the percentage of mice with skin tumors by 7 or 43%, respectively. Topical application of 1.2 or 2.5 μmol lutein with 5 nmol TPA to the backs of DMBA-treated mice twice a week for 14 weeks inhibited an average number of skin tumors per mouse by 46 or 51%, respectively. Consequently, the percentage of mice with skin tumors was reduced by 12 or 21%, respectively. Although curcumin is a very potent inhibitor of TPA-induced skin inflammation and tumor promotion, the bioavailability of curcumin is very low due to its poor absorption and very short half-life, therefore limiting its application as a chemoprotective agent against cancer. However, lycopene and lutein are two of the most abundant carotenoids in the diet and a steady and high concentration of these compounds can be established readily in

Table II Inhibitory Effects of Lycopene and Lutein on12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-Induced Skin Tumor Promotion in CD-1 Mice Initiated Previously with DMBA[a]

	6 weeks		14 weeks	
Treatment	Tumors/mouse	Percentage of mice with tumors	Tumors/mouse	Percentage of mice with tumors
DMBA + TPA	1.29 ± 0.5	33	11.33 ± 2.4	76
+ Lycopene (1.25 μmol)	0.62 ± 0.3 (52%)	29 (12%)	8.67 ± 2.1 (23%)	43 (17%)
+ Lycopene (2.50 μmol)	0.38 ± 0.2 (71%)	19 (42%)	3.00 ± 1.2 (74%)	24 (54%)
+ Lutein (1.25 μmol)	0.48 ± 0.3 (63%)	14 (58%)	6.14 ± 1.9 (46%)	29 (44%)
+ Lutein (2.5 μmol)	0.05 ± 0.1 (96%)	5 (85%)	5.52 ± 1.6 (51%)	14 (73%)

[a] Female CD-1 mice (8–9 weeks old; 30 per group) were initiated with 200 nmol of 7,12-dimethyl benz[*a*]anthracene (DMBA). One week later, mice were promoted with 2.5 nmol of TPA or 2.5 nmol of TPA together with inhibitor in 200 μl acetone twice a week for 14 weeks. Skin tumors greater than 1 mm (in diameter) were counted and recorded. Data are presented as the mean ± SE. Data in parentheses are percentage of inhibition.

the human serum or plasma by supplementation (Khachik *et al.*, 1995a, 1997d; Paetau *et al.*, 1998). As a result of these characteristics, lycopene and lutein can serve as potent cancer preventative agents in many and at a variety of endogenous target sites. Future studies are planed to investigate the effect of purified lycopene and lutein on lung, stomach, and colon tumorigenesis in mice.

Induction of Detoxication (Phase 2) Enzymes

Detoxication (phase 2) enzymes, including quinone reductase [QR; NAD(P)H: (quinone acceptor) oxidoreductase, EC 1.6.99.2], are induced transcriptionally in many mammalian cells by low concentrations of a wide variety of chemical agents and such an induction is associated with a reduced susceptibility to chemical carcinogenesis. Eight chemical classes are currently recognized as inducers by Prestera *et al.* (1993). This section reports that carotenoids and their metabolites represent a new class of inducers and that some of these compounds are very potent. Bioassay of the induction of quinone reductase activity demonstrates that there is high inducer activity from some of these carotenoids. The inducer activity of carotenoids and selected metabolites with curcumin and sulforaphane as positive controls is shown in Table III. The definition of a unit of inducer activity and the concentration required for doubling that activity (CD) is described in the experimental section. It is interesting to note that an acyclic carotenoid, lycopene, which lacks any functional group, appears to be a reasonably good inducer, suggesting that the conjugated polyene chain itself has inducer ability. If one considers α-carotene (CD = 100 μM) with 10 conjugated and one isolated double bond to be the "parent" carotenoid, then by simply altering the position of one double bond in the end groups, the phase 2 enzyme inducer potency in β-carotene (CD = 14 μM) with 11 double bonds is increased sevenfold. This, of course, should be considered in the absence of any other structural changes. However, because it is well established that the β-end groups of β-carotene are not quite in the same plane as the polyene chain in this compound, there is only a limited overlap between the π double bonds of the β-end groups with the conjugated system in this compound.

A similar increase in potency (sixfold) was observed by the introduction of hydroxyl groups in the end groups of lutein (CD = 16.5 μM) in comparison with α-carotene (CD = 100 μM) with an identical chromophoric system. An additional twofold increase in potency in comparison with lutein (CD = 16.5 μM) occurs if the allylic hydroxyl group in this compound is oxidized to give an α,β-unsaturated keton, (3*R*,6′*R*)-3-hydroxy-β,ϵ-caroten-3′-one (CD = 7.3μM). The inducer activity of (6*RS*,6′*RS*)-ϵ,ϵ-caroten-3,3′-dione (CD = 2.5 μM) with two α,β-unsaturated ketones in

Table III Phase 2 Enzyme Inducer Activities of Some Dietary Carotenoids and Their Metabolites in Comparison to Curcumin and Sulforaphane

Entry	Compound	CD[a] (μM)
1	α-Carotene	100
2	Lycopene	25
3	Violaxanthin	17
4	(3*R*,3′*R*,6′*R*)-Lutein	16.5
5	β-Carotene	14
6	2,6-Cyclolycopene-1,5-diol (90% A + 10% B)[b,c]	14
7	(3*R*,6′*R*)-3-Hydroxy-β,ε-caroten-3′-one[b]	7.3
8	(6*RS*,6′*RS*)-ε-,ε-Carotene-3,3′-dione[b,d]	2.5
9	(3*R*,3′*R*)-Zeaxanthin	2.2
10	Neoxanthin	2.0
11	Curcumin	5.0
12	Sulforaphane	0.2

[a] CD (inducer activity) is defined in the experimental procedures.
[b] Represents carotenoid metabolites.
[c] A synthetic mixture of lycopene metabolites in approximately the same ratio found in human serum.
[d] A synthetic mixture of (6*R*,6′*S*)-, (6*S*,6′*S*)-, and (6*R*,6′*R*)-ε,ε-caroten-3,3′-dione in approximately the same ratio found in human serum.

comparison with lutein (CD = 16.5 μM) is increased nearly sevenfold even if the chromophores are slightly different in these two compounds. Addition of the two hydoxyl groups and the epoxide moieties to the β-end group of β-carotene (CD = 14 μM) results in violaxanthin (CD = 17 μM) with roughly the same inducer activity. This is probably due to an increase in the inducer activity of β-carotene by introduction of the hydroxyl groups and to a decrease in activity by the removal of the double bonds in the β-end groups of this compound. However, in neoxanthin (CD = 2.0 μM), the extended conjugated polyene due to the presence of the allenic (two cumulative) double bonds, as well as the three hydroxyl groups, results in substantial inducer activity. If the hydroxylation of α-carotene (CD = 100 μM) is accompanied by the extension of conjugation to 11 double bonds, the potency is increased dramatically, as is the case in zeaxanthin (CD = 2.2 μM).

2,6-Cyclolycopene-1,5-diol (90% diol A and 10% diol B), a mixture of metabolites of lycopene with 10 conjugated double bonds, a five-membered ring system, and two hydroxyl groups has an inducer activity of CD = 14 μM and is roughly twice as potent as lycopene (CD = 25 μM). Thus, the extent of conjugation, the addition of oxygen functionality to the rings, and the presence of an α,β-unsaturated ketone are all important and apparently additive determinants of inducer potency. Although the observed

potencies do not follow the ranking for "Trolox equivalent antioxidant capacities" (ABTS* radical cation quenching) (Miller *et al.*, 1996), they are in general agreement with observed singlet quenching activities (Stahl *et al.*, 1997). Activities apparently increase with extension of the chromophore and maximum overlap of the π molecular orbitals in C=C double bonds. However, lycopene (the only acyclic compound tested) does not have the inducer potency one would predict based on the number of conjugated double bonds or ABTS radical scavenging capacity.

In the authors' system, zeaxanthin is only 10-fold less potent than sulforaphane (CD = 0.2 μM), which is the most potent naturally occurring inducer of the phase 2 enzymes. Zeaxanthin has comparable activity to benzyl isothiocyanate (data not shown) and is considerably more active than inducers such as 1,2-dithiole-3-thione (not shown) or curcumin (CD = 5 μM), both of which are currently in clinical trials by the U.S. National Cancer Institute for evaluation as chemopreventive agents (Kelloff *et al.*, 1996).

Carotenoids, therefore, appear to have considerable potential to induce phase 2 detoxication enzymes and may have potential significance as means of detoxifying xenobiotics. Some of the major dietary carotenoids and their metabolites found in serum (Khachik *et al.*, 1997c), as well as those that are apparently not detected in the blood (Khachik *et al.*, 1997b), such as neoxanthin and violaxanthin, have now been evaluated in Hepa 1c1c7 cells as described earlier. The relevance of these findings is bolstered by the fact that serum and tissue concentrations of carotenoids are in the micromolar range and are therefore comparable to those required to produce phase 2 enzyme induction.

CONCLUSION

In the course of 5 years, the authors have repeatedly emphasized the importance of other dietary carotenoids in addition to β-carotene and presented evidence for the nutritional significance of this important class of nutrients in the prevention of cancer (Khachik *et al.*, 1992c, 1995a, 1997b,c,d) as well as macular degeneration, a degenerative eye disease (Khachik *et al.*, 1997a). This was initially based on our knowledge of the widespread distribution of carotenoids in fruits and vegetables as well as the consistent presence of these compounds and their metabolites in human serum, milk, and tissues at relatively high concentrations. In addition, in

* 2,2'-Axinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

several human bioavailability and metabolic studies with selected carotenoids, such as lutein, zeaxanthin, and lycopene, the authors established the *in vivo* oxidation of these carotenoids and proposed metabolic pathways for the formation of their oxidative metabolites. Until recently, the presumed mechanisms of action for the protective role of carotenoids were their antioxidant function and their ability to upregulate connexin 43 gene expression responsible for gap junctional communications.

The authors have presented preliminary evidence for the presence of the optical stereoisomers of dietary lutein and zeaxanthin in human plasma and have provided additional evidence to substantiate earlier proposed oxidative–reductive pathways for the metabolism of these compounds in humans. They have also reviewed the most recent findings with regard to the mechanism of action of carotenoids and their metabolites by upregulation of the gene expression of gap junction proteins.

However, perhaps the most fascinating findings of the studies reported here are the discovery of three other important biological properties of carotenoids and their metabolites that may relate to their role in cancer chemoprotection: (1) anti-inflammatory property, (2) antitumor promoting property, and (3) induction of the detoxication (phase 2) enzymes. It is also remarkable that among the carotenoids found in human serum, (3*R*,3′*R*)-zeaxanthin (dietary) exhibits the highest activity by these two unrelated mechanisms of action. The mechanisms described here also revealed that various carotenoids and their metabolites exhibit different degrees of activity in a structure-dependent manner. Furthermore, in some cases, these activities are more pronounced with carotenoid metabolites than with dietary carotenoids.

Therefore, future studies with carotenoids should focus on developing an understanding of the bioavailability, metabolism, function, and mechanisms of action, efficacy, and interaction of all the serum carotenoids. These studies will allow us to carefully assess the potential protective effect of carotenoids and their metabolites against cancer and ultimately develop a mixture of purified carotenoids as chemopreventive agents for human supplementation. Such a mixture should closely resemble the relative distribution of carotenoids in the serum of healthy individuals consuming a diet rich in fruits and vegetables.

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