CAROTENOIDS AND OTHER DIETARY ANTIOXIDANTS IN FREE RADICAL RESEARCH: PROTECTION OF HUMAN RETINA HOMOGENATE AGAINST PHOTOCHEMICAL AND METAL-INDUCED LIPID AUTOXIDATION

by

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Carotenoids and other dietary antioxidants in free radical research: Protection of human retina homogenate against photochemical and metal-induced lipid autoxidation.

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Abstract

Title:

Carotenoids and other dietary antioxidants in free radical research: Protection of human retina homogenate against photochemical and metal-induced lipid autoxidation.

Dietary antioxidants including, carotenoids, tocopherols, ascorbate, and retinol are of current interest as "chemopreventive" agents in cancer, coronary heart disease, age-related macular degeneration, and other diseases. The human retina is an important target in free radical pathology, due to its anatomy, biochemistry, function, and its constant exposure to photochemically-induced oxidative stress. Lipid peroxidation of human retina homogenate can be induced by either photo-sensitization (rose Bengal and light) or metal-catalysis (iron and ascorbate). Tissue injury was estimated by the formation of lipid peroxides as assessed by the thiobarbituric acid (TBA) assay. The systems chosen are potential models for certain eye injuries, diseases, or the natural processes of aging. Ascorbate (1 mM) is the superior antioxidant in oxidative stress protecting human retina homogenate against metal-induced injury by 98% and against photosensitized injury by 68%. α-Tocopherol (1 mM) and β-carotene (.05 mM) protected against iron/ascorbate-induced damage by 34% and 18% and against photosensitized damage by 37% and 26%. Retinol (0.1 mM) protected only against the photosensitized system. Potencies of the carotenoids varied widely, with α-carotene > canthaxanthin > astaxanthin > lycopene > β-carotene > lutein ≥ zeaxanthin. The relatively low protection by β-carotene confirms suggestions that other carotenoids have been neglected in studies of protection and in chemoprevention trials. Lutein and zeaxanthin did not reach significance ($p \leq 0.05$) at

.05 mM, however they did reach significance at .01 mM. Lutein and zeaxanthin, the two major carotenoids of the retina accumulate especially at the *macula lutea* (or "yellow spot"). Their relative lack of antioxidant activity or singlet oxygen quenching ability would serve to ensure their conservation, preserving them to function as filters. By attenuating light in the macular area, they would decrease production of the species that initiate light-induced lipid peroxidation. α-Tocopherol was a more effective antioxidant at the higher partial pressures of oxygen. Oxidized β-carotene at ambient PO_2 increased production of MDA. This finding may have particular importance to studies finding a higher incidence of cancer in subjects receiving β-carotene supplementation. Endogenous lutein is positively correlated with zeaxanthin, as are total carotenoids and γ-tocopherol, but not α-tocopherol or retinol. Our results encourage speculation that, in the human retina, phototoxic reactions and free radical-mediated damage would be attenuated by the presence of high levels of ascorbate. Tocopherols, carotenoids, and retinol may protect to a lesser degree. In either event, the current data support and provide a mechanistic basis for epidemiological studies of protective actions of antioxidants in visual disorders.

for

Mike and Kyle

and

Katherine

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I would like to extend my gratitude to my committee members Dr. Allan Davison, Dr. Bruce Dunn, and Dr. Miriam Rosin for their guidance throughout the experimental phase of my work, assistance and valuable editorial comments in manuscript preparation, and especially for their patience, throughout my degree. I would also like to thank Dr. Diane Walker for harvesting the retinas and John Fee for his expertise in collecting the high pressure liqid chromatography data. Warmest appreciation to my lab associates Brian Bandy, Jim Moon, Luoping Zhang, Ann-Marie Gilbert, Suiping Zhou, Xuefeng Yin, and Anna Li for their friendship, help, and moral support over the years. Special thanks go to my mom and dad for their love and (financial!) support. Sincerest gratitude to Chris Rousseau and Paul Rousseau for their kindness and generosity. Finally, and most importantly, my greatest love and appreciation go to Mike and Kyle. They were always there for me, thanks guys.

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General Introduction.

This thesis includes a general introduction, three published articles, three manuscripts that will be submitted for publication as four separate articles, and a general discussion. The first five chapters are reviews of the photochemistry, therapeutic actions, nutrition, and biochemistry of carotenoids. Those familiar with these aspects of carotenoids may skip to chapter 6 which contains the experimental core of the thesis.

Chapter 1. Rousseau, E.J.; Davison, A.J.; Dunn, B. Protection by β-carotene against photochemical damage and singlet state oxygen. *Trends in Photochemistry and Photobiology*. 1:259-264; 1991.

Chapter 1 reviews the effects of photochemical damage and singlet state oxygen *in vitro and in vivo*. β-carotene has 11 conjugated double bonds which makes it an effective scavenger of singlet state oxygen. Possible mechanisms of action in protection against photochemical stress are discussed. The draft manuscript was prepared by myself, with editorial comments by Dr. Davison and Dr. Dunn.

Chapter 2. Rousseau, E.J.; Davison, A.J.; Dunn, B. Protection by β-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: Implications for carcinogenesis and anticarcinogenesis. *Free Radic. Biol. Med.* 13:407-433; 1992.

Chapter 2 reviews β-carotene and other carotenoids and their actions as a free radical scavengers and antioxidants protecting against oxygen-mediated stress *in vitro* and *in vivo*. There is a focus on the processes involved in carcinogenesis and possible mechanisms of protection by carotenoids. Interactions with other carotenoids and dietary antioxidants are discussed. The draft manuscript was prepared by myself, with editorial comments by Dr. Davison and Dr. Dunn. Dr. Davison prepared the graphics and illustrations.

Chapter 3. Davison, A.J.; Rousseau, E.J.; Dunn, B. Putative anticarcinogenic actions of carotenoids: Nutritional implications. *Canad. J. Physiol. Pharmacol.* 71:732-745; 1993.

Chapter 3 provides an update on nutritional aspects of carotenoids (other than vitamin A activity) with specific relevance to anticarcinogenesis. Epidemiological and laboratory studies have lead to widespread supplementation with β-carotene in humans. β-Carotene supplementation is discussed and suggestions are given for future studies which will allow recommendations to be made with more confidence. The draft manuscript was prepared by Dr. Davison and myself, with editorial comments by Dr. Dunn. Dr. Davison prepared the graphics and illustrations.

Chapter 4. Carotenoids: Biochemistry and Antioxidant Mechanisms.
Manuscript prepared for publication in *The Canadian Journal of Biochemistry*.

Chapter 4 discusses the chemistry and biochemical nature of carotenoids and possible mechanisms of action. *In vitro* studies provide information on β-carotene oxidation, chemical reactivity, chemical instability, interactions, and mechanisms. Experimental and methodological problems inherent to carotenoid research are discussed. The draft manuscript was prepared by myself, with editorial comments by Dr. Davison and Dr. Dunn. Dr. Dunn contributed a section on the chemical instability of carotenoids and retinoids.

Chapter 5. The human retina as a research tool in free radical research.
Manuscript prepared for publication in *Free Radical Biology and Medicine*.

This chapter discusses the anatomy and physiology of the human retina, its dependence on retinol and carotenoids, mechanisms of the visual process, and responses to oxygen-mediated stress and light. This review is designed to assist those considering using human retina as an experimental tool by describing pitfalls and precautions. The draft manuscript was prepared by myself, with editorial comments by Dr. Davison and Dr. Dunn, and Dr. Rosin.

Chapter 6. Protection of human retina homogenate against light and Fenton-induced oxidative stress: A role for carotenoids and other dietary antioxidants.

Manuscript prepared for publication in *Free Radical Biology and Medicine* and *Investigative Ophthalmology*.

Chapter 6 describes experiments using human retina as a target tissue. We developed two systems designed to induce different forms of oxygen-mediated stress in a saline/retina homogenate. In photochemically-induced oxidative stress, rose Bengal in the presence of light, produces mainly singlet oxygen and some peroxyl radicals. In Fenton-induced oxidative stress, ferric iron/ascorbate produces peroxyl, hydroxyl, and superoxide radicals and some singlet oxygen. These systems attempt to model eye injuries, diseases, or the natural processes of aging. Protection by dietary antioxidants is examined, with a focus on the different carotenoids. The draft manuscript was prepared by myself, with editorial comments by Dr. Davison and Dr. Dunn, and Dr. Rosin. Dr. Walker harvested the retinas and J. Fee contributed a section on the HPLC methods, and performed the HPLC analysis.

General Goals of the Study.

Much interest has been generated on the topic of antioxidants and prevention of disease. Today, carotenoids are the focus of a number of *in vitro* experiments, clinical assessments, and epidemiological studies. Chapters 1 to 4 are intended for those interested in reviewing past carotenoid research. The complex nature of carotenoid research demands rigorous controls, precautions, and standardization of experimental procedures for reliable and reproducible results. In the current study, we use human retina as an experimental tool and describe the benefits and pitfalls. Human tissue gives us a more relevant model for humans than solvent, model membrane, cell, or animal systems. Human retina was chosen for its physiologic and biochemical properties, constant exposure to oxygen-mediated stress, and accumulation of certain carotenoids. The following sections attempt to establish a role for dietary antioxidants, with a focus on carotenoids, in free radical-mediated processes relevant to human disease.

CHAPTER 1

In: Trends in Photochemistry and Photobiology 1:259-264, 1991.

Protection by β-Carotene Against Photochemical Damage and Singlet State Oxygen

by

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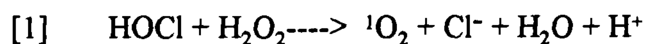
ABSTRACT

Singlet state oxygen is formed in living systems as a result of impact of far UV light, dye sensitized activations of oxygen, and enzymic activation of oxygen. The hazardous features of singlet state oxygen are: removal of spin restriction and increased reactivity with biomolecules, sufficient lifetime to cause damage, and high reactivity with biomolecules with conjugated double bonds. Plants in particular require protection against singlet oxygen, and β-carotene has evolved a system of 11 conjugated double bonds which makes it the most effective known scavenger of singlet state oxygen. Presumably, the energy transitions required for singlet quenching are readily accommodated. Its demonstrated biological role is protection of plant photosynthetic apparatus against light mediated damage. β-carotene is not known to be an essential nutrient in animals, yet it nevertheless has a number of protective and beneficial functions. β-Carotene protects against photooxidative dermatitis in porphyrics. Even in normal individuals β-carotene administration protects against erythemic response to UVB radiation. In chewers of tobacco products, β-carotene diminishes indices of genotoxicity, and causes regression of pre-malignant leukoplakias. In experimental animals, β-carotene protects against chemical, transplanted, and UVB induced tumors. The reported evidence of β-carotene as a possible anti-neoplastic agent could be related to its ability to scavenge free radical species in addition to singlet oxygen.

1. GENERATION OF SINGLET OXYGEN.

Singlet oxygen ($^1O_2$)is generated by several methods. It is thought to be biologically generated by dismutation of superoxide (O_2^-) by phagocytic cells during the respiratory burst to destroy invading microorganisms. Myeloperoxidase is a principle enzyme found in polymorphonuclear leukocytes (Schultz and Kaminker 1962) and has microbiocidal action in the presence of H_2O_2/halide (Agner 1972). Equation [1] shows a possible mechanism.

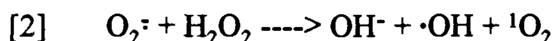


[1] $HOCl + H_2O_2 \text{---->} {}^1O_2 + Cl^- + H_2O + H^+$

Singlet oxygen is sometimes formed as an unwanted by-product *in vivo*. Erythropoietic protoporphyria, an inborn metabolic disorder, leads to the accumulation of porhyrins in the skin. Exposure to light results in singlet oxygen formation causing eruptions and scarring of the skin (Mathews-Roth 1986).

The presence of luminescence is often used as an indication of the presence of singlet oxygen. The xanthine/xanthine oxidase system produces a chemi-luminescent species (Stauff *et al* 1963). Microsomal lipid oxidase dependent NADPH oxidation by liver microsomes produces chemiluminescence (Howes and Steele 1971). Production of singlet oxygen has been detected during the oxidation of arachidonic acid by prostaglandin endoperoxide synthase (Cadenas and Sies 1983). Lipoxidase-catalyzed linoleate oxidation generates chemiluminescent species (Mamedov *et al* 1969). In all of the mentioned studies, there is evidence both supporting and refuting the enzymatic generation of singlet oxygen in these systems.

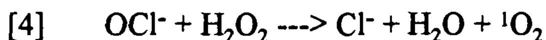
Physical generation methods include electrical discharge (Ingraham and Meyer 1985), microwave discharge, and thermal decomposition of the endoperoxide 3,3'-(1-4-naphthylidene) dipropionate ($NDPO_2$) (Mascio *et al* 1989). Singlet oxygen is also chemically generated *in vitro*. Xanthine oxidase and acetaldehyde are proposed to generate singlet oxygen by a Haber-Weiss mechanism as in equation [2] Kellogg and Fridovich 1975).



[2] $O_2^{\cdot -} + H_2O_2 \text{ ----> } OH^- + \cdot OH + {}^1O_2$

Hydrogen peroxide and hypochlorite ion (Halliwell and Gutteridge 1985a) may be generating singlet oxygen by a mechanism similar to the phagocytic burst.

[3] $Cl_2 + OH^- \text{ ----> } Cl^- + OCl^- + H^+$



[4] $OCl^- + H_2O_2 \text{ ---> } Cl^- + H_2O + {}^1O_2$

Decomposition of perchromate ion in aqueous solution generates ${}^1O_2$, $O_2^{\cdot -}$, and $\cdot OH$ (Halliwell and Gutteridge 1985b).

The best studied and most biologically relevant method of singlet oxygen generation is by photosensitization. Light energy of a given wavelength is transferred onto a sensitizer molecule. This molecule enters an excited triplet state and transfers this energy to an oxygen molecule, creating the singlet state. *In vitro* sensitizer molecules are usually dyes such as acridine orange, methylene blue, toluidine blue, and rose Bengal.

In vivo sensitizers include riboflavin, FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide), chlorophyll *a*, chlorophyll *b*, bilirubin, retinal, and some porphyrins (Halliwell and Gutteridge 1985a).

2. β-CAROTENE IS ONE OF THE MOST EFFICIENT QUENCHERS OF SINGLET OXYGEN.

β-carotene is an efficient quencher of $^1O_2$ with rates 50% (Ingraham and Meyer 1985) of the diffusion limit of 3×10^{10} M^{-1} sec^{-1} (Foote *et al* 1970). Carotenoids can react to quench the chlorophyll triplet state. Carotenoids also undergo reactions with $^1O_2$, resulting in triplet carotenoid and triplet dioxygen as in equation [5]. β-Carotene's low solubility in aqueous solution causes problems in experiments *in vitro*.

[5] $^1O_2 + {}^1\beta\text{-carotene} \dashrightarrow {}^3O_2 + {}^3\beta\text{-carotene}$

3. ORGANIC REACTIONS OF SINGLET OXYGEN.

Damage initiated by the singlet oxygen molecule is described as a Type II mechanism. The triplet state of the dye sensitizer molecule can directly damage organic molecules. This is termed a Type I photosensitization mechanism. Singlet oxygen combines chemically with molecules or transfers excitation energy by *quenching* mechanisms. The most interesting reactions involve compounds with carbon-carbon double covalent bonds. Such bond formations are found in many biological molecules

such as chlorophyll, carotenoids, cholesterol, bilirubin, and the fatty acid chains of membrane lipids. Compounds with conjugated double bonds react with singlet oxygen to produce endoperoxides. Compounds with one double bond undergo an ene-reaction i.e., the double bond shifts to a different position and the $^1O_2$ molecule is added. This reaction produces an allylic hydroperoxide and is the main mechanism in lipid peroxidation.

Photosensitization reactions with *in vivo* molecules can cause cytotoxic and genotoxic damage leading to mutations or death of the organism. Light exposure to certain mutant forms of photosynthetic bacteria (Griffiths 1955, Mathews-Roth and Sistrom 1959, Kunisawa and Stanier 1958) causes lethal damage, photo-stimulation of the molecule retinal in the retina of the eye can cause injury (Halliwell and Gutteridge 1985a), and defects in porphyrin metabolism can cause eruptions and scarring of the skin upon exposure to light (Mathews-Roth 1986). Singlet oxygen can cause protein damage by oxidizing methionine, tryptophan, histidine, and cysteine residues (Halliwell and Gutteridge 1985a). Singlet oxygen causes loss of transforming capacity and strand breaks in plasmid and bacteriophage DNA (Mascio *et al* 1989). The nucleoside guanosine reacts with singlet oxygen. Guanosine may be the site of the genetic damage that occurs with light stimulation in the presence of intercalating dyes (Ingraham and Meyer 1985).

4. BIOCHEMISTRY OF CAROTENOIDS.

Carotenoids have the general empirical formula $C_{40}H_{56}$ and are chemically related to terpenes and terpenoids ($C_{10}H_{16}$). Acetyl CoA is the basic carbon fragment used in the structure of carotenoids. Animals endogenously produce and dimerize 15 carbon fragments to form squalene and steroids. In contrast, plants and *Protista* produce 20 carbon fragments which are dimerized to the 40 carbon carotenoid phytoene. The colourless phytoene goes through dehydrogenation and cyclization to become the coloured carotenoid β-carotene (Simpson and Chichester 1981). Various organisms biosynthesize some 600 naturally occurring carotenoids, with production estimated at 10^8 tons per year.

5. CAROTENOIDS ARE LIPID SOLUBLE MOLECULES.

Carotenoids are fat-soluble molecules, with the exception of the water-soluble carotenoid, crocetin. In the digestive system of animals, carotenoids are broken down by esterases, lipases, and proteases, then solubilized by bile salts. Carotenoids are found associated with lipid fractions of blood and plasma and esterified or are complexed with protein. β-Carotene is found in plasma membranes, where its protective functions may be most important.

6. β-CAROTENE IS A SYMMETRICAL MOLECULE.

β-carotene is a symmetrical molecule consisting of two β-ionone rings extended with a 22 carbon polyene fragment. β-Carotene has 11 conjugated double bonds and 10 methyl groups. Carotenoid structures are determined by a combination of spectrometry and chemical reactions. Today analytical methods include thin layer and open column chromatography and HPLC (high-performance liquid chromatography). Chemical methods often result in oxidative degradation of carotenoids, however HPLC is a rapid and non-destructive method of analysis.

7. VITAMIN A ACTIVITY IS RELATED TO MOLECULAR STRUCTURE.

β-carotene is the most active vitamin A (retinol) precursor. β-Carotene in animals is oxidatively cleaved by β-carotene dioxygenase and theoretically produces two molecules of retinol. Other carotenoids, collectively known as xanthophylls, have additional functional groups such as hydroxyl, carbonyl, carboxylic acid, ester, epoxide, glycoside or ether. Introduction of oxygenated groups on the ring or chain decreases the vitamin A activity. β-Carotene is found in the all-trans formation. Acids or alkalis cause cis-trans isomerization of β-carotene, making it a less potent vitamin A precursor. Addition of methyl groups to the 2,2' position of the β-ionone ring, or replacing the methyl with ethyl groups also decreases the vitamin A activity. In α-carotene, shifting the double bond from the 5',6'-position to the 4'5'-position results in a 50% decrease in activity (Simpson and Chichester 1981).

8. CHEMICAL REACTIONS OF CAROTENOIDS: THE DOUBLE BOND FORMATIONS ARE HIGHLY REACTIVE.

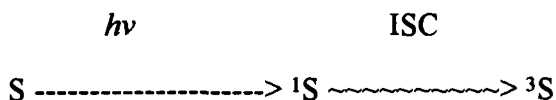
The most active sites of carotenoid molecules are the double bonds. Halogenation or hydrogenation causes a reduction of all double bonds (including the benzene rings), carbonyl, and epoxy groups. Ozonolysis causes oxidative degradation of β-carotene to form geronic acid. Chromic acid and permanganate oxidation produces apo-carotenoids (short chain) and some ketone formations. Oxidation of β-carotene in toluene (excess oxygen) results in a zero order reaction, with the site of attack being the 5'6'-double bond (El Tinay and Chichester 1970).

9. PROTECTION AGAINST PHOTOCHEMICALLY GENERATED ACTIVE OXYGEN SPECIES.

Four possible mechanisms of carotenoid protection are suggested: [1] a physical filtration system against light, [2] systems that quench photosensitizer triplet states, [3] systems that act as preferred substrates in photosensitized reactions, and [4] systems that act to repair or stabilize membranes (Krinsky 1971). Research to date has shown that the most probable mechanism of protection is [2], and should also include singlet oxygen quenching (Mathews-Roth and Krinsky 1987).

9.1 Creating the triplet sensitizer species.

Triplet sensitizer is formed in the presence of a sensitizing dye and light. Singlet sensitizer ^1S has a short lifetime of ~10^{-11} seconds and dissipates energy by interacting with solvents, fluorescing, or undergoing ISC (intersystem crossing) to produce triplet sensitizer (^3S) (Krinsky 1979).



9.2 Carotenoids quench the triplet sensitizer state.

Carotenoids can protect by quenching the triplet sensitizer ^3S before the production of activated oxygen species, as in Figure 1-mechanism A (Krinsky and Deneke 1982). β-Carotene levels in intact chloroplasts may be as high as 2 x 10^{-2} M, where it is estimated that only 10% of triplet chlorophyll (^{3}Chl) would survive quenching (Krinsky 1979). The quenching rate of triplet chlorophyll *a* (^{3}Chl) by β-carotene is accompanied by energy transfer (E_T) of 29 kcal. This is similar to the rate of ^{3}Chl quenching by ground state oxygen. Theoretically, carotenoids should only be able to protect if the concentration of carotenoid greatly exceeds the concentration of oxygen. β-Carotene protects at very low concentrations (~10^{-4} M) (Foote *et al* 1970), therefore it is also quenching singlet oxygen (Foote 1976).

9.3 Carotenoids react physically with singlet oxygen.

Carotenoids can protect against Type II reactions (oxygen present) by quenching singlet oxygen as in Figure 1-mechanism B. One molecule of β-carotene quenches up to 1000 molecules of singlet oxygen (Foote 1976). Maximum protection is conferred by carotenoids with a minimum of 9 conjugated double bonds (Foote *et al* 1970, Mathews-Roth *et al* 1974). Approximately 10^5 molecules of singlet oxygen are quenched by β-carotene for each one that escapes to react with biomolecules, such as histidine (Foote 1976).

9.4 Carotenoids react chemically with singlet oxygen.

Carotenoids are involved in chemical reactions with singlet oxygen as in Figure 1-mechanism C. Carotenoids act as preferred substrates and are oxidized to [Car-OO•]. Little information is available on this pathway.

9.5 Carotenoids scavenge radical intermediates.

Carotenoids can protect against Type I reactions by chemically scavenging radical intermediates as in Figure 1-mechanism D. These free radicals are capable of propagating chain reactions and causing further damage to the system. The radical intermediates produced by hydrogen or electron abstraction ($R^{\bullet}/R^{+\bullet}/R^{-\bullet}$ depend on the type of sensitizer and the nature of the substrate. The resulting carotenyl radical [Car•] can react with oxygen to produce a carotene peroxyl radical [Car-OO•].

10. PROTECTIVE ACTION OF CAROTENOIDS IN VITRO.

10.1. Singlet oxygen quenching varies with the number of double bonds in the carotenoid molecule.

Singlet oxygen quenching by carotenoids *in vitro* is a function of the length of the conjugated polyene chain. Decreasing the number of conjugated double bonds from 11 to 9 corresponds to a 75% decrease in the efficiency of quenching. The greatest loss is between 9 and 7 conjugated double bonds, with a further decrease to ~$2x10^8$ $M^{-1}sec^{-1}$. A decrease in double bonds from 11 to 9 and 9 to 7 corresponds to a 7% and 38% loss of protection of chlorophyll (Foote *et al* 1970). Carotenoids with a minimum of 9 conjugated double bonds provide efficient protection from photodynamic damage (Mathews-Roth *et al* 1974).

10.2 Carotenoids protect liposomes from singlet oxygen produced photodamage.

Carotenoids protect liposomes in aqueous suspension from UV-induced/methylene blue sensitized lipid peroxidation and lysis. Canthaxanthin and β-carotene delay lysis of liposomes by 50% (Anderson and Krinsky 1973). Protection increases with greater carotenoid concentrations up to 10 mmol carotenoid per mole phospholipid. At these high concentrations, lipid soluble carotenoids form micelles rather than incorporating into the liposomal membrane. Similarly, β-carotene protects by 33% against singlet oxygen generated by radio-frequency discharge (Anderson *et al* 1974).

11. CAROTENOIDS PROTECT BACTERIA AGAINST PHOTODYNAMIC DAMAGE.

Rhodopseudomonas spheroides is a mutant strain of non-sulfur purple bacterium which lacks coloured carotenoid pigments. Without the carotenoids, this bacterium is destroyed by photosensitization in the presence of oxygen and light (Griffiths 1955). *Sarcina lutea* is a yellow bacterium that survives the presence of light and oxygen, whereas the colourless mutant form is destroyed (Mathews-Roth *et al* 1959). *Corynebacterium poinsettiae* is a coloured wild type which is unaffected by oxygen and the photosensitizing dye, toluidine blue. Under these same conditions, the colourless mutant displays lethal aerobic photosensitivity (Kunisawa and Stanier 1958). Possibly, the carotenoids are protecting the wild type bacteria from the cytotoxic effects of singlet oxygen.

The combination of UVA/air/8-MOP (8-methoxypsoralen) causes photomutagenesis of *S. typhimurium*. β-Carotene at 10 and 100 μg/ml protects by 50% and 70%, respectively. 8-MOP in nitrogen causes 65% less photomutagenesis than in air. β-Carotene did not protect. Evidently, in a two-step reaction, 1) an 8-MOP-DNA photoadduct forms and 2) singlet oxygen forms *in situ*. β-Carotene protects at the second step (Santamaria *et al* 1988).

12. ERYTHROPOIETIC PROTOPORPHYRIA: SYMPTOMS OCCUR ON EXPOSURE TO VISIBLE LIGHT.

The dangers of UVB or the sunburn spectrum (290-320 nm) are well documented. Sensitivity to the visible spectrum (380-560 nm) can be caused by erythropoietic protoporphyria (EPP), characterized mainly by abnormally elevated levels of protoporphyrin IX in erythrocytes, feces, and plasma. Onset of symptoms usually occurs in childhood and includes a burning sensation of the skin, erythema, and edema on exposure to visible light (Mathews-Roth 1986).

13. CAROTENOIDS PROTECT HUMANS AGAINST PHOTOSENSITIZED REACTIONS.

Carotenoids protect green plants and photosynthetic bacteria against photosensitized reactions. The porphyrins present in EPP have a similar structure to the porphyrin group in chlorophyll. This observation led to the proposition that carotenoids could also protect humans against photosensitivity diseases such as EPP. Some 84% of EPP patients treated with β-carotene increased their tolerance to sunlight by a factor of three (Mathews-Roth 1986). In normal patients, β-carotene increased the minimal erythema dose of UVB radiation by a statistically significant amount (Mathews-Roth *et al* 1972).

14. CAROTENOIDS MAY PROTECT AGAINST PHOTOSENSITIZED REACTIONS BY INACTIVATING SINGLET OXYGEN OR FREE RADICALS.

Porphyrin-induced photosensitized reactions *in vitro* are oxygen dependent processes which can generate free radicals (Golstein and Harber 1972). It is known that activated oxygen species can cause cellular damage (Davison *et al* 1988), however it cannot be conclusively proven that free radicals are responsible for the symptoms of EPP (Mathews-Roth 1986). Vitamin E, the chain-breaking anti-oxidant also demonstrates protection against lethal hematoporphyrin photo-sensitization of white mice (Moshell and Bjornson 1977). Today it is widely accepted that carotenoids protect against singlet oxygen and possibly free radical damage in photo-sensitized reactions such as occur in patients with EPP.

15. β-CAROTENE AS A POSSIBLE CHEMOPREVENTATIVE AGENT.

In addition to its singlet oxygen scavenging capacity, β-carotene may protect against genotoxicity and cytotoxicity by acting as an anti-oxidant against free radicals. β-Carotene (10^{-5} M) protects chinese hamster ovary (CHO) cells against phagocyte and enzyme generated oxygen metabolites as demonstrated by a 27% and 34% decrease in sister chromatid exchanges respectively (Weitberg *et al* 1985). In experimental animals β-carotene protects against chemical (Schwartz and Shklar 1988), transplanted (Rettura *et al* 1982), and UVB induced (Epstein 1977) tumors. In chewers of tobacco products,

β-carotene diminishes indices of genotoxicity (Stich and Rosin 1984), and causes regression of pre-malignant leukoplakias (Stich *et al* 1988).

16. SUMMARY.

This review has examined the photo-protective capacities of β-carotene as related to singlet oxygen quenching. The theory that β-carotene is also a free radical scavenger and displays a protective effect against neoplastic transformation has generated much interest. There are still many important unanswered questions in the area of carotenoid research that need to be explored.

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LEGENDS TO FIGURES

FIGURE 1:

Mechanisms of carotenoid protection. Carotenoids (CAR) can quench triplet sensitizers "A", quench $^1O_2$ "B", chemically react with $^1O_2$ "C", or react with radical intermediates "D". "C" and "D" presumably result in the bleaching of the carotenoid pigment. ISC = intersystem crossing; S = sensitizer; R = an oxidizable substrate.

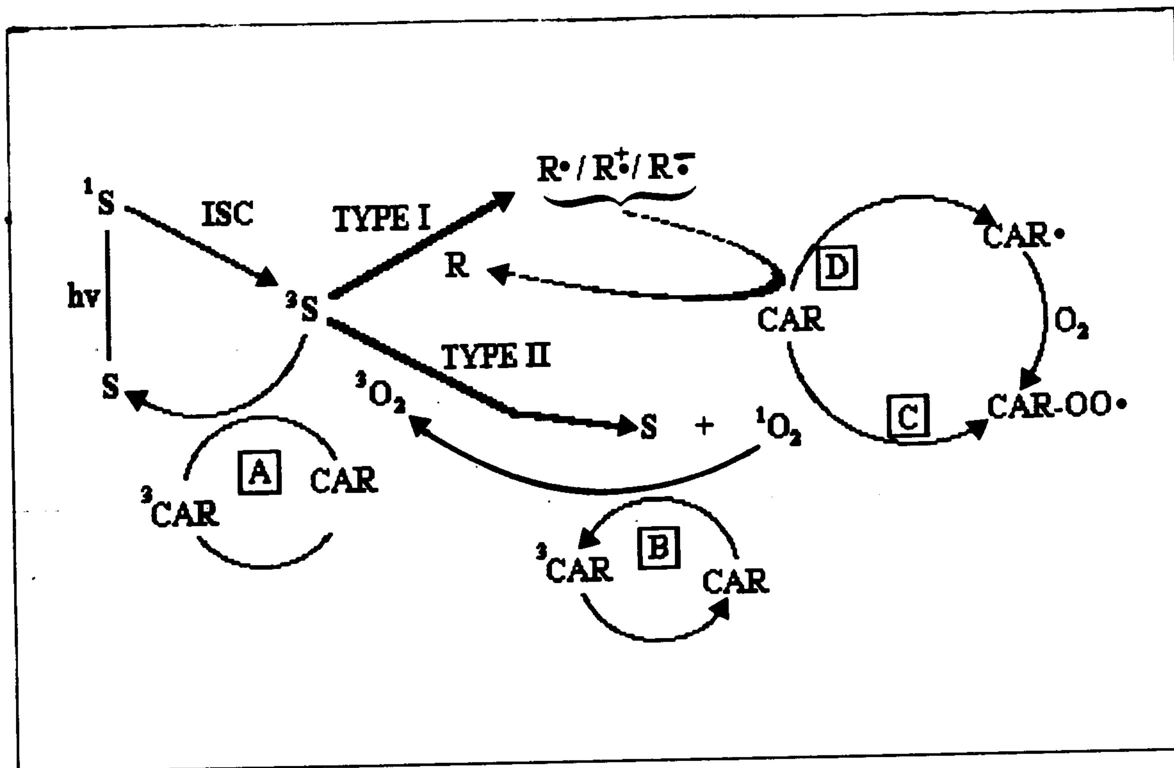

FIGURE 1

CHAPTER 2

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PROTECTION BY β-CAROTENE AND RELATED COMPOUNDS AGAINST OXYGEN-MEDIATED CYTOTOXICITY AND GENOTOXICITY: IMPLICATIONS FOR CARCINOGENESIS AND ANTICARCINOGENESIS

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Abstract—β-Carotene protects against photooxidative dermatitis in porphyric humans and mice by quenching of photoactivated species. Other actions of β-carotene in vivo are explained on the basis of its ability to scavenge free radicals in vitro. For example, in guinea pigs treated with CCl_4, β-carotene decreases pentane and ethane production. Epidemiological studies link low serum β-carotene levels to elevated risk of lung and other cancers, and in intervention trials, β-carotene diminishes preneoplastic lesions. However, the dose/response relationships are not well established, and antineoplastic mechanisms await clarification. Given a radical quenching mechanism, β-carotene should block tumor promotion, but more typically the site of action is progression and an even later role in invasion has not been ruled out. Some antineoplastic actions of carotenoids (such as increased rejection of fibrosarcomas in mice) are attributed to immunoenhancement; others may reflect conversion to retinoids and subsequent gene regulation. Carotenoids other than β-carotene may act at an earlier stage of carcinogenesis or be more effective as anticarcinogens at certain target sites. As scavengers of hydroxyl radicals, canthaxanthin and astaxanthin are more effective than β-carotene. Canthaxanthin is sometimes more effective than β-carotene in chemoprevention, but it is sometimes completely ineffective. Lycopene quenches singlet oxygen more than twice as effectively as β-carotene. However, the antineoplastic actions of lycopene or astaxanthin remain untested. Explorations of the interactions of carotenoids with other nutrients are just beginning. Dietary fat increases absorption of carotene but decreases antineoplastic effectiveness. Research is hampered by technical problems, including the unavailability of rigorous controls, the instability of carotenoids, and the heterogeneous phase structure induced by hydrophobic compounds in aqueous media. Areas of current controversy and promising approaches for future research are identified.

Keywords—β-Carotene, Carotenoids, Free radicals, Antioxidants, Carcinogenesis, Chemoprevention, Genotoxicity, Cytotoxicity

INTRODUCTION

In assessing dietary modulation of disease processes, focus shifted over the years from the recognition in the late 1800s that diseases could be prevented by replacement of missing components, to concerns in the 1960s regarding toxic food constituents. From this latter viewpoint, certain groups at low risk for cancer (such as Seventh Day Adventists) were protected by their low exposure to toxic agents, most dramatically meat fats and food additives. Recently the pendulum has swung back to recognizing that much of the impact of diet on disease can be attributed to the presence or absence of dietary protective agents. Among the dietary "chemopreventive" agents with antioxidant activity, carotenoids, ascorbate, and tocopherol have been intensively investigated.

This review is a perspective on the interface between the chemical properties of β-carotene, on the one hand, and its biological actions on the other. We begin with the biological relationship of carotenoids to oxidative stress. Some of the protection by carotenoids in isolated cells, tissue components, and intact mammals can be accounted for on the basis of singlet oxygen quenching activity. Next we consider actions

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of carotenoids in vitro which imply that in addition to quenching singlet oxygen, carotenoids are chain-breaking antioxidants, particularly at low partial pressures of oxygen, and we summarize the protective actions of carotenoids in cells and animals. The concluding sections review the antineoplastic actions of carotenoids in cultured cells, protection against carcinogens and tumor promoters in animal experiments, epidemiological and human intervention studies, and the potential of β-carotene as a chemopreventive agent. Finally, we outline a perspective on the most important unanswered questions and suggest priorities for future research.

Up-to-date reviews are available elsewhere for more intensive discussions of protection by carotenoids against photochemical damage and singlet state oxygen,[1] oxygen-derived active species,[2] protection by carotenoids in cellular and animal systems,[3] the safety of β-carotene,[4] studies of antioxidant nutrients and disease prevention,[5] and epidemiological studies of carotenoids and cancer.[6] This review is intended to complement the existing literature, and so we focus on the protective actions of β-carotene against oxidative stress in animals and humans, emphasizing the relationship between chemical reactivity and biological function.

BIOLOGICAL ROLES AND CHEMOTHERAPEUTIC POTENTIAL OF CAROTENOIDS

Overview and perspective

Historically, β-carotene has been referred to as provitamin A because of its ability to metabolize in animals to vitamin A. β-Carotene is oxidatively cleaved to form two molecules of retinaldehyde, a minor fraction of which irreversibly oxidizes to retinoic acid, and the remaining amount is reduced to retinol. In humans this is an inefficient pathway, with β-carotene delivering one sixth the vitamin A potency of an equivalent amount of oral retinol.

The biological role of carotenoids can no longer be limited to production of retinoids and protection of the photosynthetic apparatus of plants against light-mediated damage. β-Carotene is protective in a dozen or more cellular, animal, and human disorders well summarized in a review by Krinsky.[3]

The biological functions of β-carotene are as follows:

1. It is metabolized to retinol and retinoic acid.
2. It prevents photosensitized damage in bacteria, animals, and humans.
3. It prevents psoralen-induced ultraviolet A light phototoxicity.
4. It decreases bacterial revertants in bacterial mutagenesis tests.
5. It decreases sister chromatid exchanges (SCEs) and malignant transformation in cultured cells.
6. It inhibits UV- and chemically induced tumors in rodents.
7. It decreases premalignant lesions in humans.
8. It modifies the immune response.

Krinsky[2] has also reviewed the antioxidant activity of carotenoids. Here we attempt to define the current status of the interface between the antioxidant activity of carotenoids and their biological actions. We assess the extent to which the therapeutic benefits of carotenoids are explicable in terms of their chemical reactivities.

Oxidative stress links β-carotene to human health disorders

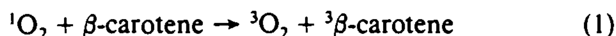
Oxygen-derived active species damage DNA, structural proteins, carbohydrates, enzymes, and especially the lipid components of membranes. Oxidative stress reportedly mediates disorders such as Alzheimer's disease,[7,8] ataxia telangiectasia,[9] atherosclerosis,[10] Parkinson's disease,[11] cataracts,[12] aging,[13,14] and carcinogenesis.[15–17] β-carotene protects against free radical-mediated oxidative damage. A study on cystic fibrosis has linked increased free radical production to low serum β-carotene.[18] In tests for therapeutic or protective actions, β-carotene has been the carotenoid of choice, used almost exclusively.[5,19] This emphasis on β-carotene may be misplaced, since although it is the most efficient at generating retinol, β-carotene is by no means the most abundant dietary carotenoid, nor is it universally the most effective antioxidant or singlet oxygen quencher.

β-Carotene is one of the most efficient quenchers of singlet O_2

Four possible mechanisms are available for protection by carotenoids against singlet oxygen:[20] (1) physical filtration of ambient far-UV light, (2) quenching of singlet oxygen or photosensitizer triplet states, (3) substitution of carotene for more vital cell constituents as preferred targets of photosensitization reactions, and (4) repair or stabilization of membranes. The second of these mechanisms rests on the firmest experimental foundations.

β-Carotene quenches singlet oxygen with an efficiency represented by a rate constant close to diffusion limits ($k_q = 1.4 \times 10^{10}\ M^{-1}\ s^{-1}$). Lycopene, an open-chain carotenoid related to β-carotene, is the

most efficient biological quencher of singlet oxygen ($k_q = 3.1 \times 10^{10}\ M^{-1}\ s^{-1}$).[21] Singlet oxygen quenching in vitro is a function of the length of the conjugated polyene chain.[22] Protective potency in photosynthetic organisms decreases with a decrease in double bonds from 11 to 9 and 9 to 7 corresponding to a 7% and 38% loss of protection of chlorophyll.[23] Carotenoids react with singlet oxygen, resulting in triplet carotenoid and triplet dioxygen (ground state). The multiplicity of double bonds allows carotene to act as a sink for a range of energy quanta in collisions with energized molecules containing electrons in antibonding orbitals above ground state. These conjugated π-orbitals also allow facile decay of the molecule to ground state, without luminescence. Decay in this way raises the vibrational energies of the carotenoid bond systems, resulting in conversion to heat rather than emitted light. The original carotenoid molecule is regenerated, so that β-carotene is not consumed when it quenches singlet oxygen.[2]



$$^1O_2 + \beta\text{-carotene} \rightarrow {}^3O_2 + {}^3\beta\text{-carotene} \quad (1)$$

$$^3\beta\text{-carotene} \rightarrow \beta\text{-carotene} + \text{heat} \quad (2)$$

β-carotene protects cells and tissues against photoactivated metabolites or xenobiotics

Light exposure is lethal to certain mutant (carotenoid-deficient) photosynthetic bacteria.[24-26] In the human eye, photostimulation of the rod cell pigment, retinal, produces singlet oxygen and potential injury to the rod cells.[27] In vitro, human lens proteins are oxidized by singlet oxygen to produce products similar to those found in the aging lens.[28] Singlet oxygen damages proteins by oxidizing methionine, tryptophan, histidine, and cysteine residues.[28] In vitro, β-carotene can prevent some of these indices of light-induced damage to elements of the retina. However, a protective role for carotenoids in age-related macular degeneration remains to be established. Orally administered canthaxanthin accumulates in the retina, but the current view is that its presence there is not deleterious.[29]

Accumulation of porphyrin metabolites can cause severe "dye-sensitized" skin lesions upon exposure to light.[30] Cutaneous inflammation is also induced by reactive species generated by UV-visible irradiation of rose bengal.[31] Application of rose bengal (1 nmol) and 120 min of light exposure increases vascular permeability and polymorphonuclear (PMN) leukocyte accumulation in rabbit skin three- or fourfold, and β-carotene (0.1 nmol) protects by 75–100%. Rose bengal or disodium 3'3'-(1,4-naphthylidene) dipropionate endoperoxide ($NDPO_2$) generates singlet oxygen, causing loss of transforming capacity in bacteriophage M13 and ϕX174 DNA, respectively.[32] Singlet oxygen generated by microwave discharge causes strand breaks in plasmid DNA pBR322 and ϕX174.[32] The nucleoside guanosine is a likely locus of genetic damage that follows light stimulation in the presence of intercalating dyes.[33] The ability of β-carotene to suppress the genotoxicity of singlet oxygen may explain some of the reported anticancer effects of β-carotene in vivo.

CAROTENOIDS SCAVENGE ACTIVE SPECIES OTHER THAN SINGLET OXYGEN

N-bromosuccinimide accelerates autooxidation of β-carotene 6.5-fold.[34] *N*-bromosuccinimide decomposes spontaneously, generating allylic radicals ($L^\bullet$), which in the presence of oxygen yield peroxyl radicals ($LOO^\bullet$). Consistent with this, the trichloromethylperoxyl radical ($CCl_3OO^\bullet$) bleaches β-carotene at a rate of $1.5 \times 10^9\ M^{-1}\ s^{-1}$ (Ref. 35), and β-carotene at 10 mM protects linolenic acid against xanthine oxidase/acetaldehyde initiated lipid autooxidation.[36]

In 1982 Krinsky and Deneke[37] suggested that carotenoids quench not only singlet oxygen but a variety of radical species. In this study, β-carotene protected liposomes against lipid autooxidation mediated by superoxide and hydroxyl radicals. β-Carotene also protected liposomes against lipid peroxidation and lysis caused by Fe^{2+}-generated radicals ($LO^\bullet$ and $LOO^\bullet$).[37] As evidence that this has biological significance, β-carotene at 5 μM prevents sister chromatid exchanges in Chinese hamster ovary cells exposed to oxidant stress.[38]

Confirmatory studies soon followed, spurred by the demonstration that β-carotene was a particularly effective chain-breaking antioxidant at oxygen tensions less than 150 Torr (discussed later).[39] In contrast, biological protection was greater at increased partial pressures of oxygen (PO_2 of 80%). Thus, β-carotene was more effective at high partial pressures of oxygen in protecting nonpigmented yeast against duroquinone-generated superoxide radicals.[40]

Carotenoids scavenge oxyhalides, sulfite, and Fenton-generated radicals

Carotene is destructively oxidized by oxychloride, oxybromide, and oxyiodide radicals, all generated in the lactoperoxidase/H_2O_2/halide system.[41] β-carotene also protects against these same radicals when they are used to induce lipid deterioration in muscle tissue.[42] Carotene protects methylthioketobutyric acid against

fragmentation (measurable as ethylene release) induced by an active species similar to the hydroxyl radical, formed by diesel soot particles in the presence of cysteine and hydrogen peroxide.[43] Sulfite radical ions, the first intermediates in the autooxidation of sulfite to sulfate, rapidly bleach the carotenoid crocin ($k = 1.0 \times 10^{10}$ M^{-1} s^{-1}) at pH 7.[44] In competing for those radicals, crocin is efficient in comparison with other tissue targets such as nucleic acids or nucleosides. The rate constants for crocin are typically less than or equal to 1.0×10^7 M^{-1} s^{-1} (ref. 44).

Carotenoids quench enzymically generated active species

Reactions of carotenoids with enzymically generated active species have been reviewed by Krinsky.[2] Many of these reactions are coupled oxidations of unsaturated fatty acids. Thus, β-carotene is enzymatically bleached by lipoxygenases[45–47] and peroxidases.[48] Reportedly, β-carotene protects red blood cells against cholesterol hydroperoxidase-induced hemolysis through its action as a chain-breaking antioxidant.[49]

Establishing radical scavenging by carotenoids demands careful controls

There are many assertions of radical-scavenging actions of carotenoids distinct from singlet oxygen quenching. However, there has been less direct evidence that β-carotene protects biological structures against free radicals. Where protection by added carotenoids has been reported, convincing controls were usually lacking, although some studies had good internal controls.[22,39,44,50] The controls particularly needed are kinetic comparisons of carotene with nonspecific chain breakers like albumin, thiols, or the allylic hydrogens of polyunsaturated fatty acids. Lacking these, carotenoids may "protect" merely by providing an alternative target for active species, neither better nor worse than other nonspecific polyunsaturated substances.

The problems of obtaining a suspension of (hydrophobic) carotenoids suitable for use in aqueous media have evaded resolution. The techniques used to date (sonication in buffers, preparation of micelles in sodium dodecyl sulfate, the use of carotenoid-enriched plasma, detergents, or organic solvents) all introduce additional problems not easily circumvented.

To make matters worse, carotenoids are easily degraded, and therefore special precautions and controls are needed to ensure integrity during manufacturing, collection, storage, and isolation for assay.[51,52] The ability of carotenoids themselves to undergo autooxidation introduces further complications.[52] For example, when oxygen consumption is used to assess target oxidation, carotenoids, like other lipids, can contribute to oxygen consumption by their own autooxidation. β-Carotene autooxidation is readily measurable at 460 nm, particularly at 760 Torr, both as loss of absorbance and as a contributor to oxygen consumption.[39] Moreover, studies of lipid autooxidation may be confounded by the oxidation products present in outdated carotenoid stock supplies. In addition to initiating and accelerating oxidations,[53] these oxidation products may elevate a nonspecific endpoint (e.g., thiobarbiturate reactive substances) by their presence.

In vivo, certain protective actions are consistent with radical scavenging

β-Carotene suppresses induction of superoxide dismutase by corn oil in rats, suggesting that it moderates oxidative stress in vivo.[54] Pigmented carotenoid-containing bacteria were more resistant to H_2O_2-generated hydroxyl radicals than a mutant colorless strain.[55] Among several carotenoids present, one (a xanthophyllic carotenoid, X5) was considered responsible for this bacterial antioxidant activity, since it disappeared upon addition of H_2O_2 (ref. 55).

As further evidence for a broader antioxidant activity in vivo, β-carotene levels in blood decrease following high-dose radiochemotherapy.[56] Since singlet species are a relatively minor product of tissue X-irradiation, the results suggest that radicals rather than singlet oxygen are being scavenged by β-carotene. Carotene also contributes in many other situations where better established radical scavengers are protective. Postoperative administration of β-carotene (like other antioxidants, including ascorbic acid and alpha-tocopherol) increased mean skin flap survival from 67% (saline-injected controls) to 89%.[57] Again, β-carotene, like ascorbate, glutathione (GSH), or tocopherol decreases pentane and ethane production in vitamin C-deficient guinea pigs injected with carbon tetrachloride.[58] The mechanism of free radical scavenging by β-carotene is still uncertain, although many of the products formed are known (discussed later).

Carotene sometimes fails where radical scavengers might be expected to protect

β-Carotene does not invariably protect against oxygen-mediated stress. β-Carotene failed to slow autooxidation of linoleic acid in sodium dodecyl sulfate micelles (760 Torr oxygen).[59] Moreover, β-carotene

in the absence of alpha-tocopherol increases oxidation of decolorized soybean oil.[53] Either β-carotene or alpha-tocopherol partially protected mice against the experimental neurotoxin MPTP (*N*-Methyl-4 phenyl-1,2,3,6-tetrahydropyridine).[60] However, neither protected dopaminergic nigrostriatal neurons in marmosets injected with low doses of MPTP.[61] β-Carotene did not protect against phototoxicity of chlorpromazine toward bovine retinal pigment of epithelial cells.[62] Nor did carotene protect against ethanol-induced gastric ulceration in rats, although other scavengers protected. Superoxide dismutase, thiourea, 1-phenyl-3-(2-thiazolyl)-2-thiourea, dimethyl sulfoxide, various inorganic compounds (elements of the first and second subgroups and of the sixth group of the periodic table), and sulfhydryl-containing substances protected in a dose-related manner.[63]

Carotenoids did not protect humans against ultraviolet light (UVA or UVB) or psoralen-induced UVA (PUVA) erythema.[64] Moreover, UVB-induced unscheduled DNA synthesis was not decreased by carotenoid pretreatment. On this basis psoralen-induced UVA erythema may not involve active oxygen species, or carotenoids may not be able to quench these species effectively in vivo. Similarly, carotenoids did not affect repair of UVB-induced mutagenic lesions in DNA.[64] In human volunteers, canthaxanthin failed to decrease the incidence of micronuclei induced in oral mucosal cells by smokeless tobacco, where β-carotene plus retinol protected.[65]

At least one carotenoid, crocin, is not very effective as a superoxide scavenger.[66] Superoxide dismutase protects under conditions where crocin does not protect. In steady-state X-irradiation or pulse radiolysis experiments, water-soluble crocin scavenges hydroxyl radicals and hydrated electrons, but not superoxide. Bleaching of crocin resulted primarily from secondary reactions with alkyl or peroxyl carotenoid radicals.

Publication of negative results has always been important to the progress of research. At this stage of research on β-carotene when euphoria prevails and many experiments are in progress, it is unethical to withhold negative data. It is only in the context of the negative findings that the significance of positive findings can be properly assessed.

Among the carotenoids, β-carotene is not the most effective radical scavenger

An important area requiring clarification is the biological importance of β-carotene relative to other carotenoids. Thus, although β-carotene is only one of many carotenoids found in foods and in plasma, other carotenoids have received little attention. The main reason for emphasizing β-carotene is the historical observation that it is the most efficient among carotenoids in its ability to be metabolized to vitamin A. Where contributions of various carotenoids to radical scavenging have been compared, β-carotene was commonly less effective than those carotenoids which possess oxo groups at the 4 and 4′-positions in the beta-ionine ring like canthaxanthin and astaxanthin (Fig. 1).[67] β-Carotene and zeaxanthin, which possess no oxo groups, were less effective in retarding hydroperoxide formation, and they autooxidized faster.

β-Carotene is a better chain terminator at low partial pressures of oxygen

At least some of the antioxidant actions of β-carotene were probably missed by workers who looked for it at ambient partial pressures of oxygen. Burton and Ingold gave a major impetus to the protective actions of carotenoids when they discovered that β-carotene is a particularly efficient antioxidant at partial pressures of oxygen less than 150 Torr.[39] This property was first demonstrated in the ability of β-carotene to protect either tetralin or methyl linoleate (in chlorobenzene) against autooxidation by radicals formed by the thermal decomposition of azo(bis)isobutyronitrile (AIBN). The data imply that β-carotene is well equipped to protect at the diminished partial pressures of oxygen that prevail intracellularly, particularly in active muscle, heart, or solid tumors. Its actions may complement protection by alpha-tocopherol, which is more effective at higher partial pressures of oxygen.

The dependence of protection by β-carotene on oxygen concentration arises from the kinetics of the intervening reactions. β-Carotene is reactive enough toward peroxyl radicals ($ROO^\bullet$) that it competes successfully with the allylic hydrogens of polyunsaturated fatty acids in membranes.[68] In the presence of peroxyl radicals, β-carotene produces a carbon-centered carotenyl radical ($\beta\text{-car}^\bullet$) reaction (Eq. 3), which in the absence of oxygen is an efficient chain terminator (Eq. 5).

$$\beta\text{-carotene} + ROO^\bullet \rightarrow \beta\text{-car}^\bullet \quad (3)$$

$$\beta\text{-car}^\bullet + O_2 \leftrightarrows \beta\text{-car–OO}^\bullet \quad (4)$$

$$\beta\text{-car}^\bullet + ROO^\bullet \rightarrow \text{inactive products} \quad (5)$$

The presence of oxygen, however, allows chain propagation. This is because the carotenyl radical reacts reversibly with oxygen to yield a chain-propa-

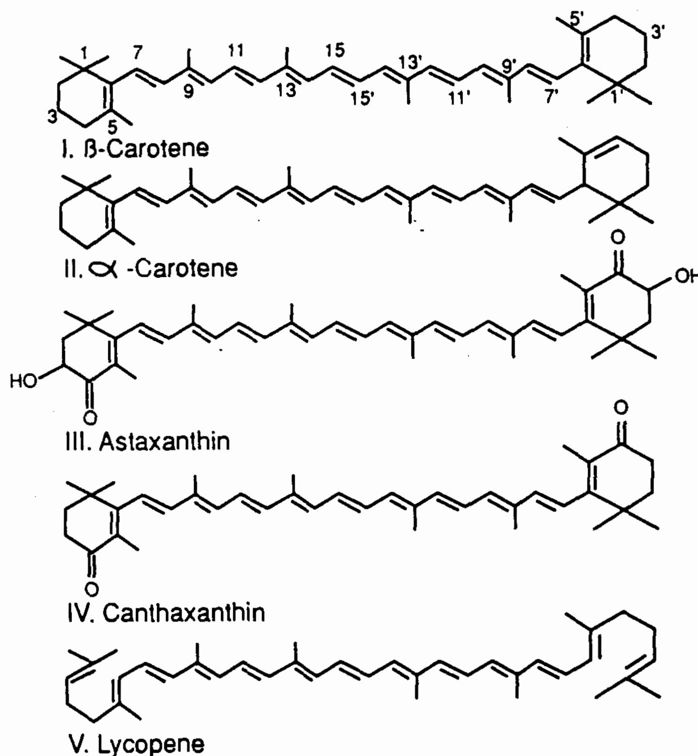


Fig. 1. Part 1. Carotenoid structures. The structures of β-carotene (I), alpha-carotene (II), astaxanthin (III), canthaxanthin (IV), lycopene (V).

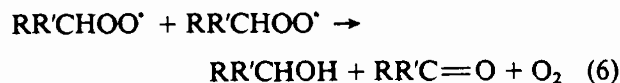
gating species, the β-carotene peroxyl radical (β-car–OO$^{\bullet}$), which triggers further oxidations (Eq. 4).

At low partial pressures of oxygen, the equilibrium of Eq. 4 shifts to the left, decreasing the concentration of chain-propagating β-carotene peroxyl radicals and increasing the concentration of chain-terminating carotenyl radicals. Whether propagation or termination predominates depends on the reaction kinetics of the carotenyl radicals in comparison with the β-carotene peroxyl radicals. The predominant fate of the carotenyl radicals at low partial pressures of oxygen is removal by another peroxyl radical in Eq. 5, leading to chain termination. This reaction is more facile than termination by a reaction between two peroxyl radicals where the products are unstable. The end result is that only at low partial pressures of oxygen is β-carotene an effective chain-breaking antioxidant.[68] That β-carotene protects better at low oxygen concentrations has been substantiated in a study by Vile and Winterbourne.[69]

Products of radical scavenging by carotenoids indicate attack at multiple sites of the β-carotene molecule

The more reactive among the free radicals attack somewhat indiscriminately; it should not therefore be surprising that more than 20 products result from free radical attack on the β-carotene molecule.[52,70,71] Regardless of the means used to induce carotene oxidation, overlapping mixtures of products were obtained. Predictably, the distribution of products changes with the means used to induce the oxidative stress. Free radical attack on the alicyclic ring led to epoxides bridged across the ring near the side chain (5,6 epoxide), or bridged between the ring and the side chain (5,8 epoxide). Free radical attack on the side chain led to formation of carbonyl compounds, an aldehyde if scission occurred distal to an even-numbered (non-methylated) carbon and a ketone if distal to an odd-numbered (methylated) carbon (Fig. 2). Under nitrogen, the major products were nevertheless oxygenated: aldehydes, ketones, and epoxides.[72] Thus, even "under nitrogen" most termination reactions were mediated by oxygenated species and presumably dependent on traces of residual oxygen in the reaction system or derived from radical-mediated solvolysis.

Peroxides, hydroperoxides, and alcohols were not significantly present among the oxidation products. The lack of alcohol products implies that termination occurs by processes other than the peroxyl-peroxyl reaction:[71]



$$RR'CHOO^{\bullet} + RR'CHOO^{\bullet} \rightarrow RR'CHOH + RR'C{=}O + O_2 \quad (6)$$

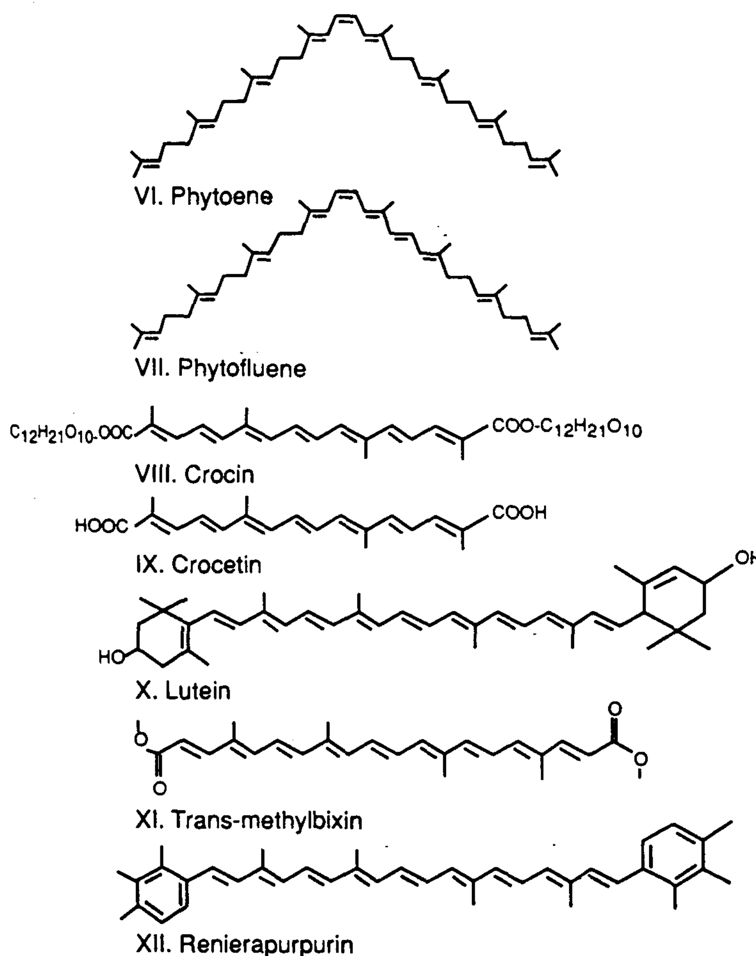


Fig. 1. Part 2. Carotenoid structures continued. Phytoene (VI), phytofluene (VII), crocin (VIII), crocetin (IX), lutein (X), trans-methylbixin (XI), renierapurpurin (XII).

The autooxidation mechanism for β-carotene is remarkably different from that of the polyunsaturated fatty acids. "Peroxidation" is a misnomer for this process, which depends on H-abstraction from an allylic carbon as a central propagating step. β-Carotene's chain is conjugated rather than allylic. The distribution of products reveals that the dominant process in carotene oxidation is electrophilic attack by propagating peroxyl radicals (or oxygen itself) on the alicyclic ring or conjugated side chain.[71] Confirmation that carotenoids other than β-carotene protect by similar mechanisms is not yet available.

BIOLOGICAL PROTECTION BY CAROTENOIDS

Carotenoids are transported by lipoproteins and may protect them against oxidation

Carotenoids are absorbed from the small intestine via the lacteal route and transported in human plasma exclusively in lipoproteins.[73] Some 75% of all plasma β-carotene and lycopene associates with LDL (low density lipoproteins), with the remaining 25% associated with HDL (high density lipoproteins). Lutein is equally distributed between the two types of lipoproteins.[74] Conflicting reports associate 79% of β-carotene with LDL, 8% with HDL, and 12% with VLDL (very low density lipoproteins).[75] The carotene content of LDL is low, 0.15 ± 0.09 nmole β-carotene per milligram of LDL, representing only one molecule of β-carotene per two or three LDL particles.[50]

It is debatable whether β-carotene in plasma is present in sufficient concentrations to be considered an antioxidant. On the one hand, the total (peroxyl) radical-trapping antioxidant parameter (TRAP) of human blood plasma is fully accounted for by contributions from urate, plasma proteins, ascorbate, and vitamin E.[76] It is noted that β-carotene concentration in plasma is too low to be a contributory factor in TRAP. On the other hand, some chain-breaking antioxidant activity was not accounted for,[76] and this may be more evident at lower partial pressure of oxygen. Moreover, oxidation of LDL initiated by copper

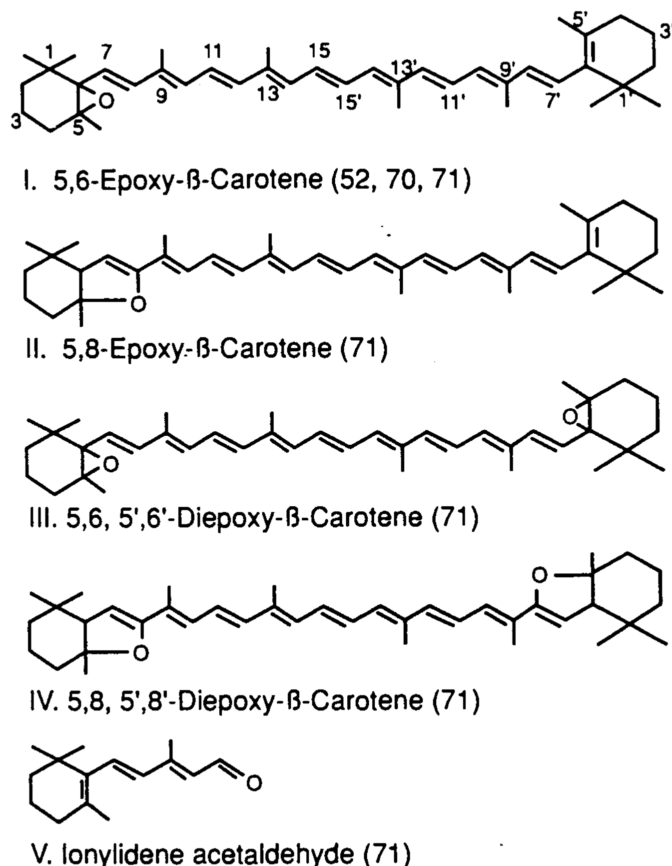


Fig. 2. Part 1. The products of β-carotene oxidation. 5,6-Epoxy-β-carotene (I), 5,8-epoxy-β-carotene (II), 5,6-5'6'-diepoxy-β-carotene (III), 5,8-5'8'-diepoxy-β-carotene (IV), β-ionylidene acetyladehyde (V).

ions is delayed until all β-carotene has been oxidized.[50] This implies that LDL are protected by the small amounts of carotene they contain. At first glance it seems paradoxical that one molecule of β-carotene can protect more than one LDL particle. However, this is not physically impossible if β-carotene or, more likely, chain-propagating intermediates can exchange between colliding LDL particles. The average period between collisions of LDL particles must then be brief in comparison with the interval between initiation and termination of a particular chain reaction sequence within the LDL. The presence of other antioxidants in LDL at higher concentrations helps to provide conditions under which carotenoids can protect optimally.

Carotenoids act synergistically with other antioxidants

Parallels between the chemical properties of β-carotene, ascorbate, and other antioxidants do not comprise a convincing argument that carotene shares the same actions in vivo since vitamins C, E, and β-carotene may well act synergistically. α-Tocopherol is reportedly the primary chain-breaking antioxidant at ambient oxygen tensions, with β-carotene predominating at low partial pressures of oxygen.[39] In vitro, alpha-tocopheroxyl radicals in the aqueous phase are recycled by ascorbate, regenerating alpha-tocopherol.[77] In experimental animals, supports for synergism between β-carotene and alpha-tocopherol is at best ambivalent. However, in guinea pigs, deuterium-labelled alpha-tocopheroxyl acetate failed to spare β-carotene.[78] These findings argue against synergism between β-carotene and alpha-tocopherol. Moreover, chicks fed a vitamin E-deficient diet containing canthaxanthin showed symptoms of vitamin E deficiency at the usual time.[79] Nevertheless, their livers were more resistant to Fe-induced thiobarbituric acid reactive substance (TBARS) formation than controls lacking canthaxanthin. More detailed investigation revealed that dietary canthaxanthin induced resistance to lipid peroxidation mainly by enhancing membrane alpha-tocopherol levels and only marginally by providing weak direct antioxidant activity.

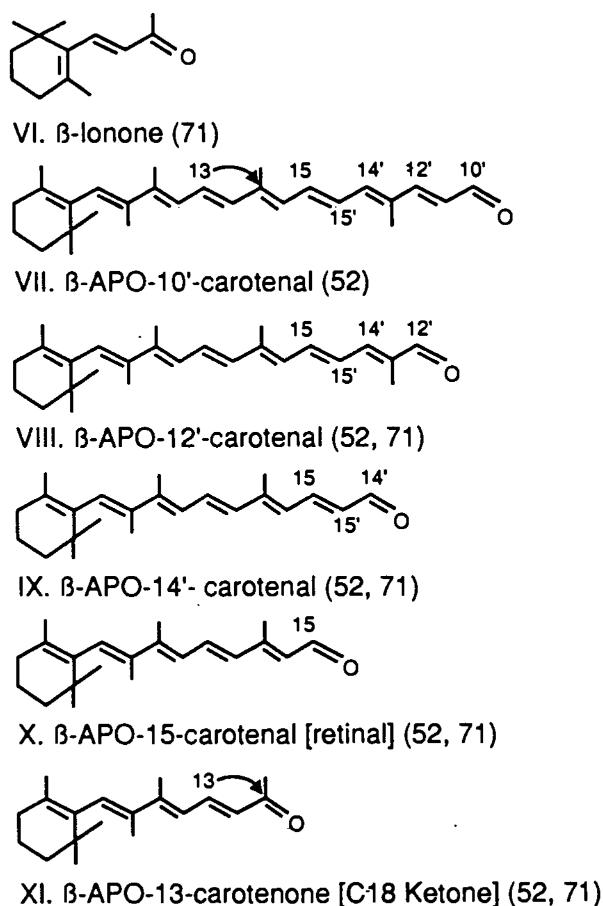


Fig. 2. Part 2. The products of β-carotene oxidation, continued. β-ionone (VI), β-apo-10'carotenal (VII), β-apo-12'carotenal (VIII), β-apo-14'carotenal (IX), β-apo-15 carotenal (retinal) (X), β-apo-13-carotenone (C18 ketone) (XI).

β-Carotene ameliorates photosensitivity in dye-sensitized photooxidative tissue injury

Exquisite sensitivity to wavelengths in the visible absorption spectrum of porphyrins (380–560 nm) is a major cause of pathology in patients with erythropoietic protoporphyria. The disorder is characterized by elevated levels of protoporphyrin IX in erythrocytes, feces, and plasma. Symptoms usually start in childhood upon exposure to visible light and include a burning sensation in the skin, erythema, and edema. Some 84% of patients treated with β-carotene increased their tolerance to sunlight by a factor of three.[30] In normal subjects too, β-carotene significantly increased the minimal erythemal dose.[80] In contrast, however, another study found no protection by carotenoids against UVA, UVB, and psoralen-induced UVA erythema.[64]

Porphyrin-photosensitized reactions in vitro are oxygen dependent,[80] and the resultant activated oxygen species damage cells,[8] although it is difficult to prove that oxygen-derived active species are responsible for the symptoms of erythropoietic protoporphyia.[30] Vitamin E and β-carotene presumably act as singlet oxygen quenchers or chain-breaking antioxidants when they protect against lethal hematoporphyrin photosensitization of white mice.[82]

CAROTENOIDS PROTECT AGAINST NEOPLASTIC TRANSFORMATION IN CELLS AND ANIMALS

β-Carotene and canthaxanthin protect against genotoxicity and neoplastic transformation

Many studies in vitro (Table 1) and in intact animals confirm that β-carotene inhibits genotoxicity. Thus, β-carotene protects against cyclophosphamide-induced mutagenesis in *Salmonella typhimurium*.[83] It also protects against benzo(*a*)pyrene- and mitomycin C-induced chromosomal damage in bone marrow of mice[84] and against photogenotoxicity in mouse skin.[85,86] Consistent with protection of the genome, β-carotene or canthaxanthin inhibit 3-methylcholanthrene induced neoplastic transformation in C3H/10T$\frac{1}{2}$ cells (discussed later).[87,88] β-Carotene (10^{-5} M)

Table 1. The Effects of Carotenoids in in vitro Studies

Carotenoid	Action	Method of Induction	Target	Effect	Reference
Bc	chromosome breaks	1. benzo(a)pyrene 2. mitomycin C	mouse bone marrow	1. protective- by 41-61% 2. protective by 44-71%	Raj and Katz 1985 (84)
Bc	lipid peroxidation	superoxide radicals hydroxyl radicals	liposomes	protective- reduces lipid peroxide formation by 40%	Krinsky and Deneke 1982 (37)
Bc	SCE's	1. TPA stimulated HP 2. Hx/X system	chinese hamster ovary cells	1. protective- reduces SCE's by 26% 2. protective- reduces SCE's by 53%	Weitberg et al. 1985 (38)
Bc	autoxidation		tetralin/methyl linoleate	increased protection at PO2 <150 Torr compared to 760 Torr	Burton and Ingold 1984 (39)
Bc	lipid peroxidation	adriamycin	rat liver microsomes	inhibited peroxidation by 50% at 162 Torr and 70& at 4 Torr	Vile and Winterbourne (69)
Bc	oxidative injury	duroquinone	R. mucilaginosa (yeast)	protective- completely prevented growth inhibition by hyperoxia (80% O2) or duroquinone	Moore et al. 1989 (40)
Bc	lipid peroxidation	reperfusion	ischemic skin flaps (rat)	protective- increased flap survival by 22%	Hayden et al. 1987 (57)
Bc	phototoxicity	chlorpromazine and UV- light	retinal pigment epithelial cells (bovine)	not protective	Persad et al. 1988 (62)
Bc/Cx	neoplastic transformation	1. MCA 2. X-rays	C3H/10T1/2 cells	1. protective- Cx > 4x more effective than Bc 2. protective- Cx 34% more effective than Bc	Pung et al. 1988 (87)
Carotenoids	neoplastic transformation	MCA	C3H/10T1/2 cells	in decreasing order of potency: Cx, Bc, alpha-carotene, lycopene. Renierapurpurin and bixin not effective	Bertram et al. 1991 (88)

β-Carotene (Bc), canthaxanthin (Cx), ultraviolet (UV), sister chromatid exchange (SCE), human phagocyte (HP), hypoxanthine/xanthine (Hx/X), 3-methylcholanthracene (MCA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), *N*-methyl-N'-nitro-*N*-nitrosoguanidine (MNNG).

protects the genome of Chinese hamster ovary (CHO) cells against phagocyte- and enzyme-generated oxygen metabolites as demonstrated by a 27% and 34% decrease in sister chromatid exchanges (SCEs), respectively.[38] There is great experimental variability in the systems used to assess chemoprotection by carotenoids. In vitro, this is due in part to nonuniformity in delivery of the hydrophobic carotenoid in an aqueous culture medium. In vivo, variability among individual animals (or subjects) contributes, as does our ignorance of the other confounding variables.

Carotenoid-rich foods protect animals against neoplastic transformation

Early studies used carrots as a source of carotene. Subcutaneous tumors and skin tumors were induced by injections or topical application of dimethylbenzanthracene.[89] Under both methods of induction, rats offered carrots ad libitum developed fewer tumors than the animals receiving a normal diet. Similarly, rats treated with diethylnitrosamine survived 72% longer than controls if fed 160 g of carrots per week.[90] Carrots contain several carotenoids, with β-carotene contributing 5–51% of the total.[91] Current studies usually employ purified carotenoids. For example, Epstein injected hairless mice with a solution of β-carotene or placebo and then exposed them to UVB radiation (290–320 nm).[85] After 12 months, the β-carotene injected animals developed 27% fewer tumors greater than 4 mm^3 and 35% fewer tumors greater than 50 mm^3.

Mice fed two types of β-carotene-supplemented diets were initiated with 7,12-dimethylbenz(*a*)anthracene, promoted with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and then maintained on the same diet.[92] Mice fed a 3% diet of β-carotene beadlets had a 25 and 29% decrease in tumors, and those fed crystalline β-carotene had similar decreases, 22 and 39%, respectively. Over a range of tumorigenic induction and promotion protocols, β-carotene consistently suppresses neoplastic transformation.

β-Carotene has antitumor activity independent of conversion to retinol

Chemoprevention by retinoids is much less consistent than protection by carotenoids. Retinoid (and carotenoid) effects are specific to the experimental conditions used, and in some instances retinoids have increased neoplastic transformation.[93] Band and coworkers have reviewed dose-timing relationships in the anticarcinogenic actions of retinoids.[94] Antitumor activity is mainly associated with the promotional stage, although some studies have indicated protection at the initiation stage of carcinogenesis. Retinoids may well exert their effect by moderating cellular proliferation and differentiation through their gene regulatory actions. The initial assumption that antitumor activity of β-carotene depended on conversion to a retinoid proved unfounded when carotenoids without retinoid activity (like canthaxanthin and phytoene) protected. Protection by these carotenoids strengthens the hypothesis that carotenoids protect by antioxidative mechanisms.

In vitro, canthaxanthin was four times as protective against 3-methylcholanthrene induced transformation as β-carotene, with an ED_{50} (effective dose for 50% protection) of 2×10^{-7} M compared with 9×10^{-7} M.[87] Similarly, β-carotene and canthaxanthin at 3×10^{-6} M protected C3H/10T$\frac{1}{2}$ cells against X-ray induced neoplastic transformation by 60% and 91% when added 1 week after irradiation.[87] Recently, the same system was used to determine the potency of diverse carotenoids. In decreasing order of effectiveness, canthaxanthin, β-carotene, alpha-carotene, and lycopene protected in a dose-dependent manner, while renierapurpurin and bixin were ineffective in protecting against 3-methylcholanthrene induced production of transformed foci.[88] Clearly, where canthaxanthin protects against neoplastic transformation, any role for retinoids is excluded. In vitro studies help to define mechanisms of protection by carotenoids.

In vivo, carotenoids without retinoid activity (canthaxanthin and phytoene) also protect. Ten weeks after starting carotenoid administration, skin tumors were induced in hairless mice by (1) UVB only, (2) dimethylbenzanthracene and croton oil application, and (3) dimethylbenzanthracene and low-dose UVB.[95] β-Carotene and canthaxanthin delayed development of UVB-induced skin tumors by 2 weeks, while phytoene delayed development by 8 weeks. With a combination of dimethylbenzanthracene and UVB, β-carotene and canthaxanthin delayed tumor formation by 2 and 7 weeks respectively, but phytoene had no effect. In contrast, only β-carotene was effective against chemical induction with dimethylbenzanthracene and croton oil, decreasing incidence of skin tumors by 70% and delaying tumor appearance by 1 week.

β-Carotene injected locally into dimethylbenzanthracene-induced tumor sites cause regression of oral carcinomas in hamster buccal pouches.[96] Here, canthaxanthin causes significant regression of tumor burden, whereas 13-*cis*-retinoic acid had no effect. β-Carotene supplementation increases activity of the intestinal carcinogen-metabolizing enzyme, aryl hydrocarbon hydroxylase in rats.[97] Hepatic aryl hydrocar-

bon hydroxylase was, however, not significantly increased, possibly due to the metabolism of dietary β-carotene to active vitamin A in the intestinal mucosa. Regardless of inconsistencies in the aforementioned studies, one thing is clear: Carotenoids do not always require conversion to the retinoid form to protect against UVB radiation.

The multistage model of carcinogenesis: at what stages do carotenoids act?

Few studies using experimental animals have sought to determine at what stage of tumorigenesis (initiation, promotion, progression) β-carotene acts (Table 2). β-Carotene administered 6 weeks prior to UVB irradiation failed to prevent skin cancers in hairless mice.[98] In contrast, when administered after irradiation, it protected by 45%. Under these conditions, β-carotene protected during the progression phase in neoplastic transformation,[98] but when present only during irradiation, no significant protection was seen. These conclusions were supported by a study with the direct carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which reacts with DNA independently of light and oxidative processes and induces gastric carcinomas in rats.[99] Neither β-carotene nor canthaxanthin protected against MNNG-induced preneoplastic lesions which reflect the promotional stage of carcinogenesis. However, they protected against progression of dysplasias to early and infiltrating carcinomas by 65 and 67%, respectively.[99] Combining these two studies, the carotenoids tested did not protect in the initiation or promotion stage; instead they protected during the progression stage.

Protection by β-carotene against tumorigenesis and growth after tumor transplantation must be attributed to blockage of seeding or invasion. In such a study, β-carotene protected female C3H/HeJ mice against inoculation with C3HBA tumor cells.[100] β-Carotene decreased the incidence of tumors after transplantation by 70–80%, and of those tumors which did develop, the size of the tumors was 70–80% less. β-Carotene also increased the survival times of the tumor-bearing animals by 60–90%.[100]

Canthaxanthin administered to rats 3 weeks prior to induction with dimethylbenzanthracene protects against mammary cancer by 65%.[101] However, it was not protective when administered after promotion with methylnitrosourea, indicating an effect at the initiation and not the promotional stage of carcinogenesis. Retinoids are thought to be most protective at the promotional stage of carcinogenesis.[94] Possibly, the presence of oxygenated functional groups in both canthaxanthin and retinoids changes the mechanism of protection against neoplastic transformation.

Overall, the data relating to time-sequence in the antineoplastic actions of β-carotene suggest that carotenoids protect primarily against progression. Moreover, cessation of β-carotene administration is often followed by reappearance of tumors. In our literature search, only one study directly addressed this important question. Here, diets of mammary-cancer-prone mice were supplemented with β-carotene-rich algae for each of three 3-month periods: 1–4 months, 4–7 months, and 7–10 months of age.[102] No protection was found in the 1–4 month group (initiation); however, significant protection occurred in the 4–7 month group (initiation and promotion), with the greatest protection in the 7–10 month group (progression). Suppression of tumorigenesis by β-carotene appeared to last only so long as administration is maintained, so that it *delayed* rather than *prevented* tumors.[102]

Carotenoids stimulate the immune response

Suppression of tumor seeding and invasion by β-carotene is consistent with the known enhancement of immune activity by retinoids and carotenoids. Granulocytes and other cells of the immune system use oxygen radicals to destroy pathogens and tumor cells. This benefit is often accompanied by unwanted damage to healthy cells by radicals, lipid peroxides, and/or other oxidative products.[103] β-Carotene protects polymorphonuclear neutrophils and phagocytic cells against autooxidative damage. At the same time, β-carotene and canthaxanthin (vitamin A has a slight effect) accelerate destruction of tumor cells by macrophages[104] and stimulate natural killer cell activity.[105]

In addition, carotenoids, like retinoids, increase immunosurveillance. For example, β-carotene augments the action of cytotoxic T-lymphocytes.[106] For this and other reasons, oral administration of β-carotene (120 μg/d) in mice increased rejection (by 50%) of rechallenged syngeneic BALB/c Meth A fibrosarcoma cell implants.[106] This augmented immunity is specific to the tumor-specific antigen, since growth of a tumor cell line lacking this antigen (Meth 1 fibrosarcoma) was unaffected. In elderly humans, supplementary β-carotene (≥30 mg/d for 2 months) increased plasma concentrations of β-carotene. Concomitantly, this resulted in an increase in the percentage of lymphoid cells with surface markers for natural killer cells and interleukin 2 receptors.[107]

The ability of carotenoids to scavenge or quench active species suggests mechanisms for these enhanced immune responses. Enhancement of immune competence by carotenoids (like canthaxanthin) which lack vitamin A activity strongly supports an antioxidant mechanism.[108] Carotenoids protect

Table 2. The Effects of Carotenoids in Animal Studies

Carotenoid	Action	Method of Induction	Target	Effect	Reference
Bc	lipid peroxidation	carbon tetrachloride	guinea pigs	In vitamin C-deficient animals: decreased pentane and ethane production in breath by 73% each	Kunert and Tappel 1983 (58)
Bc	tumor rejection	injection of fibrosarcoma cells	mice	enhanced tumor immunity: increased rejection of rechallenged sarcoma implants by 50%	Tomita et al. 1987 (106)
Bc	carcinogen metabolism	benzo(a)pyrene	rat small intestine	enhanced AHH (aryl hydrocarbon hyroxylase) carcinogen metabolizing activity 46%	Edes et al. 1989 (97)
Bc	pre-neoplastic foci	BOP	hamster pancreas	decreased the number of tubular ductal complexes by 71%	Woutersen et al. 1988 (114)
Bc	carcinogenesis	UV light	hairless mouse skin	protective- 27% reduction in tumors >4 mm and a 35% reduction in tumors >50 mm	Epstein 1977 (85)
Bc/ Cx/ Phy	carcinogenesis	UV-B light	mouse skin	delayed developement by 2,2 and 8 weeks	Mathews-Roth 1982 (95)
Bc/ Cx	carcinogenesis	DMBA/ UV-B light	mouse skin	delayed development by 2 and 7 weeks	
Bc	carcinogenesis	DMBA/ croton oil	mouse skin	delayed development by 1 week and average number of tumors/animal by 70%	
Bc	carcinogenesis	injection of C3HBA tumor cells	female C3H/HeJ mouse skin	protective- decrease in tumor incidence by 66%; increase in latent period by 22% ; survival time by 60%; and tumor size by 74%	Rettura et al. 1982 (100)
Bc Cx	carcinogenesis	DMBA	hamster buccal pouch	protective- 98% regression of tumors;	Schwartz and Shklar 1988 (96)
Bc	carcinogenesis	DMBA/TPA	Skh mouse skin	protective- decrease in tumor incidence by 39% (crystal) and (29%) beads	Lambert et al. 1990 (92)
Cx	carcinogenesis	DMBA/MNU	rat mammary gland	protective- at initiation stage by 65% not protective against promotion by MNU	Grubbs et al. 1991 (101)

β-Carotene (Bc), canthaxanthin (Cx), phytoene (Phy), *N*-nitrosobis(2-oxopropyl)amine (BOP), ultraviolet (UV), dimethylbenz(*a*)anthracene (DMBA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), methylnitrosourea (MNU).

against immunosuppressive peroxides[108] by preserving membrane receptors and fluidity. Carotenoids may also alter the release of immunomodulatory lipids such as prostaglandins and leukotrienes.

Interactions of carotenoids with other nutrients

Driven by entropic forces governing solubility, β-carotene comes to reside primarily in the lipophilic (intramembrane) compartment of cells. Ascorbate and plasma proteins protect against free radical propagation in the aqueous environment,[76] but plasma membranes require, in addition, fat-soluble antioxidants such as vitamin E and carotenoids inside the lipid-protein bilayer.[109] There are wide variations in the ability of individuals to respond to dietary β-carotene by incorporating increased quantities into their tissues.[110] Nonsmokers, women, and lean subjects had larger increases in plasma β-carotene with oral supplementation.[111] The most reliable predictor of change in plasma β-carotene is initial concentration, reflecting the observation that some people absorb β-carotene poorly whether the source is dietary or supplemental.[111] Certain individuals, more capable of incorporating carotenoids into LDLs, may benefit from enhanced transport to target tissues and enhanced tissue protection.

Dietary fat increases absorption of β-carotene[112] but concomitantly decreases its efficacy. In a 26-week study of UVB radiation-induced tumors in mice, protection by dietary β-carotene diminished with increasing dietary fat intake.[113] UVB radiation induced an average of 2.19 tumors/mouse in the high-fat-diet group. At normal fat intakes, β-carotene decreased tumor formation by 30%. Dietary fat exacerbated tumor development, so that increasing dietary fat by 9.6% decreased time of tumor development by 20%. Concomitantly, dietary fat decreased protection by β-carotene to the point where there was no difference between a high-fat diet and a high-fat diet with β-carotene.

β-Carotene decreased the number of pancreatic carcinomas, but selenium increased the number of carcinomas in the high-fat-diet animals treated with *N*-nitrosobis(2-oxopropyl)amine.[114] Both β-carotene and selenium, however, decreased preneoplastic lesions in the pancreas of hamsters and rats. Hamsters fed a high-fat diet (20% lard) had 71% more intermediate ductal complexes and 64% more tubular ductal complexes than animals fed a low-fat diet (5% lard). Both β-carotene and selenium protected at the early stages of carcinogenesis, decreasing the frequency of basophilic foci with a diameter over 272.5 μm in rat pancreas by 51% and 67%, respectively. β-Carotene decreased the number of large tubular ductal complexes in hamster pancreas by 71% compared with high-fat controls.

Many biomolecules potentially react with β-carotene, and research on interactions with nutrients such as vitamin C, vitamin E, selenium, zinc, copper, and iron must be continued. Pharmacological doses of vitamin E decrease concentrations of β-carotene in plasma and liver of rats.[115] Results with rodents must be interpreted carefully when extrapolating to humans since rodents are poor absorbers of dietary carotenoids.[116,117] Nevertheless, data from animal studies can be extrapolated to give useful information for human intervention trials. A number of trials of protection by carotenoids in humans have been undertaken. These are outlined in the ensuing section.

β-CAROTENE DECREASES RISK OF CANCER IN HUMANS

Serum β-carotene levels predict several kinds of carcinomas

The 1970s brought many reports that retinoids could inhibit carcinogenesis in animals and cell systems exposed to oxidant stressors. A correlation between above-average blood retinol or β-carotene levels and a below-average incidence of cancer emerged in epidemiological studies. In the 1980s interest shifted toward activity of the carotenoid molecule as a protectant, independent of vitamin A activity. Among these studies, 85–90% report an inverse relationship between consumption of carotenoid-rich fruits and vegetables and certain forms of cancer.[30] From these findings came a growing conviction that carotenoids could decrease the risk of cancer in humans.[6,118,119]

In the British United Provident Association (BUPA) study,[120] β-carotene was measured in serum samples (taken several years before the diagnosis of cancer) from 271 patients with cancer of various kinds and 533 controls. Levels of β-carotene in serum of cancer patients (19.8 μg/dL) were lower than those of the controls (22.1 μg/dL). Individuals in the top two quintiles of serum β-carotene levels had a 40% lower risk of developing cancer than those in the bottom quintile. There was no relationship between β-carotene levels and site of cancer, although the association with lung cancer was the strongest. In a 12-year (1973–1985) follow-up study, the most consistent finding was the continued correlation between low plasma carotene levels and the increased risk for all cancers, especially bronchus and stomach.[51]

Other studies have linked high serum β-carotene levels to decreased incidence of lung cancer.[121–123] In tobacco-induced cancers, free radicals in smoke or ca-

techols in tar provide oxidant stress. In a study of Hawaiian men of Japanese ancestry, cancer patients' β-carotene serum levels averaged 20.9 μg/dL compared with 29.0 μg/dL in controls.[124] Serum concentrations of β-carotene were 58% lower in lung cancer patients than in controls.[125] Smokers who did not consume carrots had a threefold greater incidence of lung cancer than those who consumed carrots several times a week.[126]

Not all studies found an inverse relationship between consumption of carotenoid-rich foods and cancer. Risk of cervical cancer was unaffected by consumption of β-carotene-rich dark green or yellow/orange vegetables, fruits, or legumes.[127,128] However, dietary and serum lycopene levels indicate a strong inverse relationship with cervical intraepithelial neoplasia.[128] Papilloma viruses are major factors in the etiology of cervical cancer, and these can sensitize cultured cells to neoplastic transformation by oxidant tumor promoters.[129] Complicating variables such as these may mask protection in studies where many variables interact. This complexity should serve as a stimulus rather than a deterrent for future studies of intervention using anticarcinogens.

Epidemiological associations do not indicate cause and effect

Although low levels of plasma β-carotene are strongly associated with lung cancer, retinol is not similarly protective. Thus, β-carotene protects by a mechanism other than conversion to retinol.[119,130] Epidemiology with supporting laboratory and clinical evidence has convinced many researchers that some human cancers can be prevented with carotenoids (Table 3). Epidemiological studies establish correlations but not cause and effect. Heredity, lifestyle, smoking, diet, drug interactions, and exposure to other carcinogens are but a few of the extrinsic factors that make it difficult to draw mechanistic inferences from epidemiological studies. These limitations have prompted intervention trials designed to establish cause and effect. Accordingly, the National Cancer Institute is sponsoring 10 much-needed human intervention trials of the chemopreventive effects of β-carotene.[19]

Unlike retinoids, β-carotene is relatively nontoxic

β-Carotene is not carcinogenic, mutagenic, embryotoxic, or teratogenic in animals. In clinical trials, doses are usually between 15–30 mg β-carotene per day, although some studies have used 180 mg/d. The highest levels of administration yield serum levels of 640 to 1360 μg/dL (normal levels are 20 to 40 μg/dL).[4] Unlike retinol, which is toxic at high levels, carotenoids cause only "hypercarotenodermia," a yellowing of the skin, particularly palms and soles.[112] Occasionally, side-effects are reported anecdotally. In a study involving 61 participants receiving these supplements, 3 subjects reported blurred vision, dizziness, or fatigue. Two of these were receiving oral contraceptives, and the remaining subject was taking thiazides. Symptoms ceased upon discontinuation of the β-carotene supplementation.[112] Interactions of other drugs with high levels of β-carotene remain unknown and should be studied. Chronic administration of enormous doses of β-carotene to dogs (50, 100, and 250 mg/kg/d for 2 years) and mice (100, 250, 500, and 100 mg/kg/d) resulted in histological changes in the livers of treated animals as compared with control animals.[131] Hepatic sinusoidal cells became lined with vacuolated cells with eccentric flattened nuclei; however, these changes were not considered to be toxicologically relevant to human trials at 0.3 to 3.5 mg/kg.

In contrast, retinoid toxicity is well established and widely discussed elsewhere. Canthaxanthin supplementation has at least one side-effect. There have been several reports of a reversible, canthaxanthin-induced retinopathy in which canthaxanthin crystals become visible in retinoscopy.[29]

Cancer chemoprevention trials have focussed mostly on β-carotene

Of all the antioxidant nutrients, β-carotene has garnered the strongest evidence for an anticancer action. Beyond decreasing indices of premalignancy, the question "can β-carotene reverse premalignant states?" has been addressed. Six months' supplementation with β-carotene (180 mg/week) and β-carotene (180 mg/week) plus vitamin A (100,000 IU/week) caused a remission of oral leukoplakias by 14.8% and 27.5% respectively, compared to the placebo-treated group (3%).[132] However, in a study of maintenance therapy after high-dose retinoid treatment, low doses of 13-*cis*-retinoic acid were protective in preventing progression of oral leukoplakias to cancer, whereas β-carotene was ineffective.[133]

Most intervention trials have focused on the protective effects of β-carotene against lung cancer. The Fred Hutchinson Cancer Research Center has begun two chemoprevention pilot studies to study the effects of β-carotene (15–30 mg/d) and retinol 25,000 IU/d).[19] Target populations included asbestos-exposed workers and heavy smokers. Early positive results and lack of side-effects (other than slight yellowing of the skin) confirmed the feasibility of a larger study. A simi-

Table 3. The Effects of Carotenoids in Human Studies

Carotenoid	Disease	Target	Effect	Reference
Bc	EPP	skin	Protective- 84% increased tolerance to sunlight exposure by 3 X	Mathews-Roth 1986 (30)
1. Bc	Oral leukoplakia-tobacco/areca nut	oral mucosa	1. Protective- 14.8% remission of oral leukoplakias	Stich et al. 1984 (65)
2. Cx	same	same	2. no effect	
Bc	cancer	all sites	Protective- individuals in top 2 quintiles have 60% of the risk of developing cancer	Wald et al. 1988 (120)
Crt	cancer	all sites	Strongest association with lung and stomach cancer	Stahelin et al. 1991 (51)
Bc	cancer	all sites	Strong inverse association with lung cancer, relative odds = 4.30	Menkes et al. 1986 (123)
Bc	cancer	lung	Strong inverse association with lung cancer; relative odds = 3.4	Nomura et al. 1985 (124)
Lcp	neoplasia	cervical	Strong inverse relationship relative odds = 3.8	VanEenwyk et al. 1991 (128)
1. Bc	cancer	1. lung	1. Strong inverse association	Comstock et al. 1991 (121)
		2. melanoma, bladder	2. Suggestive inverse association	
		3. rectal	3. Non-protective- suggestive association only	
2. Lcp	cancer	1. pancreas	1. Strong inverse association	
		2. bladder, rectum	2. Weaker inverse association	
1. Bc	Oral leukoplakia-	oral mucosa	1. Not protective	Lippman and Hong 1991 (133)
2. RA	same	same	2. Protective	

β-Carotene (Bc), carotene (Crt), lycopene (Lcp), erythropoietic protoporphyria (EPP), 13-*cis*-retinoic acid (RA), relative odds (a comparison between highest and lowest quintiles).

lar study known as the Carotene and Retinol Efficacy Trial (CARET) is examining the same target population with supplementation of 30 mg β-carotene/d and 25,000 IU retinyl palmitate/d. Current intervention studies in China and Finland are examining the effects of β-carotene among other antioxidants.[19] Thousands of patients with cancer are presumably dosing themselves with β-carotene. Also, patients with ataxia telangiectasia, Bloom's syndrome, or Fanconi's anaemia are clinically receiving supplemental β-carotene. It is time now to shift the focus and determine the relative effectiveness of β-carotene and competing chemoprevention candidates and the appropriate dose regime in each disorder.

β-Carotene decreases incidence of micronuclei and induces remission of premalignancies

Each chromosome must duplicate in the cell nucleus during mitosis. During metaphase, the mitotic spindle (consisting mainly of microtubules) is formed, enabling chromosomes to line up along the equatorial plane. Microtubules attach at the chromosome's centromere at a protein structure called the kinetochore and are responsible for movement toward the spindle poles. If a section of chromosome becomes separated and has no centromere, it will fail to move with the rest of the chromosome. These extranuclear fragments, or micronuclei, are therefore used as markers of chromosome breakage in previous cell divisions and identifiers of the genotoxicity of specific carcinogens.[134-136] It is not widely realized that scission of one of the spindle microtubules gives rise to "kinetochore positive" micronuclei.[137] Protection by β-carotene against micronuclei formation therefore reflects diminished scission of either DNA or microtubules. A study of whether β-carotene decreases kinetochore positive or kinetochore negative micronuclei will give a major focus to the site of action of β-carotene. Exfoliated cells from oral mucosa provide a convenient tissue sample which reveals the extent to which the donor's incidence of micronuclei is increased by oral exposure to carcinogens or decreased by anticancer agents.[135]

Administered to betel nut/tobacco chewers, retinol (100,000 IU/week) plus β-carotene (300,000 IU/week) decreased the frequency of micronuclei to one third the original incidence.[135] β-Carotene (180 mg/week for 6 months) caused a 14.8% remission of oral leukoplakias.[132] Although canthaxanthin protected dramatically in C3H/10T$\frac{1}{2}$ cells,[87,88] canthaxanthin administration did not significantly decrease incidence of micronuclei despite its ability to quench singlet oxygen.[65]

MECHANISMS OF ACTION OF CAROTENOIDS AS ANTITUMOR AGENTS

To understand the mechanism of protection by antioxidants against carcinogenesis, the roles of oxidants in the multistage processes of neoplastic transformation must be better defined. In particular, more rigorous operational definitions of each of the stages of initiation, promotion, progression, invasion, and metastasis may allow more penetrating questions to be asked (Fig. 3). On the basis of current knowledge, carotenoids may protect by several synergistic mechanisms at any one or more of these stages of neoplastic transformation.

Potential target sites for β-carotene and related compounds in tumor initiation

The first stage of carcinogenesis is damage to the normal genetic material of the cell by radiation, chemicals, or viruses. Initiators include mutagens, genotoxic prooxidants, or UV and/or ionizing radiation. Chemical precarcinogens are often metabolized to more electrophilic "ultimate" carcinogens which covalently bind to DNA, RNA, or proteins. If DNA repair systems fail to repair the damage, DNA may be replicated on a damaged template. The resulting point mutations occasionally convert proto-oncogenes to oncogenes, or cause gene duplication, amplification, or translocation. In this model, β-carotene and related compounds can protect against initiation by detoxifying, quenching, or trapping the active intermediates which react with DNA. Protection by β-carotene against viruses has not been reported, but improved membrane integrity and increased immunocompetence from β-carotene supplementation suggests a mechanism by which carotenoids could inhibit the appearance of virally induced cancers.

Potential targets for β-carotene and related compounds in tumor promotion

The second stage of carcinogenesis, promotion, concerns the growth and proliferation of initiated abnormal cells. Operationally, promotion is ill defined, and the mechanisms are poorly understood. Promotion in humans is often reversible (until development of an autonomous tumor cell clone) and there is a long latent period, up to 40 years (i.e., in prostatic cancers).[138] The agents with the greatest potential as anticarcinogens are those which exert their effects at the promotional stage. Intervention studies with β-carotene have focused on regression of premalignant

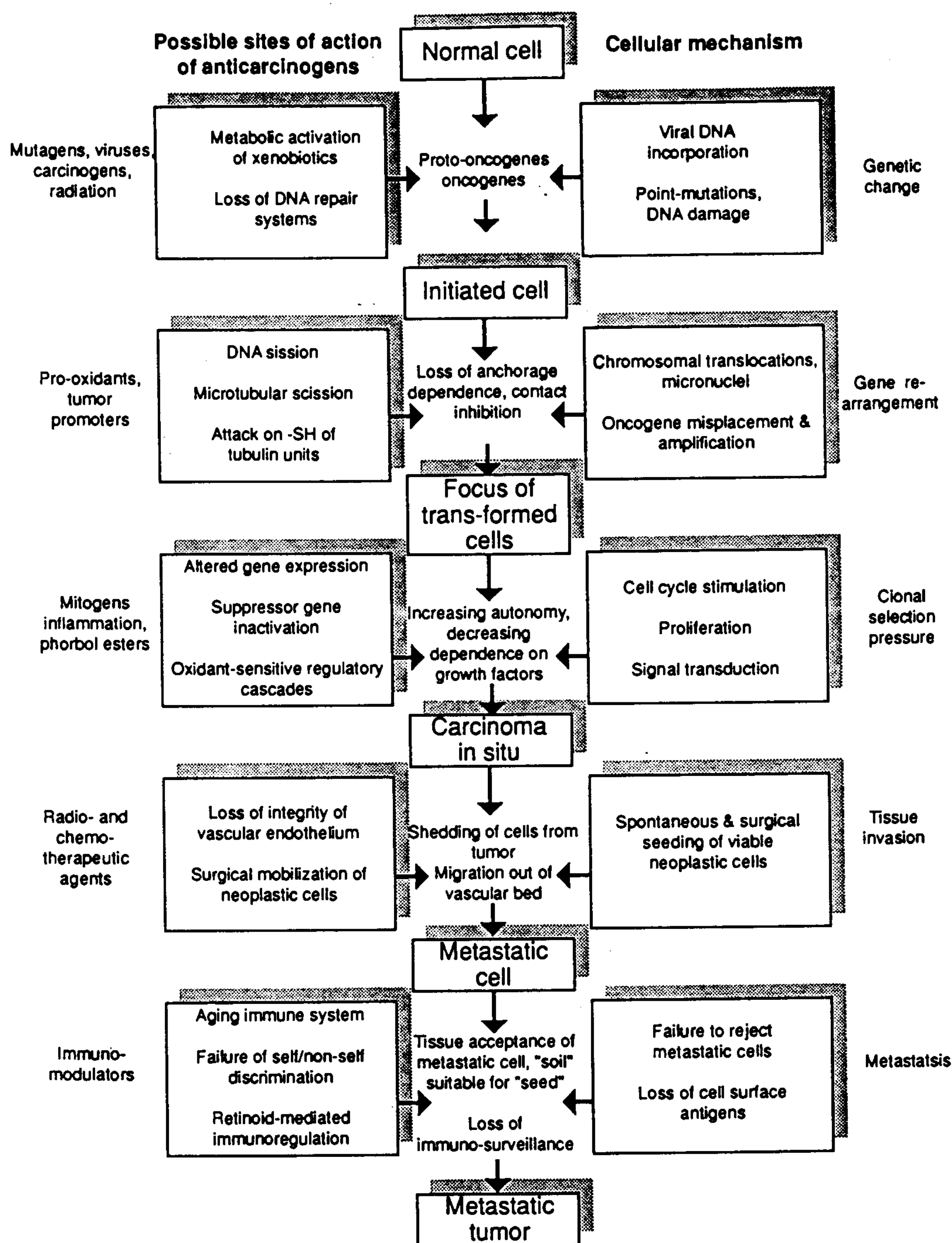


Fig. 3. Processes in carcinogenesis and anticarcinogenesis.

lesions, or slowing growth of the neoplasm such that the patient's life is extended.[138]

Mutations to mitochondria may self-propagate to increase production of oxygen-derived active species which contribute to neoplastic transformation.[14] To the extent that carotenoids block propagation of such mitochondrial mutations, they could preempt carcinogenesis. Carotenoids readily incorporate into intracellular membranes, including mitochondria. Hepatic mitochondrial membranes of chicks fed 5 g canthaxanthin/kg diet contain 0.4 ng canthaxanthin/mg phospholipid phosphorus.[139] Carotenoids (like β-carotene, canthaxanthin, zeaxanthin, astaxanthin, or lutein) with two polar groups distally situated on the molecule can span phospholipid membranes of bacteria and act like rivets to mechanically stabilize the membrane.[140] The largest incorporations are found when

1. the length of the carotenoid molecule corresponds to the width of the hydrophobic core of the bilayer;
2. the phospholipids are mixed (as in egg lecithin or from *Halobacterium*), or;
3. the phospholipids and carotenoids are from similar sources (i.e., archaebacterial type).

In mammals, carotenoids may function similarly to increase membrane stabilization. Orientation of the molecule with the polar head groups on either side of the membrane may help protect against neoplastic transformation by locally deactivating the genotoxins responsible for mitochondrial or nuclear mutations, or scavenging the released active oxygen species at the targets. That the polar heads often contain oxo groups and that epoxide formation is exclusive to the ring formations may give additional clues to possible mechanisms of protection.

Tumor promoters include saccharin (bladder), 12-*O*-tetradecanoylphorbol-13-acetate (mouse skin), and phenobarbitol and DDT (liver).[141] Many promoters, including granulocytes activated by inflammation (or phorbol esters), produce active oxygen species. The ability of carotenoids to scavenge such species provide a ready explanation for any antipromotional actions. Alternatively, the mechanism of protection could be preservation of the suppressor genes which maintain contact inhibition. Cytotoxic chemicals often weaken transfer of normal mitosis-restraining factors by destroying patency of cell gap junctions. Retinoids (and perhaps carotenoids) help maintain proper functioning of the gap junctions.[142]

Other actions of retinoids may also involve carotenoids. Ornithine decarboxylase (ODC) is an enzyme necessary in the production of putricine, a precursor of the polyamine growth factors, spermidine and spermine. ODC activity is a phenotypic change essential in skin tumor promotion. Application of TPA to mouse epidermis increased ODC activity by over 200-fold.[143] Although retinoic acid does not inhibit TPA binding to protein kinase-C (a TPA receptor),[144] it does modulate protein kinase-C activity, protecting against TPA-induced ODC gene expression.[145] Such actions of retinoic acid suggest that carotenoids may indirectly (or conceivably directly) exert an effect in the protein kinase-C cascade. Candidate mechanisms in tumor promotion are as follows:

1. general mitogenicity (with DNA becoming more exposed during mitosis), sometimes resulting from nonspecific tissue injury;
2. inactivation of suppressor genes;
3. DNA or microtubular scission (reflected in the formation of micronuclei) allowing relocation of oncogenes distal to frequently activated promoter regions;
4. mitochondrial mutations;
5. activation of the protein kinase-C cascade system.

Potential targets for β-carotene and related compounds in tumor progression

The final stage in tumorigenesis is a sequence of steps known as progression, where the cells become more autonomous, dividing in a less controlled manner. At this stage cells develop the ability to invade healthy tissue and develop metastatic foci at various sites. The mechanism of this stage is poorly understood, and it remains to be determined what, if any, protective effects carotenoids could exert. A promising avenue for exploration is revealed by the observation that prooxidants (including anticancer drugs) accelerate migration of neoplastic cells from the circulation to the interstitial fluid.[146] They do so by stripping off the endothelial basement membrane and encouraging adhesion of the neoplastic cells. Intravenous bleomycin, followed by injection of syngeneic fibrosarcoma cells in mice, increased seeding of pleural tumors threefold. To the extent that progression includes oxidative processes, antioxidants may retard invasion. β-Carotene and related compounds could protect against metastatic invasion by increasing immunocompetence or by maintaining integrity of endothelial and other barriers. In addition to potential antimetastatic effects, patients receiving chemotherapy might benefit both from the known photoprotective effects of β-carotene and the purported increase in immunocompetence.[147]

Antitumor activity may depend on carotenoid structures

Carotenoid structure and the relation to its functions as an anticancer agent need defining. Possibly, the carotenoid metabolites are exerting a protective effect. Water-soluble carotenoids such as crocetin no doubt have actions different than the lipophilic carotenoids. Colorless phytoene and phytofluene may have actions separate from the colored carotenoids. Any actions by canthaxanthin, phytoene, and crocetin (no vitamin A activity) certainly would indicate mechanisms other than retinoid function. Structural differences of carotenoids such as chain length, number of double bonds, number of alicyclic rings, presence of oxygenated functional groups, number of methyl groups, and stereochemistry all need to be addressed in terms of antitumor activity.

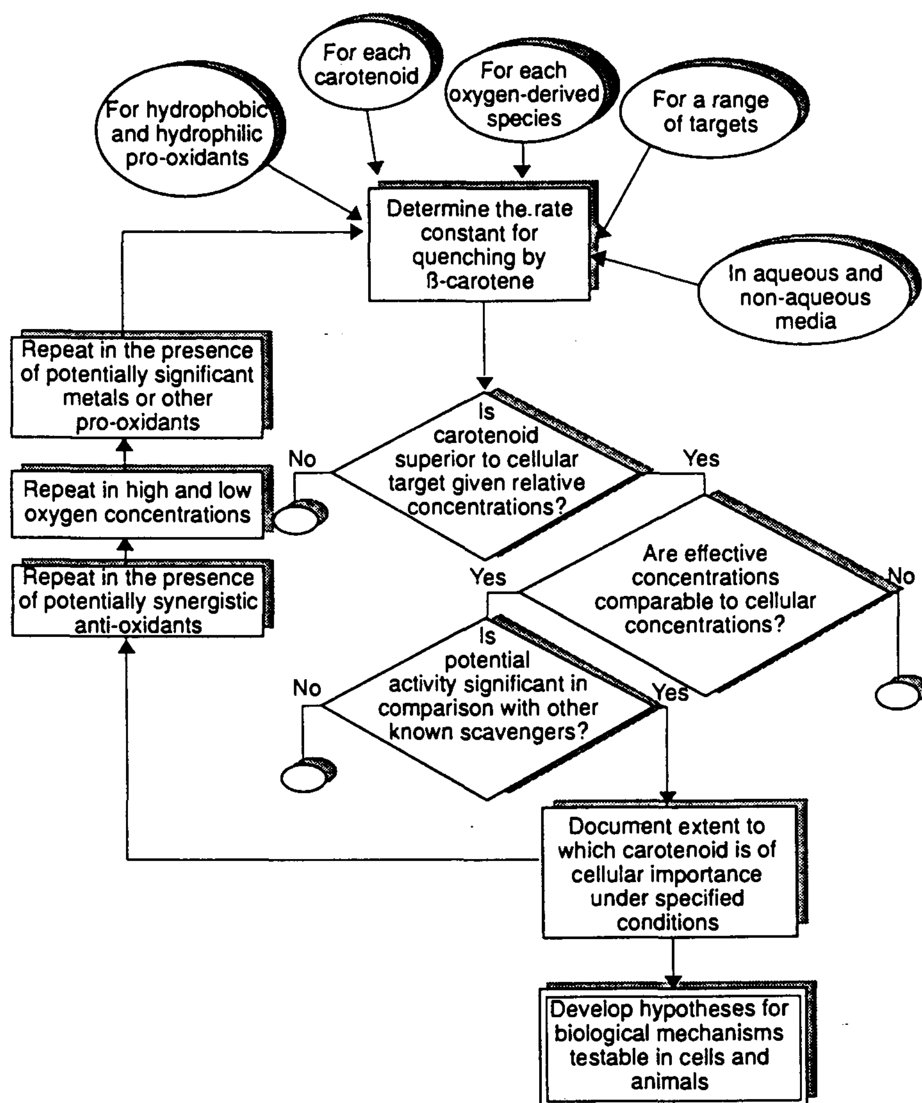


Fig. 4. Chemical approaches in cell-free systems. The flowchart indicates an in vitro approach to future studies with carotenoids.

Important questions await exploration

Much of the biochemistry of carotene has been deduced from studies in organic solvents. Studies of β-carotene in lipid solution, liposomes, heterogenous media or micelles, and aqueous dispersion are needed for comparison (Fig. 4). Liposomes with carotenoids incorporated into the membranes may be used as carriers in biological research and medicine.[148,149] These may target β-carotene specifically to the most important organs and intracellular locations.

One of the most urgent issues is comparison of the protective actions of β-carotene with other carotenoids over a range of targets, oxidant stresses, or predisposing conditions. Five comparisons of β-carotene with other carotenoids are available:

1. Lycopene—better singlet quencher (evidence of protection against cervical cancer)
2. Crocin—relatively ineffective superoxide scavenger (chemopreventive potency unknown)
3. Astaxanthin—better hydroxyl radical scavenger (chemopreventive potency unknown)
4. Canthaxanthin—better hydroxyl radical scavenger (protects against methylcholanthrene [MCA]-induced neoplastic transformation in cells, not against tobacco/areca nut-induced oral leukoplakias)
5. Phytoene—more effective against UVB-induced skin tumors in mice (scavenging characteristics unknown).

The mechanism for coupled enzymic cooxidations of carotenoids and unsaturated fatty acids is an im-

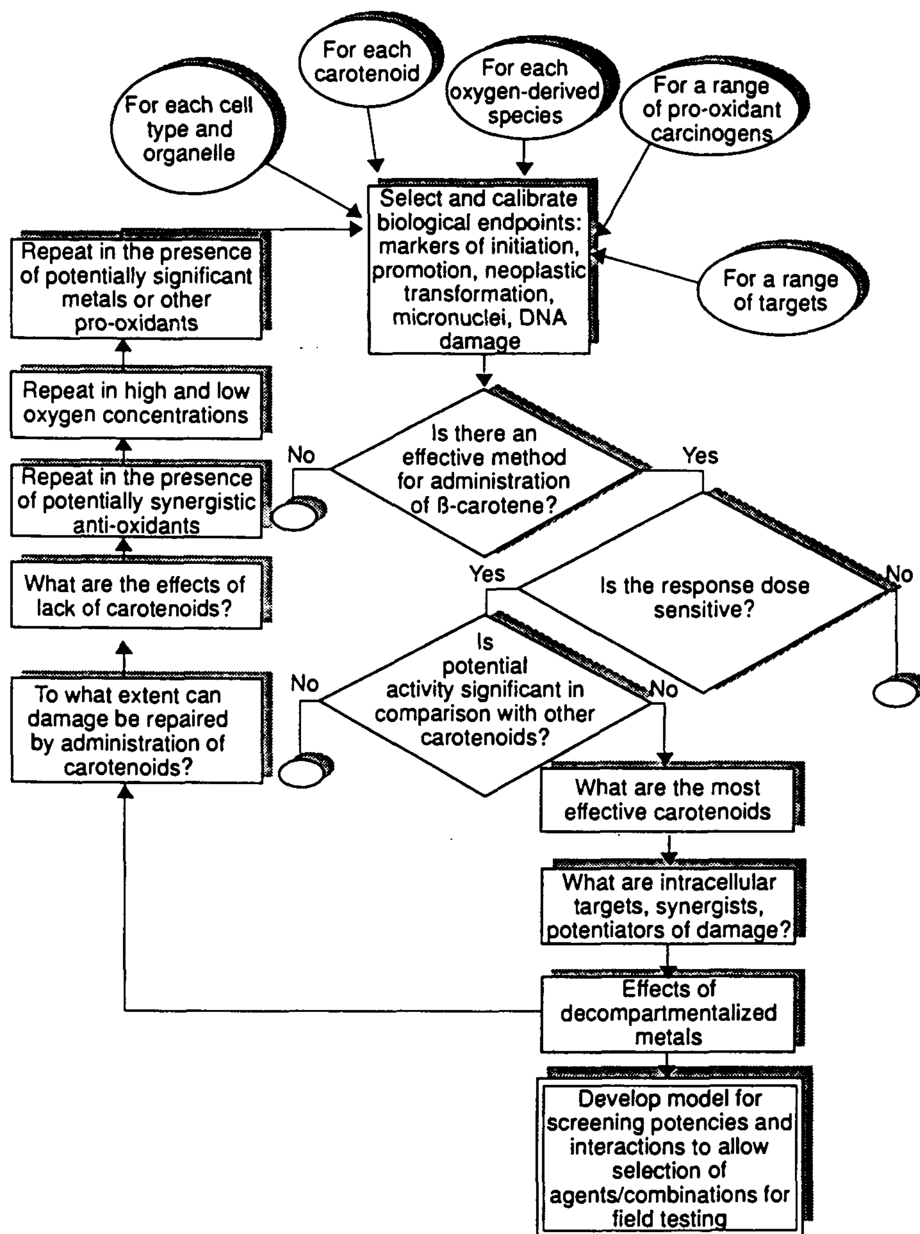


Fig. 5. Cellular approaches. The flowchart indicates a cellular/biological approach to future studies with carotenoids.

portant area awaiting systematic investigation. Another is interactions of carotenoids with antioxidant nutrients such as alpha-tocopherol, ascorbate, and antioxidant enzymes, and of protection by combinations of prooxidants against specific oxidant stressors, including peroxide-forming molecules such as cholesterol. Since β-carotene is observed to be more protective at low partial pressures of oxygen, characterization of the products formed at varying oxygen conditions should be given a high priority.

The most cost-effective answers will come from detailed studies with cultured cells and systems which simulate clinical responses in vivo (Fig. 5). Researchers must examine the impact of carotenoids and related substances in immunoenhancement and on protection of cultured cells against carcinogens, free radicals, and mutagens. The distribution of β-carotene among the cell compartments remains virtually unexplored, as does the significance of the local distribution in pro- and antioxidants within cells and cell organelles. Even then, the question of whether carotenoids in membranes serve antioxidant functions will not be answered by experiments in homogenous media like organic solvents or even plasma.

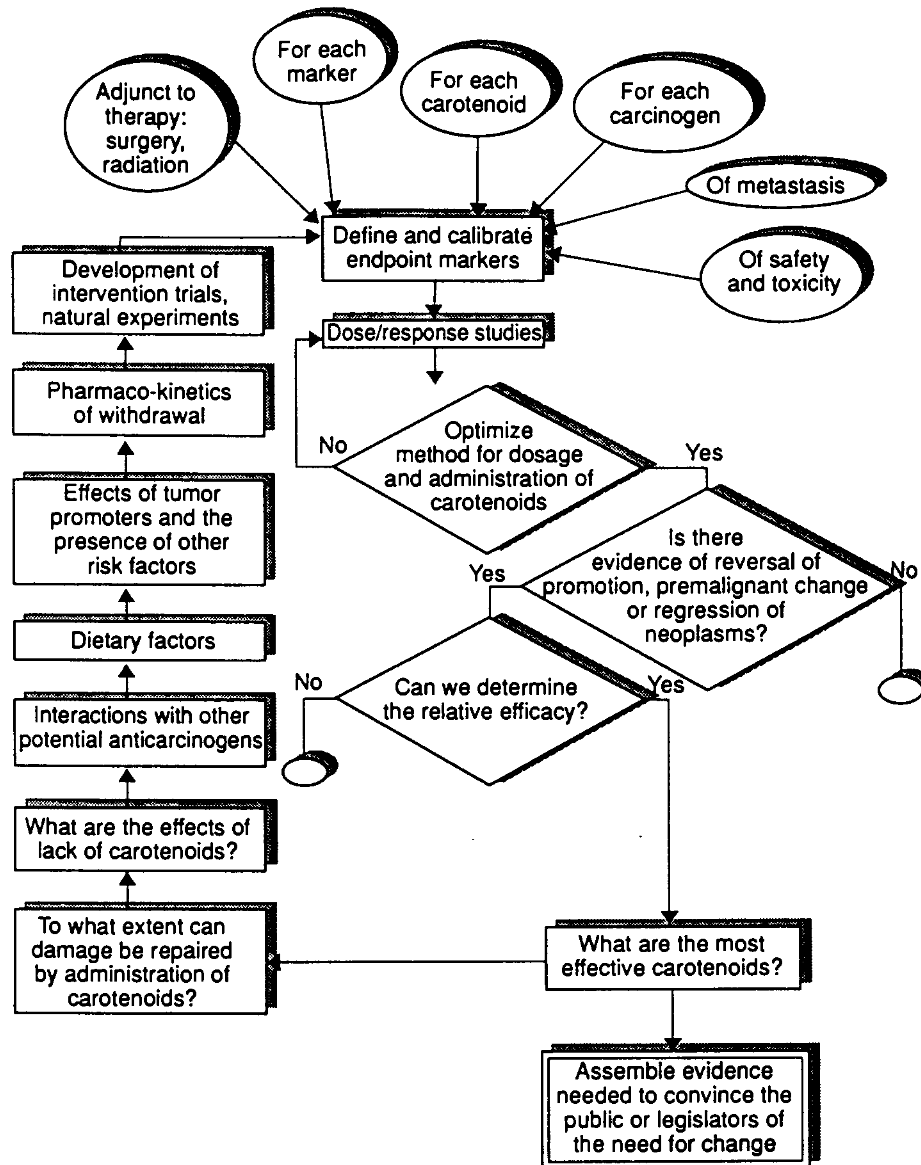


Fig. 6. Epidemiological approaches. The flowchart indicates an epidemiological approach to future studies with carotenoids.

The question of whether carotenoids are significant antioxidants in plasma cannot yet be confidently answered. On the one hand, the total antioxidant capacity of serum can be almost completely predicted from the content of urate, proteins, ascorbate, and tocopherol.[76] Carotenoid concentrations are dwarfed by those of better established and more abundant antioxidants, so it was considered unlikely that they contribute significantly in vivo. On the other hand, oxidation of the components of lipoproteins is delayed until carotenoids are fully consumed.[50] The studies relegating β-carotene to a minor role were in plasma and not replicated at intracellular PO_2, where protection is optimal. Moreover, the ability of carotenoids to act synergistically with other antioxidants may allow them to exert effects out of all proportion to their concentrations. Such a synergistic role for carotenoids can be tested only by reconstitution studies. In such an investigation, quantitative contributions of total radical-trapping activity of plasma will be compared with those of its components separately and in combination at several concentrations of oxygen, and toward a range of targets.

It is distressing that after so much research, the quantitative protective contribution of normal cellular carotenoids remains in question. Clearly, much remains to be done. Future studies should investigate protection by carotenoids against a wider range of po-

tentially free-radical mediated disorders including age-related macular degeneration, Parkinsonism, Alzheimer's disease, and cancer in individuals with chromosomal instability. Meanwhile, carotenoids are being adopted as cancer chemopreventive agents with and without official sanction by nutritional authorities. Authoritative and practical recommendations are limited by a lack of knowledge regarding dose-response relationships or interactions among protective agents. Studies of mechanisms and interactions are difficult to control adequately in intact organisms and may be best suited to studies of cultured cells and cell-free systems.

The question as to what stages of carcinogenesis are susceptible to carotenoids needs resolution. Carotenes likely protect at several stages of carcinogenesis. Protection may arise from carotenoid metabolites and not the precursor molecule itself. The relationship between carotenoid structure and function needs defining. The gene regulatory actions of retinoic acid, and its superiority to β-carotene as an anticancer agent in at least one comparison, suggests a role for gene regulation by carotenoid metabolites in some circumstances.[133] Research to determine when, if ever, retinoids mediate antineoplastic actions of carotenoids deserves a high priority.

The effects of dietary antioxidants on immunocompetence opens up an important new area of research in the chemoprevention of cancer and aging. Future studies will depend on the development of new and more effective biochemical markers and endpoints (Fig. 6). Future findings in these areas will reveal the mechanisms of neoplastic transformation in such a way as to optimize strategies for intervention.

CONCLUSION

In epidemiological studies, low plasma levels of β-carotene are correlated with increased incidence of cancer. In intervention trials, β-carotene supplementation decreases the incidence of preneoplastic lesions in at-risk human volunteers. Supplementation with β-carotene protects animals, cells, and isolated cellular constituents against chemical, transplanted, and UVB-induced damage.

Based on such evidence, one must conclude that β-carotene is an antioxidant anticarcinogen of great potential importance. Undoubtedly, elevated concentrations of carotenoids protect against oxygen-mediated cytotoxicity and genotoxicity by more than one mechanism. The relative contributions of competing carotenoids and candidate mechanisms over a range of circumstances are slowly being established. Carotenoids with oxygenated functional groups (canthaxanthin, retinoids) seem to protect at the earlier stages of carcinogenesis (initiation and promotion), while β-carotene is a stronger protective agent against progression of cancers. Such differences may hold a key to mechanisms of action of carotenoids and related compounds. Understanding these mechanisms will help develop effective regimens in cancer chemoprevention. Much remains to be done before chemopreventive regimens can be recommended with any confidence of diminishing the incidence of cancer worldwide. However, the potential benefits lend urgency to the need for better questions and better answers.

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ABBREVIATIONS

β-car˙—(β-carotene) alkyl radical
β-carOO˙—(β-carotene) peroxyl radical
^{3}β-carotene—triplet carotenoid
$^1O_2$—singlet oxygen
$^3O_2$—triplet dioxygen
˙OH—hydroxyl radical
H_2O_2—hydrogen peroxide
GSH—glutathione
HDL—high density lipoprotein
LDL—low density lipoprotein
VLDL—very low density lipoprotein
L˙—alkyl radical
LO˙—alkoxyl radical
LOO˙—peroxyl radical
MNNG—*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine
MPTP—*N*-methyl-4 phenyl-1,2,3,6-tetrahydropyridine
ODC—ornithine decarboxylase
PMN—polymorphonuclear leukocyte
TBARS—thiobarbituric acid reactive substance
TPA—12-*O*-tetradecanoylphorbol-13-acetate
TRAP—total (peroxyl) radical-trapping antioxidant activity
UV—ultraviolet light

CHAPTER 3

Putative anticarcinogenic actions of carotenoids: nutritional implications[1]

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This review provides an update on nutritional aspects of carotenoids (as distinct from retinoids), with specific relevance to anticarcinogenesis. Critical gaps remain in our knowledge of the nutritional functions of carotenoids despite an overwhelming accumulation of research data in areas tangential to human nutrition. In addition to their roles as precursors of retinol and retinoids, carotenoids have distinct functions of their own in animals and humans. In vitro they are antioxidants with a broad range of potencies. In vivo, they protect porphyrics against sunlight. The evidence for anticarcinogenic actions of β-carotene in certain specified test situations is persuasive. Nevertheless, despite a large number of studies demonstrating protection by carotenoids, the characteristics that render a given carotenoid effective and the relative efficacy of the individual carotenoids are not known. Moreover, dose–response and pharmacokinetic relationships remain virtually unexplored. Research to uncover mechanisms of protection by carotenoids is, for technical reasons, painfully slow. Epidemiological studies reveal associations but not cause and effect. To explore cause and effect, intervention trials are underway, hampered by the paucity of data regarding optimal choice of carotenoid, dosage, and regimen. The in vitro test systems that would provide this information are not available because the molecular sites relevant to the chemopreventive action of carotenoids are obscure. Each of these problems has a solution, but not a simple one. Until these are resolved, blanket recommendations regarding supplementation will remain problematic. To this point, health authorities have not recommended dietary supplementation with carotenoids. Instead, they recommend increased consumption of yellow and dark green carotenoid-rich vegetables. In the future, an individual at risk for a particular carcinogenic process may be recommended a supplement of the most appropriate anticarcinogen, specific to their individual endowment of genetic and environmental risk factors. This review emphasizes not only what is known but also what is not known. Consequently we identify priorities for research that, if undertaken, will allow such recommendations to be made or discounted with more confidence.

Key words: β-carotene, carotenoids, free radicals, antioxidants, carcinogenesis, chemoprevention.

DAVISON, A., ROUSSEAU, E., et DUNN, B. 1993. Putative anticarcinogenic actions of carotenoids: nutritional implications. Can. J. Physiol. Pharmacol. 71 : 732–745.

Cet article fait une mise à jour des aspects nutritionnels des caroténoïdes (par opposition aux rétinoïdes), en particulier en ce qui a trait à l'anticarcinogenèse. Il y a encore de graves lacunes dans nos connaissances sur les fonctions nutritionnelles des caroténoïdes, malgré une impressionnante accumulation de données dans des domaines de recherche connexes à la nutrition humaine. En plus de leurs rôles comme précurseurs du rétinol et des rétinoïdes, les caroténoïdes ont des fonctions distinctes propres chez les animaux et les humains. In vitro ce sont des antioxydants ayant une vaste gamme d'activités. In vivo ils protègent les sujets atteints de porphyrie contre la lumière du soleil. La mise en évidence d'actions anticarcinogènes du β-carotène dans certaines situations tests est concluante. Néanmoins, malgré une multitude d'études démontrant l'effet protecteur des caroténoïdes, les caractéristiques qui font l'efficacité d'un caroténoïde donné et l'efficacité relative de chacun des caroténoïdes ne sont pas connues. De plus, les relations pharmacocinétiques et les relations dose–effet demeurent pratiquement inexplorées. La recherche sur les mécanismes de protection des caroténoïdes est, pour des raisons techniques, d'une extrême lenteur. Les études épidémiologiques révèlent des associations, mais non la cause ni l'effet. Les tentatives d'exploration dans cette voie sont ralenties par la rareté des données sur le choix optimal des caroténoïdes, des dosages et posologie. Les systèmes tests in vitro qui fourniraient cette information ne sont pas disponibles parce que les sites moléculaires ayant rapport à l'action chimiopréventive des caroténoïdes sont inconnus. Chacun de ces problèmes a une solution, laquelle n'est pas simple. Jusqu'à ce que ces problèmes soient résolus, les recommandations générales sur la supplémentation demeureront problématiques. Jusqu'ici, les autorités médicales n'ont pas recommendé de supplémentation alimentaire de caroténoïdes. Elles recommandent plutôt d'augmenter la consommation de légumes riches en caroténoïdes, vert foncé et jaunes. À l'avenir, un individu prédisposé à un processus carcinogène particulier pourrait se voir recommander un supplément de l'anticarcinogène le plus adéquat, compte tenu de ses facteurs de risque environnementaux et génétiques. Cet article porte sur ce qui est connu et sur ce qui ne l'est pas encore. Conséquemment nous identifions les priorités de la recherche qui, si elle se réalise, permettront d'émettre ou de rejeter de telles recommandations avec plus de certitude.

Mots clés : β-carotène, caroténoïdes, radicaux libres, antioxydants, carcinogenèse, chimioprévention.

[Traduit par la rédaction]

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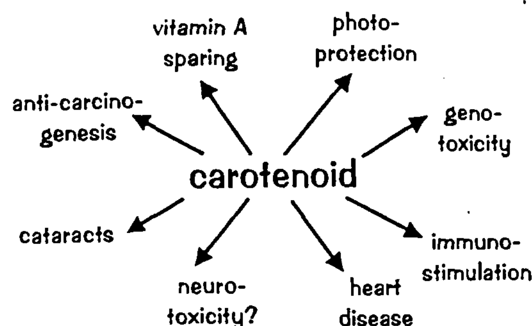

FIG. 1. Biological protection by carotenoids. The figure enumerates areas in which claims have been made for beneficial actions of carotenoids. References to these claims are given in the text or in a companion article (Rousseau et al. 1992).

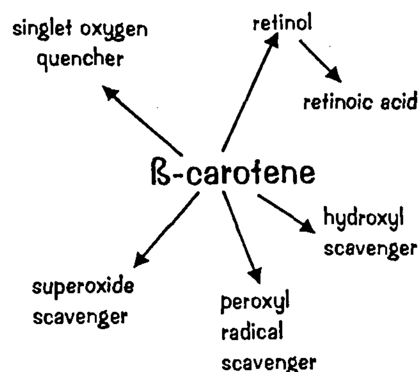

FIG. 2. Chemical actions of carotenoids. In addition to relieving the symptoms of vitamin A deficiency, most of the actions of carotenoids relate to the scavenging of oxygen-derived active species.

Introduction

Dietary supplementation with carotenoids is increasing, despite critical gaps in knowledge

The accumulating reports regarding beneficial biological actions of β-carotene are in stark contrast to the lack of detailed knowledge of mechanisms, optimal agent, or regimen. Figure 1 summarizes the range of situations in which carotenoids have been reported to confer a benefit. It should not be surprising in the light of these glittering promises that the use of carotenoid dietary supplements is accelerating long before the promises are confirmed. This is reflected as increased availability in supermarkets and health-food stores, presumably as a result of reportage in the popular press. Such overinterpretation of test results in vitro underscores important gaps in our knowledge of the specific protective roles of carotenoids in intact humans. The most effective carotenoids remain to be identified. Even if we knew which they were, we are ignorant of their pharmacokinetics, or the optimal dose–response regimes for their actions. Carotenoids have a variety of chemical actions (Fig. 2), but it is not known which of these, if any, are implicated in their biological actions.

Nutrition texts fail to provide an up-to-date perspective on nutritional aspects of carotenoids. This review seeks to provide an update and status report on nutritional aspects of carotenoids in relation to their antioxidant and anticarcinogenic actions. The current manuscript falls conveniently into three sections. In the first, we survey nutritional considerations regarding carotenoids, as distinct from their role as retinol precursors. In the second, we examine the evidence supporting an antioxidant role for carotenoids in vivo. In the final section, we examine the status of the anticancer actions of carotenoids. We outline the areas in which information is lacking and consider what nutritional recommendations can be made based on existing evidence.

Diet and cancer

Many life-style factors have positive and negative impacts on the incidence of cancer. Initial epidemiological studies revealed crude geographical distributions of human cancers explicable in terms of nutrition. For example, in Japan and China there are higher than normal incidences of stomach and esophageal cancers. In Western countries there is a high incidence of breast, prostate, ovarian, endometrial, pancreatic, and colonic cancers (Weisburger 1991). In mountainous areas of South America, stomach cancer is more prevalent than in populations living at sea level (Correa 1982). In many developing countries there is a high incidence of oral cancers (International Agency for Research on Cancer 1985). Within countries there are regional differences, and within regions there are "at-risk" populations. Studies of these groups have given only preliminary insights into the etiology of cancer. The risk factors must still be teased out with difficulty from the confounding variables: regional disparities in genetic endowment, environmental conditions, and cultural dictates of diet and life-style.

Nutritional risk factors are inferred from associations of cancer with diet. Thus, the incidence of stomach cancer increases with intake of salted, pickled, or smoked foods (Sugimura 1986). Colorectal cancer accompanies the low-fibre, high-fat diets often seen in the Western world (Weisburger and Wynder 1987). Cancer of the breast (postmenopausal), ovary, and prostate accompany high-fat diets (Weisburger 1991). Cervical cancer is linked to papilloma or herpes viruses (Munoz et al. 1989; Klein 1989) and smoking (Brock et al. 1989; Kvale et al. 1983). Cancer of the urinary tract may be linked to a high-fat high-protein diet (Weisburger 1991). Liver cancer is common in many parts of Southeast Asia, China, and Africa, where hepatitis and mycotoxins such as aflatoxins are endemic (Harris and Sun 1987). The chewing of tobacco and betel nuts results in cancers of the oral cavity, common in many developing countries (International Agency for Cancer Research 1985; Stich et al. 1984). Heavy alcohol consumption decreases cellular β-carotene levels (Stich et al. 1986), and this may be the mechanism by which alcohol excess contributes to lung cancer.

Certain dietary agents, including carotenoid-containing foods, have anticancer effects. Although high-fat diets are linked with many kinds of cancers, olive oil (rich in oleic acid and poor in polyunsaturated fatty acids) has no effect, while eicosapentenoic acid (an $\omega-3$ fatty acid found in fish oils) protects. Education, availability of a greater variety of foods, and changing life-styles have reversed many of the cancer trends of the past.

Anticarcinogens in yellow and green vegetables and fruits may include carotenoids

In the epidemiological search for anticarcinogens, the most consistent dietary finding is protection by carotenoid-rich

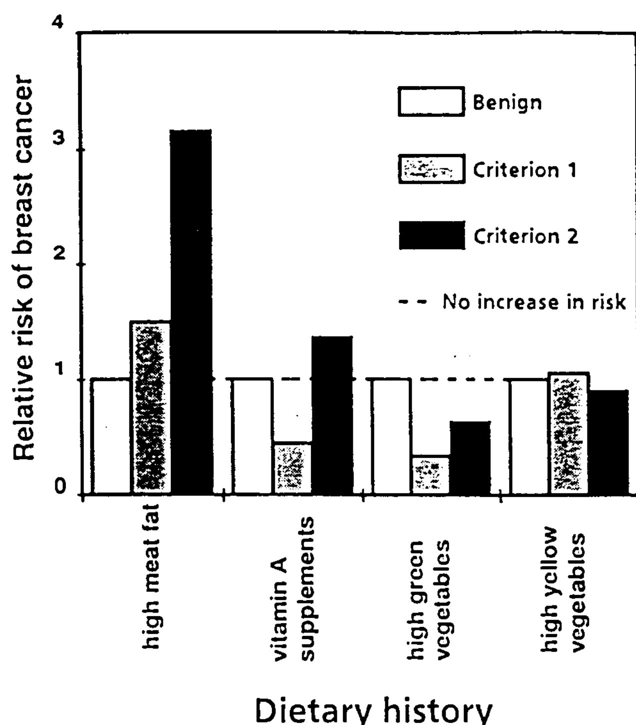

FIG. 3. Effects of dietary history on relative risk of breast cancer. Relative risk of breast cancer on the basis of two separate histopathological criteria was compared for patients with high intakes of green or yellow vegetables or meat fat, in a case-control study. Green and yellow vegetables protect against one index of breast cancer, while high animal fat diets predispose to another. Data are replotted from Hislop et al. (1990).

yellow–green vegetables and fruits against cancers of various sites, including the lung, stomach, pancreas, liver, colon, breast, and prostate (Byers and Perry 1992; Peto et al. 1981; Wolf 1982; Ziegler 1991). High dietary fats predispose to breast cancers, and a diet rich in vegetables may protect. Data of Hislop and co-workers (1990) suggest that these two factors in the etiology of breast cancer may relate to two distinct sets of pathological criteria. They administered a dietary survey to patients undergoing histological examination for suspected breast cancer. One set of histological criteria (Fig. 3, criterion 1) was not associated with a high-fat intake, but with below-median intakes of vegetables. The other set of histological criteria (Fig. 3, criterion 2) identified patients at risk because of a high-fat intake. These patients were not protected by a diet rich in vegetables (Hislop et al. 1990). Such information confirms and reinforces the mortality data, but it also reveals a complexity that will require detailed studies focusing on the underlying variables.

A more direct method than dietary surveys is to take a blood sample and test for specific nutrients. The Finnish Mobile Clinic Health Examination Survey found serum β-carotene levels in male and female controls to be 14 and 5.5% higher than in the corresponding cancer case groups (Knekt et al. 1991). As with dietary questionnaire studies, blood nutrient level studies have limitations. The serum samples are often frozen for an unspecified amount of time before analysis, and it is known that β-carotene stability varies with the method of storage. Temperature, oxygen levels, light, and the presence of acids must all be carefully controlled to minimize degradation of carotenoids. Also, unlike tissue stores of nutrients, serum levels are more sensitive to acute changes in diet, not representative of the subject's long-term habits. This has obvious disadvantages, but also advantages. For example, results of dietary questionnaires may concur better with the serum nutrient analysis results. Like dietary studies (Byers 1991; Dartigues et al. 1990; Negri et al. 1991), many serum nutrient analysis studies (Menkes et al. 1986; Nomura et al. 1985; Wald et al. 1988; Stahelin et al. 1991) find a strong association between low levels of β-carotene and an increased risk of lung cancer.

In the search to identify naturally occurring anticarcinogens, vegetables and fruits have seemed the most promising places to look. However, it is difficult to know where to start the search. Vegetables are rich sources of minerals and other essential nutrients, including ascorbate, folate, and carotenoids. Fruits are generally not rich in essential nutrients, except vitamin C and potassium. Among the essential nutrients in fruits, the most promising chemopreventive agent is ascorbate. However, we must look beyond the classical nutrients. High intakes of fruits provide fibre and displace dietary protein and fat. Moreover, fruits and vegetables are rich in the redox-active compounds: flavonoids, ellagic acid, indoles. Flavonoids represent a neglected topic in human nutrition and it would be premature to exclude them from consideration as anticarcinogens. Only a start has been made in attempting to reconstitute the anticarcinogenic potency of foods from a mixture of their components. A few individual food components have been tested, and of these, carotenoids have generated the most interest.

Section 1. Nature and occurrence of carotenoids: relationships with retinol and retinoids

Occurrence of carotenoids in plants

Over 600 distinct carotenoids occur in nature, with global production estimated at 10^8 tons per year (Isler 1971). They are the most ubiquitous of nature's pigments, and arguably the most important. A variety of carotenoids are readily isolated from deep orange, rich yellow, and dark green leafy vegetables and fruits. The most abundant carotenoid is fucoxanthin (marine algae), with lutein, violaxanthin, and neoxanthin (green leaves) being the next three major carotenoids synthesized. Plants use light as their primary energy source, and photochemical side reactions threaten the delicate machinery of life. In plants, carotenoids function as antennae in light-harvesting reactions, and as quenchers of photosensitized molecules such as chlorophyll triplet states and singlet-state oxygen. Of the hundreds of different carotenoids, about 50 are precursors of retinoids, and of these about 10 are considered nutritionally significant in humans (based on their ability to provide retinol). It is not surprising that only a fraction are vitamin A precursors. Carotenoids evolved not to meet the vitamin A needs of humans but to serve plants. The major water-soluble carotenoid is crocetin, found in high concentrations (as the ester crocin) in saffron (*Crocus sativus*) petals.

Carotenoids are formed in plants from acetyl coenzyme A

Carotenoids are derivatives of a series of hydrocarbons having the general empirical formula $C_{40}H_{56}$. In plants and animals acetyl-CoA is the "two-carbon" precursor of mevalonic acid (C_6), which undergoes decarboxylation to isopentyl pyrophosphate (C_5). Animals condense three of these isoprene units to produce farnesyl pyrophosphate (C_{15}), which then dimerizes to squalene (C_{30}) and cyclizes to steroids, including cholesterol. Although they can trimerize isopentyl

pyrophosphate, animals are incapable of de novo synthesis of carotenoids, since they cannot produce tetramers. Plants (and some fungi) produce a tetramer, geranylgeranyl pyrophosphate (a 20-carbon fragment), two of which condense tail to tail to form the carotenoid phytoene (C_{40}). Photosynthetic bacteria synthesize acyclic (purple bacteria) and cyclic (green bacteria) carotenoids, while some nonphotosynthetic bacteria produce C_{30} (staphylococci) and C_{45} and C_{50} carotenoids (flavobacteria) (Goodwin 1992). In plants, the colourless phytoene is dehydrogenated to lycopene and further cyclized to become the pigmented carotenoid β-carotene. Successive dehydrogenations and oxidations produce the hundreds of other carotenoids found in nature.

In mammals carotenoids accumulate in membranes or plasma lipids

Carotenoids make their way through the food chain and are distributed throughout the animal kingdom. They are not only found in tissues, membranes, and blood but are also responsible for the bright colours of birds, marine animals, and insects. Carotenoids give rise to a beautiful spectrum of colours from yellow to red, and when complexed with proteins, are responsible for colours ranging from greens to blues. In mammals carotenoids occur primarily in the lipid fractions of plasma and tissues, much esterified (as carboxylate esters) or otherwise complexed with protein or lipid. Molecular adducts of carotenoids with lipids and peptides are solubilized by bile salts, and hydrolyzed by esterases, lipases, and proteases in the digestive system of animals. In chicks fed canthaxanthin beadlets (5 g/kg diet for 5 weeks) the hepatic mitochondria acquired 0.4 ng canthaxanthin/mg phospholipid phosphorus (Mayne and Parker 1987). The lipids of plasma and mitochondrial membranes are rich in unsaturated fatty acids, which provide attractive targets for oxygen-derived active species, and the protective functions of carotenoids may be particularly important at these target sites.

In mammals, adipose tissue is the largest reservoir of β-carotene

β-Carotene forms about 15–30% of total serum carotenoids in humans (Bendich and Olson 1989) and concentrates mainly in the liver, adrenal gland, kidney, ovary, and fat (Stahl et al. 1992). The remaining 70–85% are other HPLC-detectable carotenoids, including α-carotene, lycopene, lutein, and cryptoxanthin (Parker 1989). Lycopene, found in tomatoes, is prominent in the plasma of ketchup aficionados, and accumulates to highest concentrations in the testes (Stahl et al. 1992). To the extent that β-carotene functions as a cellular antioxidant, tissue levels of carotenoids are of interest (Rosin 1990). Adipose tissue is the largest reservoir in humans, with 80–85% of total carotenoids; the liver stores 8–12%, muscles 2–3%, and serum ~1%, with other tissues containing the remainder (Olson 1983). However, neither adipose tissue nor liver has the highest concentrations. Tissue analyses show that the corpus luteum contains ~60 μg/g, while the adrenal gland contains ~20 μg/g. For comparison, adipose tissue and liver contain ~10 μg/g of tissue (Bendich and Olson 1989). The *cis* or *trans* isomers of carotenoids show preferential target tissues presumably reflecting selective processes in the pathways of bioaccumulation (Stahl et al. 1992).

The term vitamin A is used to include carotenoid precursors of retinol

The term vitamin A is sometimes used as synonymous with retinol, e.g., to exclude β-carotene. This, however, leaves us without a generic term. This may be of little concern to biochemists, but for each essential nutrient nutritionists need a generic term that includes all compounds that can relieve symptoms of a deficiency. We agree therefore with those who use "vitamin A" to include retinol, retinaldehyde, β-carotene, and all other compounds that can be converted to retinol. Even such a definition is problematic. For example, how should we classify retinoic acid, which relieves the skin lesions of vitamin A deficiency without relieving the night blindness, or reproductive symptoms (Wolf 1980)? Moreover, carotenoids may have biological actions quite distinct from their ability to meet the body's need for retinol.

Carotenoids may have biological actions unrelated to their ability to produce retinol

Carotenoids undeniably have chemical activities that retinoids lack. For example, β-carotene is a much more efficient singlet oxygen quencher than retinol. Its 11 conjugated double bonds provide energy transitions appropriate to the energy released when photoactive species, including singlet oxygen, decay to the ground state. Retinol lacks this ability because the sequence of conjugated bonds (spanning five carbon atoms) is too short. Do the special chemical properties of carotenoids confer a special biological role? Health and Welfare Canada sums up the evidence for a separate role for carotenoids as "not confirmed" (Health and Welfare Canada 1990). They cite nutritional experiments in which animals have been reared, sometimes for several generations, on vitamin A sufficient, carotenoid-free diets without deficiency symptoms. They state nicely that the "antioxidant properties of [carotenoids] can be used imaginatively to explain how carotenoids might protect animals against carcinogens and related noxious chemicals, but evidence that these mechanisms are more than theory is less easily obtained."

However, the matter does not rest there. At least some humans (those suffering from porphyria) receive a benefit (diminished photosensitivity) from carotenoids, which is not provided by retinoids. Photosensitization is a major cause of pathology in patients with erythropoietic protoporphyria. About 84% of patients treated with β-carotene increased their tolerance to sunlight threefold (Mathews-Roth 1986). In normal human subjects, photoprotective effects are equivocal. In one study, β-carotene significantly increased the minimal erythema-producing dose (Mathews-Roth et al. 1972). However, another study failed to show protection by carotenoids against UVA, UVB, or psoralen-induced UVA erythema (Wolf et al. 1988). β-Carotene (and vitamin E) presumably act as singlet oxygen quenchers or chain-breaking antioxidants when they protect against lethal hematoporphyrin photosensitization of white mice (Moshell and Bjornson 1977).

Plasma vitamin A levels are increased by dietary supplementation with carotenoids but not retinol

Dietary retinol within the recommended safe range does not increase serum retinol levels. Instead, absorbed retinol is esterified with long-chain fatty acids, incorporated into chylomicrons, and removed from the bloodstream by the liver. Serum retinol levels are homeostatically maintained within a narrow range by a retinol-binding protein, which delivers it to target tissues (Olson 1984). Presumably these mechanisms evolved to control the toxicity of retinoids. If very high doses of retinol are consumed, the retinol-binding capacity of plasma may be exceeded, allowing unbound retinol to spill into the circulation, a prelude to potentially lethal tissue injury. As a result of this fine homeostatic regulation, doses of retinol allowable in humans probably do not protect substantially

against free radical mediated processes. In contrast, serum carotenoids are substantially increased by dietary supplementation, potentially increasing defense mechanisms against free radicals. For example, as we shall see, carotenoid supplementation may spare plasma ascorbate.

β-Carotene may not be the most important contributor to all actions of carotenoids

Dietary sources of β-carotene are well known and include broccoli, spinach, kale, carrots, orange sweet potatoes, cantaloupe, mango, and papaya. The orange carotenoid pigments, found in highest concentrations in green leafy vegetables, are masked by green chlorophyll pigments. Oranges are high in canthaxanthin. Common sources of lycopene include tomatoes, red peppers, and red grapefruit. Dietary intakes of carotenoids amount to 5−15 mg/day, depending on method of assessment, with β-carotene contributing only 1−2 mg/day. Most of the carotenoids that lack vitamin A activity remain almost unexplored for either occurrence or activity in mammals. Lycopene and canthaxanthin are notable exceptions. Neither yields retinol in mammals, but canthaxanthin is a more effective scavenger of peroxyl radicals than β-carotene (Terao 1989) and it is sometimes anticarcinogenic (Mathews-Roth 1982) and sometimes inactive (Stich et al. 1984). Lycopene is twice as effective as β-carotene at scavenging singlet oxygen, and concentrations (0.7 μM) in human plasma are slightly higher than that of β-carotene (0.5 μM) (Di Mascio et al. 1989). Researchers have noted its potential importance as a mammalian antioxidant, and we can expect an expansion of information. A proper knowledge of which non vitamin A carotenoids are significant in the mammalian diet is contingent on availability of relatively inexpensive commercial preparations suitable for dietary supplementation.

Section 2. Antioxidant functions of carotenoids

A wide range of experimental results suggests that carotenoids are antioxidants or radical scavengers in vitro. This raises the question, does β-carotene quench radicals in vivo? As we have seen, prevention of singlet state mediated skin lesions of porphyrics confirms that carotenoids can scavenge singlet oxygen (a nonradical) in humans. It is now time to look at the much stronger evidence for antioxidant protection by carotenoids in vivo in nonhumans.

In nonhumans β-carotene meets several criteria for biological antioxidant activity in vivo

At least four lines of evidence suggest that β-carotene scavenges free radicals in vivo. First, in ascorbate-depleted guinea pigs treated with CCl_4, β-carotene supplementation diminishes exhalation of pentane and ethane (Kunert and Tappel 1983). In this respect β-carotene resembles the known antioxidants ascorbate and α-tocopherol (Doba et al. 1985). Second, β-carotene suppresses induction of superoxide dismutase in rats treated with corn oil (Blakely et al. 1988). Third, β-carotene levels in blood decline following radiochemotherapy (Clemens et al. 1989). Since radicals (and not the nonradical, singlet oxygen) likely mediate damage in these circumstances, the simplest explanation is that the carotenoid is removed in scavenging radicals. Finally, pigmented carotenoid-containing bacteria are more resistant to H_2O_2-generated hydroxyl radicals than a mutant colourless strain (Carbonneau et al. 1989). Taken together, these disparate lines of evidence combine to yield the strong inference that carotenoids are antioxidants in vivo effective against a broad spectrum of oxidant stressors and in more than one species.

Carotene protects bacteria against photodynamic damage

Rhodopseudomonas spheroides, a mutant strain of nonsulfur purple bacterium, lacks coloured carotenoid pigments. Without the carotenoids, this bacterium cannot survive the simultaneous presence of both oxygen and light (Griffiths et al. 1955). *Sarcina lutea* is a yellow bacterium that survives the presence of light and oxygen, whereas the colourless mutant form is destroyed (Mathews-Roth and Sistrom 1959). *Corynebacterium poinsettiae* is a coloured wild type, which is unaffected by oxygen and light in the presence of the photosensitizing dye toluidine blue. Under these same conditions, the colourless mutant displays lethal aerobic photosensitivity (Kunisawa and Stanier 1958). The simplest interpretation of these data is that the carotenoids are protecting the wild-type bacteria from the cytotoxic effects of singlet oxygen.

The combination of UVA light, air, and 8-methoxypsoralen causes photomutagenesis of *Salmonella typhimurium*. β-Carotene at 10 and 100 μg/mL protects by 50 and 70% (Santamaria et al. 1988). 8-Methoxypsoralen in nitrogen causes 65% less photomutagenesis than in air, and here β-carotene did not protect. Evidently, in a two-step reaction, an 8-methoxypsoralen−DNA photoadduct forms, and singlet oxygen forms in close proximity to the DNA. β-Carotene protects at this latter stage (Santamaria et al. 1988).

Carotenoids in the human retina may protect against photooxidative damage

The retina is particularly vulnerable to reactive oxygen species for the following reasons: (*i*) light is concentrated onto the photoreceptors by the lens; (*ii*) in vitro, vertebrate retinas show the highest oxygen consumption of any tissue; (*iii*) there is an abundance of mitochondria that could leak active oxygen species; (*iv*) rod photoreceptor membranes contain the greatest concentration of docosahexaenoic acid (22:6 ω−3), the most highly unsaturated fatty acid found in the human body (therefore susceptible to lipid peroxidation); and (*v*) the combination of light and oxygen is postulated to create activated oxygen species such as superoxide radical, hydroxyl radical, and singlet oxygen, resulting in phototoxicity (Handleman and Dratz 1986; Ham et al. 1984). These characteristics may combine to make the retina particularly susceptible to prooxidants. Certainly the presence in the eye of a minute sliver of iron can induce gross degeneration of the retina. The retina has been examined for antioxidants including carotenoids. Total carotenoids in the human retina ranged between 20 and 250 ng/retina, but these did not include more than traces of β-carotene. These carotenoids are not evenly distributed. The posterior central part of the retina contains a small yellow focal point of vision known as the macula. It is not known to what extent oxidative processes contribute to age-related macular degeneration, but certainly the macula is particularly rich in antioxidant pigments. The yellow colour of the macula is attributed to elevated concentrations of the main carotenoid pigments of the retina: zeaxanthin and lutein (Handleman et al. 1988). These are hydroxylated forms of β-carotene, which are not retinol precursors. Like β-carotene, they are thought to filter out much of the phototoxic blue light blue and to act as singlet oxygen quenchers. They may also function as effective chain-breaking antioxidants at the low partial pressures of oxygen found in the neural layers of the human retina (Handleman and Dratz 1986).

The Eye Disease Case-Control Study Group (1993) has evaluated the antioxidant (serum vitamins E and C, carotenoids, and selenium) status in 421 patients. Risk of age-related macular degeneration was decreased by 50% in the group with medium serum carotenoid levels and by 66% in the subjects with high serum carotenoid levels. Vitamin E, vitamin C, and selenium did not display any individual effects. If carotenoids in the retina function as antioxidants that protect photoreceptor membranes from lipid peroxidative damage, they become candidate test agents for ameliorating the effects of iron toxicity in the retina, or as protective agents in age-related macular degeneration. Similarly, low serum concentrations of β-carotene and vitamin E are found to be risk factors for senile cataract. Future controlled trials on the roles of antioxidant vitamins and eye disorders are warranted (Knekt et al. 1992).

β-Carotene administration increases plasma ascorbate in humans

In a preliminary study, we attempted to show that administration of β-carotene might increase antioxidant reserves in human plasma as assessed by the methods of Burton and co-workers (1983). Although there was no significant increase in total antioxidant activity, plasma ascorbate levels increased ($p < 0.05$) following oral β-carotene supplementation (Fig. 4). This unexpected observation deserves to be followed up. In a study of dietary antioxidant (vitamin C, vitamin E, and β-carotene) supplementation and protection of LDL (low density dipoproteins), addition of vitamin C increased plasma levels of β-carotene (Reaven et al. 1993). These results imply that β-carotene and ascorbate may be sparing each other in vivo, perhaps by diminishing oxidant processes that would otherwise consume them.

Exfoliated oral mucosal cells and plasma are accessible samples for tissue analysis

The major stores of carotenoids are not easily measured in healthy subjects or patients. Plasma levels of antioxidants are now routinely assayed in clinical chemistry laboratories, but they may not accurately reflect tissue reserves. At least one cellular sample can be obtained noninvasively. Exfoliated stratified squamous epithelial cells are conveniently obtained by brushing the inside of the mouth with a toothbrush (Cameron et al. 1989). The procedure yields 10 to 100 mg of cells (wet weight). The cells are cornified, and no longer viable, but the incidence of micronuclei can nevertheless be determined in these scrapings. Moreover, they can be analyzed for β-carotene content, and the results presumably reflect the β-carotene content of the living mucosal cells. β-Carotene levels in these cells can be correlated with other indices of the actions of genotoxins, such as percentage of cells containing micronuclei.

Several studies have examined the relationship between diet and carotenoid content of exfoliated cells. Exfoliated cells represent the carotenoid dietary intake of about 3 weeks earlier, 3 weeks being the time required for cells generated in the basement layer to migrate to the outer surface, become keratinized, and exfoliate. Plasma levels reflect carotenoid intake over the recent few weeks, whereas tissue levels indicate longer term intake along with life-style and environmental factors. These factors are difficult to disentangle, given the inaccessibility of the major pools. Pharmacokinetic studies with rodents are of limited applicability to humans, since rodents absorb carotenoids much more sparingly. Nevertheless, ready accessibility

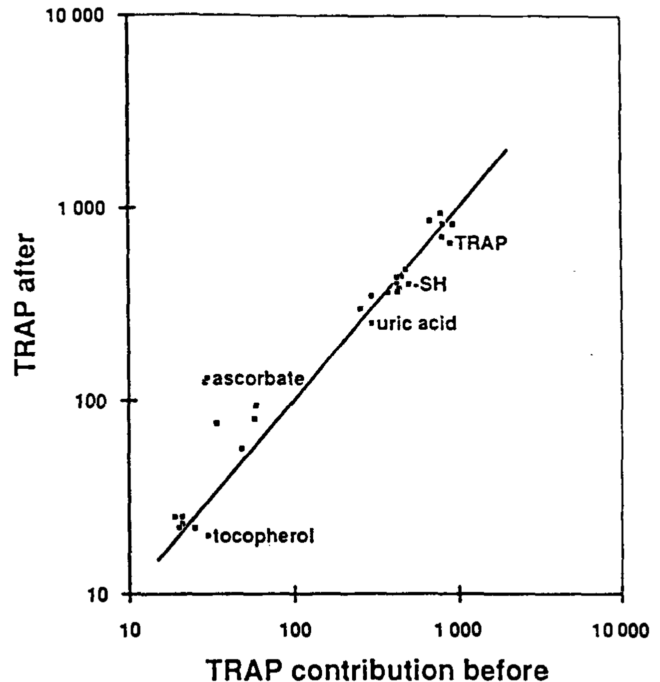

FIG. 4. Supplementation with β-carotene raises plasma ascorbate levels. Levels of plasma antioxidants 2 weeks after supplementation with 180 mg of β-carotene are plotted as a function of levels before supplementation. Total (peroxyl) radical-trapping antioxidant parameter (TRAP) units are as defined by Burton and co-workers (1983).

of carotenoid levels in skin and plasma brings the possibility of epidemiological studies to any medical clinic. This capability holds promise for future findings regarding carotenoid nutrition. To fulfill this promise and allow correlations with dietary intake of carotenoids, food tables (printed and computerized), however, will need to be updated to tabulate content, not merely of total vitamin A activity but of all of the carotenoids present in common foods.

As we shall see, the increased carotenoid levels induced by supplementation in volunteers confer on their oral mucosal cells the ability to resist genotoxicity of chewing tobacco products and other risk factors in oral carcinogenesis. Despite the use of these techniques for over a decade, we could find no study in unsupplemented humans correlating the incidence of micronuclei in exfoliated oral mucosal cells with the levels of carotenoids in these cells. Determining such relationships deserves a high priority in future research.

Dietary and life-style factors modulate absorption of carotenoids in humans

Nonsmokers, females, and lean subjects had the largest responses to β-carotene supplementation. Females reportedly absorb a greater fraction of their dietary β-carotene (Stacewicz-Sapuntzakis et al. 1986). Individuals with large body surface areas and adipose tissue reserves may require higher doses of β-carotene than normal individuals to reach a target plasma concentration (Dimitrov and Ullrey 1989). The presence of sulfides (Peiser and Yang 1979), dietary fats (Dimitrov et al. 1988), and acids such as ascorbate (Haralampu and Karel 1983) in the digestive tract also aid carotene absorption. Dietary fats, the most important of these factors, dissolve carotenoids and presumably aid carotenoid absorption in the

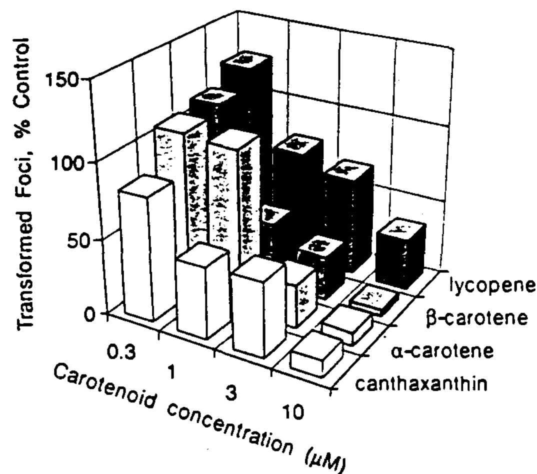

FIG. 5. Several carotenoids protect 10T½ cells against methylcholanthrene-induced neoplastic transformation. Dose–response curves. The number of transformed foci are plotted as a function of carotenoid concentration. Protection in each case is statistically significant at $p < 0.001$. Replotted from data of Bertram and co-workers (1991).

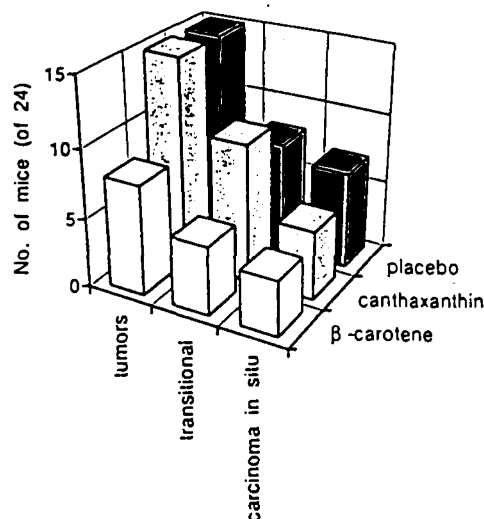

FIG. 6. Protection against nitrosamine-induced bladder tumors, by β-carotene in mice. Canthaxanthin fails to protect significantly. Three indices of carcinogenesis were followed. In comparison with placebo, β-carotene was effective for all three indices at $p < 0.05$, while canthaxanthin was ineffective by the same criterion. Replotted from data of Matthews-Roth et al. (1991).

process of their own absorption (as chylomicrons) via the lymphatic route (Dimitrov et al. 1988).

Although fats enhance carotenoid absorption, they decrease chemoprotective effects. In mice, dietary fat can nullify protection by β-carotene against tumorigenesis by ultraviolet light (Mathews-Roth and Krinsky 1984). In humans, many factors affect metabolism and excretion of carotenoids. Metabolic disorders such as diabetes and thyroid disease (Neiman and Obbink 1954) as well as anorexia nervosa (Robboy et al. 1980) tend to elevate blood β-carotene levels. Plasma β-carotene levels in chronic smokers are lower than those of nonsmokers (Ziegler 1989). Moreover, β-carotene content of exfoliated mucosal cells in heavy consumers of alcohol average 0.08 ng/10^6 cells compared with 1.24 ng/10^6 cells in nondrinkers (Stich et al. 1986). It is not impossible that the high incidence of cancer in alcoholics and smokers results from diminished tissue carotenoid content. It is in any event important to determine whether the decreased levels result from diminished intake of β-carotene, increased metabolism, or impaired absorption. Some individuals appear to be poor absorbers of β-carotene, regardless of diet or other external factors (Dimitrov et al. 1988; Gilbert et al. 1990). It is important to learn whether they are at an elevated risk for cancer.

Section 3. Antineoplastic actions of carotenoids in cells, animals, and humans

There are compelling reasons for both hope and skepticism

In this section we will contend that there is a convincing body of evidence that certain carotenoids can prevent, or at least delay, some cancers. Across a variety of species, using a host of indices of carcinogenesis and precarcinogenesis, and a broad spectrum of carcinogenic stressors, carotenoids repeatedly protect. On the contrary side, it is also clear that many cancers are not prevented by carotenoids, and that in some situations certain carotenoids protect, while others consistently fail to protect.

Carotenoids can protect against chemically induced cell transformation

Bertram and co-workers (1991) have screened eight carotenoids and antioxidants for their ability to protect against neoplastic transformation in 10T½ cells. Among these, β-carotene, α-carotene, canthaxanthin, lycopene, and α-tocopherol protected ($p < 0.05$, Fig. 5). Lutein, renierapurpurin, and bixin were ineffective.

A more limited spectrum of protective actions against bladder cancers was noted in mice treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine by Matthews-Roth and co-workers (1991). Here β-carotene protected ($p < 0.05$) against all three criteria of carcinogenesis: tumors, carcinoma in situ, and transitional cell carcinoma, but canthaxanthin failed to protect (Fig. 6). The slight protection against carcinoma in situ by canthaxanthin failed to reach significance at the $p = 0.05$ level.

Micronuclei are markers of genotoxicity and β-carotene can diminish micronucleus formation

Each chromosome in the cell nucleus must duplicate during mitosis. During metaphase, microtubules assemble from the protein tubulin and arrange themselves along the equatorial plane. Microtubules attach at the chromosome's centromere at a protein structure called the kinetochore. They align the parent and daughter chromosomes across the mitotic spindle and their capacity for motility is responsible for moving the chromosomes towards the spindle poles. If, as a result of DNA breakage, a chromosome is broken, the section that has no attached centromere will fail to move with the rest of the chromosome. Such extranuclear chromosome fragments remain in the cytoplasm of the daughter cells as Feulgen-positive bodies called micronuclei. These micronuclei can be used as markers or indices of chromosomal breakage in previous cell

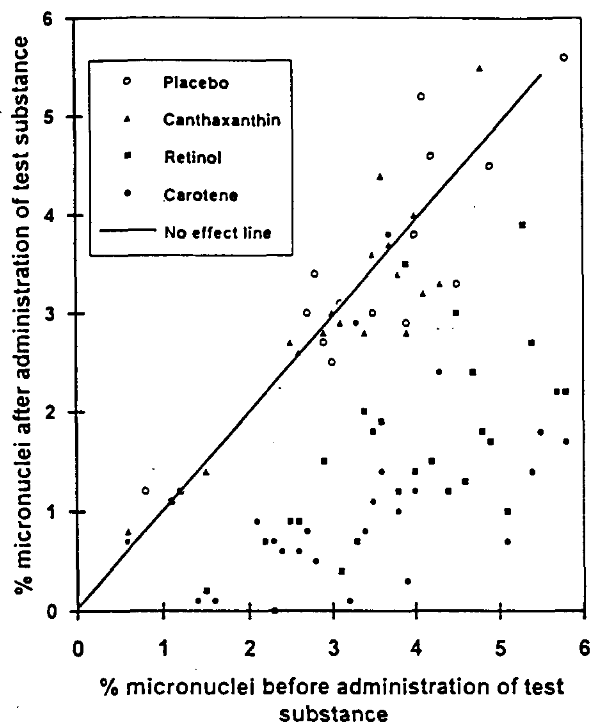

FIG. 7. β-Carotene decreases the frequency of micronuclei in exfoliated oral mucosal cells of tobacco chewers. Retinol is almost as protective as β-carotene, but canthaxanthin fails to protect. β-Carotene (180 mg/week), retinol (150 000 IU/week) or canthaxanthin (180 mg/week), or a placebo was administered for 9 weeks. Replotted from data of Stich et al. (1984).

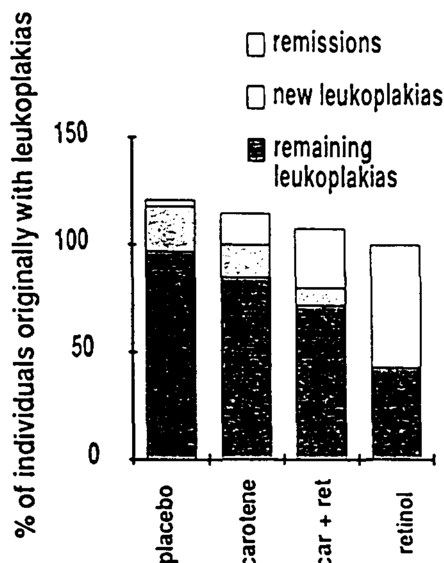

FIG. 8. Carotene induces regression of oral leukoplakias in tobacco chewers. Carotene increased the number of regressions about 5-fold, whereas retinol increased regressions over 10-fold. Remissions, new leukoplakias, and remaining leukoplakias are plotted as a function of the number of individuals with leukoplakias in each group prior to supplementation. β-Carotene (180 mg/week), retinol (200 000 IU/week) or retinol (100 000 IU/week) plus carotene (180 mg/week) (car + ret), or a placebo was administered for 6 months. Replotted from Stich et al. (1988*a*, 1988*b*).

divisions. Their formation characterizes the genotoxicity of specific carcinogens (Rosin 1990; Stich and Rosin 1984; Stich and Dunn 1986).

There is another mechanism that can also give rise to micronuclei: scission of one of the spindle microtubules. It is not widely realized that micronuclei from these two sources are readily distinguishable. Micronuclei from DNA scission contain no kinetochore, but microtubule scission gives rise to "kinetochore-positive" micronuclei (Yager et al. 1990). Protection by β-carotene against formation of micronuclei may therefore reflect diminished scission of either DNA or microtubules. A study of whether β-carotene decreases kinetochore-positive or kinetochore-negative micronuclei will be an important indicator to the site of action of β-carotene. Such a study is eminently feasible since exfoliated cells from oral mucosa reflect the extent to which the incidence of micronuclei is increased by oral exposure to carcinogens, or decreased by anti-cancer agents (Stich and Rosin 1984).

A start has been made in the human intervention trials needed to establish cause and effect. Such studies can distinguish the immediately effective agents from inert agents co-distributed with them. Stich and co-workers (1984) compared the effects of orally administered retinol, β-carotene, and canthaxanthin in protecting against tobacco-induced micronuclei in exfoliated human oral mucosal cells. β-Carotene or retinol protected substantially, but canthaxanthin was devoid of any protective effect (Fig. 7).

Carotene plus retinol causes regression of leukoplakias in tobacco chewers

The further question of whether β-carotene can protect against the later stages of carcinogenesis has also been examined by following the progression of leukoplakia. Stich and co-workers (1988*b*) administered β-carotene (180 mg/week, equivalent to 100 000 IU of vitamin A activity from retinol), β-carotene (180 mg/week) plus retinol (100 000 IU/week), or placebo to tobacco chewers who had already sustained premalignant leukoplakias. After 6 months treatment, the placebo-treated group had few remissions and a much larger number of new leukoplakias (Fig. 8). Although β-carotene induced significant remission of leukoplakias compared with the placebo group, it was less effective (statistical significance undetermined) than retinol plus β-carotene (Stich et al. 1988*b*). In another study by Stich and co-workers (1988*a*) retinol (200 000 IU/week) completely prevented new leukoplakias, and increased the number of remissions about 10-fold (to about 50% of the affected group).

In a similar study, patients with oral leukoplakias were administered 30 mg β-carotene, 1000 mg vitamin C, plus 800 IU of vitamin E daily. After 3 months supplementation, 60% ($n = 20$) of the subjects had experienced partial or complete regression of their oral leukoplakia (Brandt et al. 1992).

Carotenoids have been more consistent anti-tumor agents than retinoids

Despite the greater activity of retinol in the aforementioned study in humans, retinoids have had at best inconsistent effects in many animal studies. In some chemical induction experiments retinoids inhibited carcinogen action. Thus, retinoic

acid inhibited *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine induced bladder cancer in rats by 55% (Grubbs et al. 1977) and decreased dimethylbenzanthracene (DMBA) and croton oil induced papilloma mass in mice by 91% (Bollag and Matter 1981). Three retinoids decreased azaserine-induced pancreas tumors in rats, by an average of 72% (Longnecker et al. 1982). Retinyl acetate inhibited *N*-methyl-*N*-nitrosourea (MNU) induced mammary tumors in mice by 33% (Moon et al. 1977). In mice inoculated with tumor cells retinol increased survival time by 42% (Rettura et al. 1982).

Nevertheless, various retinoids are reportedly inactive in 1,2-dimethylhydrazine-induced colon cancer in rats (Rogers et al. 1973). DMBA and MNU induced mammary cancer in rats, and 13-*cis*-retinoic acid failed to protect (Moon 1989). Similarly, retinyl acetate failed to protect C3H-A^{vy} mouse mammary gland against DMBA and MNU (Maiorana and Gullino 1980). In several studies retinoids aggravated carcinogenesis. Hamsters fed 2400 μg retinyl acetate per week had 23% more benz(*a*)pyrene-induced respiratory tract tumors than those fed 100 μg retinyl acetate (Smith et al. 1975). Retinoids appear to demonstrate a higher degree of target organ and species specificity than carotenoids. Perhaps the actions of retinoids reflect a balance of cytotoxic and cytoprotective effects, whereas the actions of carotenoids are more purely protective, reflecting their lower dietary toxicity.

Retinoids decrease the incidence of chemically induced or transplanted tumors, but not UVB-induced skin tumors. β-Carotene is more effective against chemically induced neoplastic transformation than retinoids (Schwartz and Shklar 1988). Direct-acting mutagens, thio-TEPA (triethylene thiophosphoramide), methyl methanesulfonate, and busulfan, induce clastogenic changes in bone marrow cells of hamsters. β-Carotene gave a dose-dependent decrease in aberrant cells, but retinol did not protect (Renner 1985). β-Carotene decreased the mass of DMBA-induced carcinomas in the buccal pouches of hamsters by 98%, whereas retinoic acid was inactive (Schwartz and Shklar 1988).

The inconsistencies among and within retinoids and carotenoids are disturbing to say the least. When protection is seen, it is often dramatic and reproducible. And the failures to protect are often equally convincing. Perhaps the response depends on the basal levels of the protective vitamin in the control (placebo) groups. When basal levels are adequate, further supplementation is ineffective. For this reason, further studies of anticarcinogenesis in vitamin A deficient populations are needed. These will undoubtedly be easier with experimental animals, but the human dimension should not be neglected.

What mechanisms are available for the anticarcinogenic actions of carotenoids

The question as to whether chemoprevention by carotenoids is related to vitamin A activity cannot yet be answered with confidence. The best answer at this stage is perhaps "sometimes." As described above, several workers have compared canthaxanthin with β-carotene. The outcome should be decisive, since canthaxanthin has antioxidant potency reportedly comparable with β-carotene but lacking vitamin A activity. The results to date have been equivocal. In some experiments, canthaxanthin or α-tocopherol was as effective as β-carotene, suggesting that the effect was antioxidant in nature. In other experiments, canthaxanthin was devoid of activity, casting gloom over those who wished to attribute the anticancer effects purely to antioxidant activity.

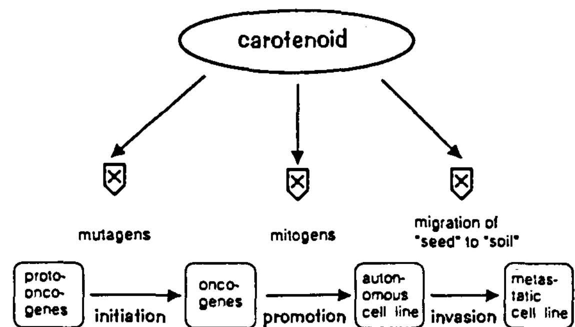

FIG. 9. Stages in carcinogenesis for protection by carotenoids. Events and causative agents are indicated for each of the three major phases of carcinogenesis: initiation, promotion (including progression), and invasion. Each of these sites contains processes that are potentially blocked by carotenoids.

The gloom may have been premature. Failure of one carotenoid to protect against oxygen-mediated stress does not rule out antioxidant mechanisms in protection by another. Perhaps once in place, both agents are antioxidants, but there are strict steric requirements if the test agent is to be delivered to the intracellular protective site. Another perspective is that as with other nutrients (pyridoxal, α-tocopherol, ascorbate, selenium, copper) the initial evolutionary benefit was that of the substance itself. However, over millennia, mechanisms (enzymes) evolved to improve efficiency. Thereafter, the substance itself retains a rudimentary activity, but the effects when facilitated by an apoprotein dwarf those of the original precursor. It is not surprising, in either event, that some benefits are associated with a detailed requirement for a specific stereochemistry. There is weak evidence that the anticancer activity of β-carotene is associated with antioxidant actions. In human squamous cell lines, inhibition of proliferation by β-carotene was associated with production of a 70-kDa protein believed to regulate cellular oxidative processes (Schwartz et al. 1990).

We have discussed the pharmacokinetics and mechanisms of these effects elsewhere (Rousseau et al. 1992). To summarize, carotenoids can act at any of the major stages of carcinogenesis: initiation, promotion, or invasion (Fig. 9). The few pharmacokinetic studies available suggest that carotenoids delay rather than prevent cancer. Moreover, where timing has been studied, protection occurs later, rather than earlier, in the sequence of events. There is no lack of potential target sites, both late and early. A few are indicated in Fig. 10.

Proper skepticism demands that negative results be given equal prominence

Progress requires dissemination not only of positive results but also of negative data. It is not only the lay public who can be carried away with euphoria regarding the benefits of β-carotene supplementation. Hard-nosed researchers are not immune to "climbing aboard bandwagons." Many experiments regarding the benefits of carotenoids are now in progress. As the results become known, it is unethical to withhold negative data. Only in the context of negative findings can the significance of positive findings be properly assessed.

Pharmacological doses of β-carotene protect against several carcinogens, genotoxins, and tumor promoters (Brandt et al.

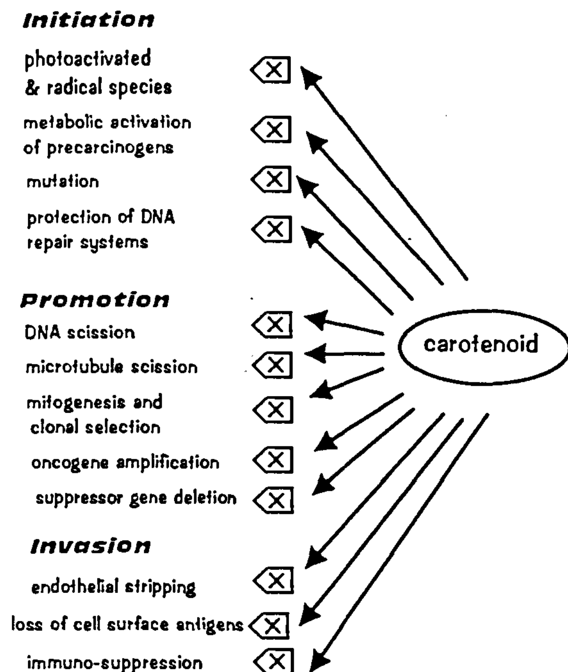

FIG. 10. Potential target sites in carcinogenesis for the actions of anticarcinogens. Selected processes that have been shown to respond either to carotenoids or to antioxidants. Such sites are attractive candidates for the chemoprotective actions of carotenoids.

1992; Malone 1991; Stich et al. 1984, 1988*b*). Nevertheless, public health authorities have preferred to recommend foods that protect against cancers rather than supplementation with specific vitamins. β-Carotene may be the most effective carotenoid precursor of vitamin A, but it is not yet known which carotenoid is the best anti-cancer agent. With the protective components not yet identified, it seems premature to selectively recommend β-carotene. Moreover, dose–response information is rudimentary at best.

β-Carotene as a dietary supplement

Those determined to take supplementary β-carotene tablets despite the absence of positive recommendations may find the established dosages and requirements difficult to ferret out. The difficulties lie in the changes in terminology of vitamin A and its derivatives over the years. In Canada, the recommended nutrient intake (RNI) for vitamin A is 1000 retinol equivalents per day. If this was all supplied as β-carotene, it would amount to 6 mg/day, about half the amount present in a medium-sized carrot. The typical Canadian intake of vitamin A is about 50% over the RNI. In clinical trials a dosage of 90 mg/day is typical, about 15 times the RNI. This dosage has been shown to cause substantial increases in the β-carotene content of exfoliated oral mucosal cells (Gilbert et al. 1990). Typical doses in β-carotene supplements found on the vitamin counters of supermarkets are 10 000 to 25 000 "international units." These large numbers are misleading because of the use of the obsolete international units. These tablets in fact contain 1000 to 2500 retinol equivalents, and in countries with more rigorous labeling standards, are marked as such. To put the labels in perspective, they contain 6–15 mg of β-carotene. Higher dosage supplementary capsules may be available in the form of Solatene® (Roche) tablets intended for porphyrics. These contain 30 mg β-carotene each, and are the form often used in supplementation studies, three capsules per day usually taken under supervision. Pharmaceutical companies should indicate that maximum shelf life of carotenoid supplements or vitamins containing β-carotene will be greatly extended if kept in the freezer in a lightproof container.

Can carotenoids be recommended as anticancer agents?

Health and Welfare Canada (1990) (Nutrition Recommendations: The Report of the Scientific Review Committee) conclude that "there is a high probability that hopes that cancer can be avoided will not be fulfilled." We remain more buoyant regarding prospects for chemoprevention in general. In support of our position we reflect that current knowledge allows about 70–80% of cancers to be avoided by life-style changes (Bertram et al. 1987). Taking experiments at face value, risk of several cancers can be diminished by over 50% by administration of specific anticarcinogens. Once optimal agents and regimens are established, an optimist might readily foresee synergism between risk avoidance strategies and chemoprevention. We see no reason to reject a future in which 90% of cancers can be classed as avoidable.

The conclusion of Health and Welfare Canada is that "there is certainly insufficient evidence to justify fortifying more foods with preformed vitamin A or recommending the use of supplements." They guardedly recommend increasing intake of carotenoid-rich foods on the grounds that it is better to err on the side of safety. Nutritional authorities should begin to consider what their positions will be when genetically modified plant foods are available, having elevated carotenoid content. These are likely to include cauliflower, rice, and root crops such as potatoes. Perhaps Health and Welfare Canada's current recommendation will still apply: "In cancer prevention, the importance of even small benefits, the importance of acting early in life, and the long delay before positive evidence will be obtained ... could justify positive recommendations based on inconclusive data. There is no risk in consuming generous amounts of foods rich in carotenoids."

Even if we are more optimistic, it is difficult to disagree with these recommendations. The case for supplementation with carotenoids is now stronger than at the time of the Health and Welfare Canada report, but still inconclusive. Any blanket recommendation that vitamins be used in pharmacological quantities still poses many difficult questions, even for an agent as safe as β-carotene. For example, will there eventually be a recommended nutrient intake (RNI) or recommended dietary allowance (RDA) for carotenoids for the general population? Will carotenoids be recommended as a pharmacological agent for those at elevated risk for cancer or its recurrence? RNIs are based on scientific evidence and are reviewed every 5 years. Nutritional committees are already recommending increased intakes of an antioxidant vitamin in oxidatively stressed individuals, as in the recommendation of increased vitamin C intake for smokers. The need of smokers for α-tocopherol and carotenoids deserves to be examined.

At present, educating the general public to the advantages of consuming carotenoid-rich foods seems preferable to instituting an RNI for β-carotene. Health and Welfare Canada has

already adopted such a recommendation, and some third-world countries have attempted to increase consumption of yams as an anti-cancer measure. Governmental attempts to change established eating patterns are more likely to succeed if a traditional food crop can be replaced by another of the same kind with increased carotenoid content. One can predict an increase in genetic experimentation and micropropagation of plant foods. Such experiments may yield carotenoid-rich varieties of crops such as rice. The new yellow cauliflower and pink-fleshed honeydew melon may turn out to be early steps in this direction.

The opposition of the health authorities to carotenoid supplementation can be predicted to be ineffective against the all too human desire for a "magic bullet" against cancer. Increased use of supplements (as judged by the popular press and in the availability of carotene tablets in supermarkets and health-food stores) is "not necessarily desirable, merely inevitable." Carotenoids have been added to cheese and margarine for years as colouring agents. The Habitant Soup Company and presumably others are starting to add β-carotene to canned foods. β-Carotene is now appearing in breakfast cereals and as an ingredient of "multivitamin" tablets. Such unauthorized self-experimentation brings with it the benefit of providing a useful new experimental group in epidemiological case-control studies, but makes it more difficult to find untreated controls.

Research on nutritional prevention of cancers must be taken forward to the biochemical level

Future studies should focus on interactions between combinations of chemopreventive agents at specific stages of carcinogenesis for a range of carcinogens. Carotenoids might slow the progression of cancer more or less effectively in conjunction with other substances present in fruits and vegetables. Mechanisms of carotenoid protection are poorly understood; therefore more research on reactions of individual carotenoids with specific radical species is required. In vitro models must be established to allow screening of mixtures at a variety of doses for the better design of human intervention trials. The effectiveness of the various carotenoids against specific cancers needs to be extended. For example, high serum lycopene levels are associated with a decreased risk for pancreatic (Burney et al. 1989) and bladder (Helzlsouer et al. 1989) cancer. Transport of carotenoids and retinoids by lipoproteins needs to be delineated more precisely. Recommendations for further research in this area are given in more detail elsewhere (Rousseau et al. 1992).

Broad experimentation with carotenoids seems justified on the basis of the positive results to date

Despite the accelerating frequency of reports of beneficial actions of carotenoids, there seems little danger of the field becoming overcrowded. Each report that carotenoids protect against, e.g., hypertension or heart disease raises additional questions that clamor for solution. The number of studies that have been completed pales into insignificance in comparison with those that remain to be done: haemochromatosis, allergies, infections, retinitis pigmentosum, and age-related macular degeneration. High-risk populations must be targeted, and "natural experiments must be identified." Chemoprevention trials must be prioritized in terms of benefit and risk (Bertram et al. 1987). The National Cancer Institute is sponsoring 10 human intervention trials to study the chemopreventive effects of β-carotene (Malone 1991). Such intervention trials should establish dose–response relationships over a variety of carotenoids. Those engaged in carotenoid research seem recipients of the legendary curse: "May you live in interesting times." Our perspective is that much of the euphoria will not survive closer investigation. However, not all of the dramatic results will vanish with close scrutiny, and the small fraction which persists will more than justify the enormous effort and cost. In the future a much smaller number of people may be consuming a β-carotene supplement, but hopefully they will do so with greatly increased confidence that it is providing a specific benefit.

Optimal dosage and regime need to be established for chemopreventive actions of individual carotenoids

Establishing the most promising carotenoids will be greatly facilitated by identification of the biochemical site of its action. This would allow comparison of carotenoids for potency in the primary chemical event. Pending a chemical test system, it is important to define biological test systems, including intact animals, cultured cells, and biological isolates. In each system we must determine the extent to which protection by carotenoids mirrors their protective potencies in animals and patients. Until such systems are established, there is no alternative to the present lengthy and expensive clinical trials with micronuclei and other "markers," preneoplastic lesions, and eventually the incidence of neoplasms as end points. Before long-term administration of purified carotenoids can be recommended, lifetime detrimental effects must be extrapolated from long-term toxicity studies. Clearly we are many years away from being able to make convincing recommendations, and this lends urgency to the research tasks currently underway.

Conclusions

In the first section, we surveyed the nature and occurrence of carotenoids. In the second we showed that carotenoids have at least some functions in mammalian cells that are quite distinct from their roles as precursors of the retinoids. In the third, we established at least a limited antioxidant role for carotenoids in vivo and presented evidence for and against an antineoplastic role for carotenoids. In the final section we support the recommendations of Health and Welfare Canada, that diets rich in carotenoid-containing foods can be unequivocally recommended. We have identified a number of priorities for research, which if undertaken will allow future recommendations to be made with more confidence.

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CHAPTER 4

Carotenoids: Biochemistry and Antioxidant Mechanisms

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Abstract

β-Carotene is protective in certain cancers, cardiovascular disease, age-related macular degeneration, cataracts, and other diseases known to have free radical-based pathologies. Chemoprevention trials are currently underway and β-carotene is being added to numerous consumer products. This widespread use demands a search to fully understand carotenoid antioxidant and oxidation mechanisms. β-Carotene oxidation products have the potential to increase lipid autoxidation processes within cell membranes, increasing free radical-mediated processes. β-carotene is unstable when shelf-stored under normal conditions, and degrades to oxidation products. No longer confined to the laboratory, this problem has now become an issue for the general public. *In vitro* studies provide much needed information on products of β-carotene oxidation, mechanisms, chemical reactivity, chemical instability, interactions, and bioavailability. *In vivo*, bioavailability and antioxidant potency of carotenoids depends upon membrane partitioning and interactions with other cellular constituents, and we need to know if oxidation products also accumulate in tissues. Studies involving carotenoids are subject to an array of experimental and methodological problems which can be attenuated by controlling experimental conditions. Standardization for methods of storage, handling, and testing will make information more reliable and relevant.

Introduction.

Evolution has provided humans with a compliment of antioxidant enzymes such as superoxide dismutase, catalase, Se-GSH-Px, and PLOOH-GSH-Px. Endogenous non-enzymatic antioxidants include the quinones, bilirubin, uric acid. Metal-binding proteins such as ceruloplasm, transferrin, and lactoferrin decrease the production of Fenton-reaction radicals, whereas haptoglobin and hemopexin (which bind hemoglobin and free heme, respectively) protect against H_2O_2 reactions that form ferryl species. Free radical-mediated oxidative damage to cellular membranes, nuclear material, proteins, and tissues is linked to a variety of pathological conditions such as Alzheimer's disease, atherosclerosis, and cancer. These and other disorders, such as age-related macular degeneration, may be caused by the breakdown of antioxidant defenses.

Dietary antioxidants include the lipid-soluble carotenoids and tocopherols, as well as water-soluble ascorbic acid. β-Carotene appears to have many functions independent of its conversion to retinoids. We focus here on the antioxidant functions of carotenoids, discuss what is known about their chemistry, reactions to oxygen-mediated stress, interactions with other antioxidants, biological functions as related to structure, and how these mechanisms may apply *in vivo*. Finally, we will discuss potential problems in carotenoid research and possible resolution.

Carotenoids protect plants and animals.

Carotenoids protect plants and bacteria against light-induced damage by quenching singlet state oxygen and sensitizing pigments in the triplet state. Of the over 600 carotenoids in nature, less than 50 are involved with human nutrition, and of these only about 10 have been used for experimental study. Oral β-carotene in high doses protects patients with light-sensitivity disorders like erythropoetic protoporphyria, by quenching active states produced in porphyrin-photooxidation reactions (Mathews-Roth, 1986). Several reviews have discussed the actions of carotenoids as singlet oxygen quenchers (Rousseau et al., 1991) and antioxidant scavengers of active oxygen species (Krinsky, 1989; Canfield, 1992). Nutritional studies with animals and epidemiological studies with humans have been discussed (Davison et al., 1993). Biological actions and possible mechanisms of anticarcinogenesis and antioxidant activity are discussed in a recent review (Rousseau et al., 1992). Carotenoids are associated with increased protection in an array of disorders and diseases such as age-related macular degeneration (Eye disease case-control study group, 1992); cataracts (Knekt, 1992), cancer (Knekt, 1991), and atherosclerosis and heart disease (Physicians Health Trial, 1992). However, some studies have found no significant correlation between serum carotenoids levels and age-related macular degeneration (West et al., 1994), and others report and increase in lung cancer with β-carotene supplementation (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). This disparity in β-carotene effect may have more to do with experimental conditions than the true antioxidant potency of the molecule.

Carotenoid reactions.

Characterization of products from β-carotene oxidation reactions.

Many studies of the stability of β-carotene involve heat treatment and processing in the food and agricultural sciences. Recent studies include β-carotene autoxidation (Handelman, 1991; Mordi, 1991), peroxyl radical oxidation (Handelman, 1991; Kennedy, 1991; Kennedy, 1992), and carotene-oxygen radical reactions (Conn, 1992). These have contributed much needed information on mechanisms of the reactions of carotenoids with activated species. Moreover, the oxidation products are now fairly well characterized (reviewed in Rousseau et al., 1992).

Self-initiated autoxidation of β-carotene.

Self-initiated autoxidation to β-carotene with molecular O_2, 30°C, in the dark, in benzene or tetrachloromethane, produced an array of epoxides, aldehydes and ketones (Mordi et al, 1991). After several hours, a complex mixture of shorter chain compounds appeared. Interestingly, after 24 hours, all the β-carotene was consumed but oxygen uptake continued for another 24 hours. This indicates that the oxidation products themselves retain chemical activity. The oxidation products were identified by UV, GC/MS, HPLC, and NMR. Other studies of spontaneous autoxidation of β-carotene report similar products (Handelman, 1991; Mordi, 1993). These results concur with our finding that oxidized β-carotene dramatically increased lipid autoxidation in human retina homogenate (MSc thesis, Rousseau, 1994). The products of self-initiated autoxidation under these conditions may be similar to shelf-aged oxidation products.

Peroxyl radical-initiated oxidation of β-carotene.

Peroxyl radical reactions with β-carotene in hexane result in production of 5,6-epoxy-β-carotene, 15,15'-epoxy-β-carotene, and several unidentified polar products (Kennedy, 1991). The same investigators looked at reactions of peroxyl radicals with β-carotene in liposomes, and with the exception of 15,15'-epoxy-β-carotene, found similar products. β-Carotene depletion and product formation at 160 (ambient PO_2) and 760 Torr (high PO_2) are similar. β-Carotene depletion and product formation at 12 Torr (low PO_2) are 57 and 50% of higher partial pressures of oxygen (Kennedy, 1992). Apparently, at low partial pressures of oxygen, fewer oxygen molecules are available for the production of peroxyl radicals to participate in chain reactions. This may have significance at the low oxygen tensions found in some tissues.

β-Carotene is oxidized at multiple sites.

At normal PO_2, free radical attack on the β-ionone ring produces epoxides bridged across the ring near the side chain (5,6 epoxide), or bridged between the ring and the side chain (5,8 epoxide), with diepoxides also being formed. Free radical attack on the conjugated polyene chain produces an array of carbonyl compounds (aldehydes and ketones). Similarly, at 15 Torr, the major products were oxygenated (Kennedy, 1992). Thus, even under low PO_2 conditions, termination reactions were mediated by oxygenated species.

The evidence suggests that β-carotene protects by radical addition.

The main products of β-carotene oxidation (ketones, aldehydes, and epoxides) as well as the absence of certain products (peroxides, hydroperoxides, and alcohols), suggest that termination does not occur by the peroxyl-peroxyl reaction seen as Eq.[1]

$$RR'CHOO\cdot + RR'CHOO\cdot \text{------->} RR'CHOH + RR'C{=}O + O_2 \quad [1]$$

Unlike the oxidation of polyunsaturated fatty acids, which proceeds by H-abstraction from an allylic carbon, oxidation of β-carotene proceeds by electrophilic attack by propagating peroxyl radicals or oxygen on the conjugated side chain or alicyclic ring (Mordi, 1991). Carotenoids other than β-carotene have not been subjected to oxidation product analysis, although one group included β-apo-8'-carotenol and retinol (Mordi, 1993). Possibly, xanthophylls (carotenoids with oxygenated functional groups) react with these radicals by a different mechanism.

Superoxide produced by pulse radiolysis in an aqueous system containing β-carotene (solubilized in the neutral detergent Triton X-100) or lycopene (in hexane) resulted in the formation of carotenoid radical anions (Conn, 1992). The authors suggest a β-carotene-superoxide radical complex formed by addition of superoxide (or HO_2 the protenated form) rather than by electron transfer from superoxide. Lycopene, the open chain analogue of β-carotene, undergoes a reversible electron transfer with the superoxide anion, suggesting that lycopene does not undergo superoxide radical addition. These findings concur with the hypothesis of Burton on the process of peroxyl radical addition to the alicyclic ring structure of the β-carotene molecule (Burton, 1989). This would make β-carotene, and possibly other carotenoids with alicyclic structures, an important antioxidants in the autoxidation of lipid membranes.

Chemical reactivity of carotenoids depends on experimental conditions.

Chemical reactivities of carotenoids *in vitro* depends on: 1. partial pressures of oxygen; 2. chemical structures of the carotenoids; 3. interaction with other antioxidants (Palozza and Krinsky, 1992a); 4. and experimental protocol including radical species and method of generation, solvents (whether aqueous or non-aqueous system), targets, assays, lab conditions, storage, and history of the carotenoid.

Much of the work has been done using peroxyl radical-generating azo-compounds. Peroxyl radicals (ROO•) are less reactive than hydroxyl radicals (OH•), and are therefore able to diffuse greater distances and reach specific targets, such as plasma and intracellular membranes. Peroxyl radicals are among the most biologically damaging molecules because of their ability to induce lipid peroxidation reactions within biomembranes. They participate, for example in the toxicity of xenobiotics and oxygen-mediated processes in human disorders. Carotenoids are efficient scavengers of peroxyl radicals. *In vivo*, additional variables include interactions with other antioxidants, peroxyl scavenging and metal-chelating properties of plasma proteins, and the effects of membrane partitioning, which are idiosyncratic to the individual subject or species. Chemical responses of carotenoids in relation to different experimental conditions are discussed in the following sections. For this reason, care must be taken in the interpretation of *in vitro* results when implying biological relevance. However, information on mechanisms of action of carotenoids must be derived *in vitro*, and the following are reported findings with relevance to biological systems.

1. Response to different oxygen pressures.

In 1984 Burton and Ingold reported that β-carotene was a better antioxidant at low partial pressures of oxygen and this finding has since been confirmed in a variety of systems (Vile and Winterbourne, 1988; Moore, 1989; Kennedy, 1992). β-Carotene can exhibit prooxidant activity at high partial pressures of oxygen (Burton, 1989).

β-Carotene + ROO· ------------> ROO-β-Carotene· **[2]**

ROO-β-Carotene· + O_2 <--- ---> ROO-β-Carotene-OO· **[3]**

ROO-β-Carotene· + ROO· -----> inactive products **[4]**

Surprisingly, β-carotene, unlike α-tocopherol, reacts directly with ROO· by radical addition rather than by H abstraction. The result is a resonance-stabilized, carbon-centered radical (ROO-β-Carotene·). Increased oxygen pressure forces reaction [3] to the right, causing ROO-β-Carotene· to autoxidize. This new peroxyl species (ROO-β-Carotene-OO·) acts as a prooxidant, propagating a chain reaction. However, lower oxygen pressures forces reaction [3] to the left, lowering the prooxidant action of ROO-β-Carotene-OO·, and increasing the concentration of the less reactive ROO-β-Carotene· species. In addition, ROO-β-Carotene· can react with ROO· to form inactive products as in reaction [4]. We reported that under increased oxygen pressure, β-carotene (stored under argon, in the dark, at -80° C, for less than 1 year) contained

oxidation products, and increased lipid autoxidation of human retina homogenate (MSc thesis, E. Rousseau, 1994). Possibly, reports of β-carotene's prooxidant effects at high partial pressures of oxygen are due to contamination with oxidation products. Studies looking at the effects of different partial pressures of oxygen should ensure fresh carotenoid samples checked by HPLC for oxidation products.

2. Bioavailability and chemical reactivity are related to carotenoid structure.

Carotenoids include the hydrocarbon carotenes and xanthophylls, their oxygenated derivatives. Heat-treatment of foods increases the bioavailability of the hydrocarbon carotenes (β-carotene, α-carotene, lycopene) and decreases the bioavailability of xanthophylls (lutein, canthaxanthin, astaxanthin, epoxy carotenoids) (Glynn, 1990). Temperature effects on carotenoids need clearer delineation. Antioxidant potency and chemical reactivity depend on carotenoid structure and polarity. The most hydrophobic carotenoids include the carotenes (no oxygenated functional groups) lycopene and β-carotene. Xanthophylls are more polar than the carotenes, with crocetin being water-soluble. C3H/10T½ cells exhibit a greater uptake of polar carotenoids compared to nonpolar carotenoids (Bertram, 1991). The most polar carotenoid (lutein) exhibits the highest uptake, but is not as effective against methylcholanthrene-induced damage as canthaxanthin, β-carotene, α-carotene, or lycopene.

Some carotenoids, like lycopene and β-carotene (with 13 and 11 double bonds) are deeply pigmented red-orange, reflecting greater absorption of phototoxic blue light than some of the xanthophylls. Both these carotenoids are superior singlet oxygen quenchers (Di Mascio, 1989). Astaxanthin and canthaxanthin are more active scavengers of AMVN-

induced peroxyl radicals than β-carotene or zeaxanthin (Terao, 1989; Lim, 1992). These authors suggest that the presence of oxo groups at the 4 and 4' positions enhance peroxyl radical scavenging in this system. Similarly, two recent studies find that carbonyl and hydroxyl groups increase radical scavenging potencies. Astaxanthin protection against Fe^{2+} damage to linoleic acid is 10 and 100 times greater than other carotenoids and α-tocopherol, respectively (Miki, 1991). Astaxanthin is 100 to 500 times more protective than α-tocopherol against Fe^{2+}-induced lipid peroxidation of mitochondrial homogenates from the livers of vitamin E-deficient rats (Kurashige, 1990).

Astaxanthin is widely distributed in most animals, especially marine fish and shellfish. Under certain conditions, astaxanthin, with its polar oxo and alcohol group, is a carotenoid of superior scavenging capabilities compared to β-carotene. On this basis, astaxanthin should be compared with β-carotene in studies of lipid peroxidation, and its oxidative products should be described. When there are commercial sources of pure astaxanthin for experiments, chemoprevention trials using astaxanthin will be possible. Carotenoid structure affects the polarity of the molecule and thus its orientation within lipid membranes. Studies should include an array of carotenoids with different structures and functional groups to ensure a range of polarities.

Carotenoids span lipid bilayers in model membranes and bacteria.

Ambi-polar molecules such as carotenoids, tocopherols, and cholesterol partition (along with components of the lipid bilayer such as phospholipids) with the hydrophobic chains to the center of the bilayer and the more polar heads towards the outer and inner aqueous interface. Organic radicals (R·, RO·, and particularly ROO·) react with

membranous components. Peroxyl radicals (due to the polar nature of the oxygen group) may partition towards the hydrophilic membrane-water interface. The azo-initiated production of 15,15'-epoxy β-carotene in hexane (Kennedy, 1991) could not be duplicated in liposomes (Kennedy, 1992). This suggests that β-carotene orients or spans across the lipid bilayer where the 15,15' bond (centrally located within the membrane) was not accessible to peroxyl radicals approaching from the hydrophilic membrane surface (Kennedy, 1992). Similarly, bacterial carotenoids containing one or two terminal polar groups reportedly span the membrane bilayer acting as molecular rivets, to increase membrane rigidity (Ourisson, 1989). If similar partitioning occurs in human systems, we can come closer to understanding the mechanisms of action of carotenoids and other dietary antioxidants.

Astaxanthin may have special antioxidant qualities.

Astaxanthin (in phospholipid membranes) could situate across the membrane, its hydrophobic isoprenoid chain within the nonpolar center of the membrane and polar heads at the membrane surface in contact with the intra and extracellular aqueous phases (Lim et al, 1992). Theoretically, a molecule such as astaxanthin could act as a carotene and protect against intramembranous lipid peroxidation chain reactions by ROO• addition, and scavenge ROO• by hydrogen abstraction like α-tocopherol. If the hydroxyl group on astaxanthin's ring can function like the phenolic •OH of tocopherol (to prevent chain propagation by H donation), then it is also subject to repair by ascorbate. Possibly, this explains the superior antioxidant activity of astaxanthin compared to tocopherol and other carotenoids (Miki, 1991; Kurashige, 1990). In addition, perhaps the more polar xanthophylls are capable of scavenging aqueous phase oxygen radicals (superoxide,

hydroxyl) and active oxygen species (hydrogen peroxide and singlet oxygen) immediately at the membrane surface. The more polar xanthophylls would compliment the antioxidant activity of the more hydrophobic carotenes such as β-carotene, α-carotene, and lycopene, and provide more complete protection of biological systems.

Carotenoids protect LDL.

Non-polar micronutrients are transported in LDL (low density lipoproteins). Components of LDL (polyunsaturated fatty acids, cholesterol, and apoprotein B) can undergo oxidative attack, initiating phagocytosis by macrophages. The oxidized LDL accumulates beneath the endothelial wall, forming a fatty streak. This streak forms the site of an atherosclerotic plaque, the major factor in coronary heart disease. The Physicians Health Trial reports that dietary carotenoids are inversely related to coronary heart disease and stroke (Physicians Health Trial, 1992). Similarly, higher plasma β-carotene levels are associated with a lower risk of angina. As low density lipoproteins (LDL) are the major carrier of carotenoids in humans, their presence may protect against oxidation of LDL. The mechanism of protection is likely similar to those that occur in model membranes, bacteria, tissue homogenates , and even *in vivo* situations. Antioxidant function is contingent upon how the carotenoid partitions within the system.

Interactions with other antioxidants.

β-Carotene and α-tocopherol are lipid-soluble antioxidants that protect non-aqueous fractions such as membranes and lipoproteins. Some studies have found that

β-carotene and α-tocopherol spare or prolong the protective effects of one another (Terao 1980; Palozza, 1991; Esterbauer, 1989a; Esterbauer, 1991). A recent *in vitro* study has suggested a synergistic interaction between β-carotene and α-tocopherol, where in combination they confer greater protection to rat liver microsomes than the sum of the individual antioxidants (Palozza and Krinsky, 1992). A mixture of β-carotene, reduced glutathione, α-tocopherol, and ascorbic acid worked synergistically to protect hamsters against (7,12-dimethylbenz(a)anthracene (DMBA) induced oral tumors (Shklar, 1993). Perhaps they act together providing overall protection at the different oxygen tensions found throughout a biosystem.

Vitamin C (ascorbate) and plasma proteins protect aqueous fractions against free radical damage. Ascorbate is the first line of defense against Cu^{2+}-induced lipid peroxidation of LDL, sparing α-tocopherol and β-carotene until it is consumed (Esterbauer, 1989b). Regeneration of α-tocopherol by ascorbate *in vitro* has been postulated (Niki, 1982; Sato, 1990). Similar reactions with β-carotene are dubious because its antioxidant mechanism is thought to be by radical addition rather than H abstraction. Possibly, the increased antioxidant activity of astaxanthin is attributable to ascorbate regeneration of its hydroxyl group. Whatever the relationship between carotenoids and other antioxidants, it is apparent that higher levels of carotenoids confer protection against the effects of oxygen-mediated stress in a variety of systems.

In vivo conversion of β-carotene to retinol proceeds by excentric cleavage.

Retinol is formed only from carotenoids with pro-vitamin A activity, with the most nutritionally active including β-carotene, α-carotene, and β-cryptoxanthin. Conversion of

these carotenoids by animals (and some microorganisms) is the only known source of retinol in nature. Interestingly, fish are able to metabolize carotenoids without vitamin A activity such as astaxanthin, canthaxanthin, and isozeaxanthin to β-carotene, which in turn can be converted to retinol (Olson, 1989).

Proponents of the central cleavage theory (Goodman and Huang, 1965; Olson and Hayaishi, 1965) claimed that incubation of β-carotene with preparations of intestine and liver yielded retinol as the only product. The enzyme, thus named β-carotenoid-15,15'-dioxygenase, was isolated from the intestines of several species, including man. More recently, similar procedures using rat intestinal mucosa failed to produce evidence of enzymatic central cleavage of β-carotene (Hansen and Maret, 1988). The use of rodent models in carotenoid experiments may be questionable as rodents are found to be absorb carotenoids sparingly.

Others have supported the excentric cleavage theory (Wang et al, 1991). In a comprehensive study, homogenates of intestinal mucosa from human, monkey, ferret, and rat were incubated with β-carotene (Tang et al. 1991). Characterization of β-apo-13-carotenone and β-apo-14'-carotenal was determined by HPLC, UV/visible spectra, reduction to the corresponding alcohol, oxime formation, and by mass spectrometry. Direct proof of an enzymatic excentric cleavage mechanism for β-carotene was finally provided.

These *in vitro* studies with intestinal homogenate give a glimpse at the possible mechanisms that occur *in vivo*. In light of the evidence, it is likely that both excentric and central cleavage pathways occur. Conversion mechanisms are possibly carotenoid, species, and even tissue specific. Conversion is known to occur in liver and several other organs in addition to the intestine. It would be interesting to know if the carotenoids

lutein and zeaxanthin, which are accumulated in the human retina, are converted on site to 11-*cis* retinal. This retinoid is part of the visual pigment rhodopsin, and adequate concentrations are necessary for optimal functioning of the retinal photoreceptors.

Potential problems in experimental studies with carotenoids.

There are many pitfalls for the unwary entering the uncertain realm of carotenoid research. In this section we outline some experimental problems, and suggest which are intractable, and which are susceptible to solution if proper precautions are taken. A large number of controls and precautions are necessary if painfully obtained data are not to be tainted with awkward questions. Unfortunately the research archives are littered with spurious data and unfounded inferences resulting from failure to take painful but necessary precautions. We outline hereunder some of the more obvious pitfalls.

Chemical instability and limited shelf-life of carotenoids and retinoids.

Those using carotenoids for supplementation, or as laboratory standards should be aware that crystalline β-carotene, like β-carotene in solution, is susceptible to air- and light-induced oxidation. This is not fully prevented by the usual precautions: refrigeration to -80°C. under nitrogen or argon, desiccants, amber containers, and yellow-filtered laboratory lighting. We have tested unsealed bottles of β-carotene subjected to prolonged (5 years) storage which contained as little as 5% carotene, the remainder of the material apparently being oxidative or other breakdown products. According to undocumented reports, there is a chemical hazard associated with a propensity for spontaneous ignition as

explosion in freezer-stored β-carotene warmed to room temperature. Preliminary testing indicates that the oxidized β-carotene contains thiobarbituric acid reactive substances (TBARS), and may increase peroxidation of lipids (MSc thesis, Rousseau, 1994). The stability of β-carotene under various experimental conditions has been analyzed (Scita, 1992a; Scita, 1992b).

HPLC analysis indicates that the degradation products are more polar than β-carotene, and are bleached, in that they have a much decreased visible absorption relative to the parent compound. Unless crystalline β-carotene is unequivocally pure, analytical standards and stock solutions for experimental work should not be prepared by weight. At the very least, it is advisable to check the purity of all carotene used in experimental work. A rough measure of the purity of crystalline β-carotene may be obtained by completely dissolving a small weighed sample of β-carotene in hexane and measuring its visible absorption. Samples containing bleached degradation products will have less absorption than that calculated from the extinction coefficient. Normal or reverse-phase chromatography by thin-layer or HPLC can be used to directly separate and visualize polar degradation products from non-polar β-carotene. However, when attempting to estimate the level of impurities using chromatography it must be remembered that degradation products are bleached, and have relatively little visible absorption per unit weight. Up-to-date and comprehensive methods of separation and quantitation of carotenoids can be found in a recent review (Packer, 1992).

Physical instability of carotenoid preparations in aqueous media.

Another problem is the instability of carotenoid suspensions in aqueous media. In the simplest approach, the carotenoids may be dissolved in a hydrophobic solvent, and rapidly injected into the aqueous medium through a syringe, with rapid stirring. If the medium contains lipid, this may be acceptable. For example, if the medium contains micelles or cell membranes, the injected carotenoid should partition itself mainly in the lipid phase. In the absence of stabilizing detergents or stably suspended lipids, micellar aggregation occurs readily, especially if a plastic container having a hydrophobic surface is used. All too easily the colloidal state may be broken, especially in the presence of electrolytes like buffer salts. A few nanograms of β-carotene can become a microscopic globule on the side of a test-tube while the experimenter unknowingly pipettes out a solution from which almost all the carotenoid has dissipated. Controls should include measurement of the fractions of the carotenoid which can be recovered from the medium and target after addition. We turn now to methods available for producing stable preparations of carotenoids in aqueous suspension.

Methods for stabilization of carotenoids in hydrophilic (aqueous) media.

Four methods are available for suspension of a carotenoid of interest to an aqueous medium such as a culture medium. Be aware, however, that none fully replicates the chemical circumstances by which carotenoids find their intracellular locations *in vivo*.

1) Carotenoids and liposomal components may be dissolved in a hydrophobic solvent, evaporated under nitrogen and the residue suspended in an aqueous detergent solution

with vortexing. The original method used chloroform as a solvent and dodecyl sulfate as a detergent (Anderson and Krinsky, 1973). Traces of acid in the chloroform, however, may degrade the carotenoid. More recent methods dissolve β-carotene in tetrahydrofuran (THF), add fatty acids in hexane, and evaporate to dryness under argon (Canfield, 1990). The film is suspended in buffered bile, with shaking and sonication until micelles are formed. These methods are to be recommended only with caution. Care must be taken in any attempt to make a saturated solution of carotene in moderately polar solvents such as alcohol or DMSO. If more crystalline material is used than will completely dissolve, the solvent can selectively dissolve the more soluble polar carotenoid degradation products, lowering the purity of the material in solution. Among the solvents well tolerated by biological systems, tetrahydrofuran is reportedly the best solvent for β-carotene.

2) Small amounts of a detergent may be added to the carotenoid in hydrophobic solution. The solution is evaporated to dryness under vacuum and the residue forms an aqueous dispersion in buffer, which is then filtered (Aziz et al, 1971; Kanner and Kinsella, 1983; Ekstrand and Bjorck, 1986). Alternatively, pre-stabilized preparations of β-carotene beadlets manufactured for dietary supplementation (a product of Hoffmann-LaRoche) combine carotenoids with a protein matrix. This too forms a stably dispersed suspension if mixed with aqueous media and filtered (Bertram, 1991). The use of stabilizers partly solves the problem of physical instability. However, even if the procedure is standardized the liposomes or micelles are likely to come out of dispersion and either float on the surface or attach as globules to the wall of the container. Attempts to solve the difficulty by using sufficient concentrations of stabilizers simply add another problem, that of controlling for any biological actions of the stabilizer. The stabilizing agents in the beadlets are not well defined, but a mixture of the non-carotene ingredients may be obtainable from the manufacturer for use in control experiments. In addition, the

protein matrix itself may exert effects independent of the carotenoids. Only β-carotene and canthaxanthin are available, which limits experimental conditions.

3) β-Carotene enriched plasma can be obtained from animals fed diets supplemented with β-carotene (Alam and Alam, 1983; Alam et al, 1990). Plasma from supplemented animals probably deserves to be used more frequently. Note however, that the problem of controlling for biological activity of the stabilizers (natural in this case) persists. Even the use of plasma from unsupplemented animals does not provide an unequivocal control, since the supplementation may have modified levels of active agents (such as growth factors) in the plasma.

5) Carotenoids dissolved in THF can be injected into culture medium. Carotenoids delivered to cells by this method are readily incorporated and the THF results in a low solvent toxicity. The last method, using THF for direct delivery of carotenoids in biosystems, appears to have overcome some of the problems associated with delivering hydrophobic carotenoids in aqueous media (Bertram, 1991). We have successfully delivered carotenoids to saline homogenates of human retina using THF as a solvent (MSc thesis, E. Rousseau, 1994).

A paucity of information is available on the relative merits of these alternate methods. The question of how to add carotenoids to aqueous media is a major unsolved problem in the use of carotenoids *in vitro*, and deserves a high priority in current research. Moreover, studies of the actions of oxidants and antioxidants in tissue homogenate suspension are just beginning.

Discussion

Since the recognition that free radicals and oxygen-mediated stress are associated with many human disorders, the field of antioxidant chemistry has opened new avenues of research. "Carotenology" is certainly one of the most challenging areas of antioxidant research, just by virtue of its ubiquitous nature, the numerous varieties of carotenoids, and its restrictive experimental protocols. Proper storage and research techniques will increase reliability and relevance of experimental results. Mechanisms of action are far from complete,however, it appears a main function is to scavenge chain-carrying peroxyl radicals and that xanthophylls may be superior to carotenes as antioxidants. Questions on membrane partitioning of different carotenoids, interactions with other antioxidants, and chemical reactivities within polar and nonpolar environments need to be answered. Current research must be expanded to carotenoids other than β-carotene and commercial sources must be made available. The increasing number of human disorders being attributed to low intakes of carotenoids has prompted many people to self-prescribe β-carotene. Therefore, it is imperative that we delineate actions of carotenoid antioxidant activity, and also describe the possible hazards of β-carotene oxidation in dietary supplements.

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CHAPTER 5

The human retina as a research tool in free radical research.

by

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Abstract.

Much of the free radical research to date has been in organic solvents, micelles, liposomes, and microsomes. Human retina homogenate is a valuable test medium for free radical research with high *in vivo* relevance to human systems. Retina membranes, by virtue of their high content of the most unsaturated fatty acid found in the human body, and constant exposure to light, are potential targets for active oxygen species and lipid autoxidation. The evidence suggests that damage to retina photoreceptors and associated structures occurs by free radical-mediated processes. Photochemical and metal systems induce lipid autoxidation of retina homogenate by different active oxygen species. Damage to retina homogenate by different systems can be compared and mechanisms of protection by different antioxidants studied.

Introduction.

This chapter reviews carotenoid and retinoid functions, focusing on their roles in the anatomy and physiology of the human retina. The mechanism of vision is discussed. We examine evidence for free-radical based pathology in diseases of the retina and associated structures, and for roles of the antioxidant vitamins. Of special interest are the carotenoids, which possibly serve multiple functions within the human eye. The presence of (only) zeaxanthin and lutein within the retina, and especially the macula, raises questions as to their function. Active oxygen species created in the photochemical and Fenton (metal) reactions induce lipid autoxidation of human retina by different processes. Human retina can be a test medium for a range of free radical research studies of significance to human systems. This information is designed to assist those considering using human retina in future research.

Section 1.

Roles of retinoids and carotenoids in the eye.

Sources of retinoids and carotenoids.

Some 15% of the 600 known carotenoids are potential precursors to vitamin A. β-carotene, a carotenoid with the highest known provitamin A activity, is found in yellow-orange vegetables and fruits and dark leafy green vegetables. There are two theories of carotenoid cleavage. In central cleavage, β-carotene for example, is oxidatively cleaved at the center of the molecule (15,15' bond), creating two molecules of retinol (Goodman and Huang, 1965; Olson, 1989). Excentric cleavage suggests that bonds other than the 15,15' bond are cleaved (Tang et al, 1991; Wang et al, 1991) in the conversion to retinol. In either event, the retinol is then esterified and stored in tissues. Retinol (vitamin A) cannot be synthesized *de novo* by animals or plants. Animal requirements for retinol are met by ingesting animal tissues or indirectly by consuming carotenoids from plant sources. Blood levels of retinol are tightly regulated by cellular retinol binding proteins (CRBP) at around 2.5 μM. Higher levels are toxic. Plasma carotenoids are mainly transported in LDL (low density lipoprotein), and have serum concentrations that vary widely but average around 1.187 ± .617 μM for carotenes and .812 ± .275 for xanthophylls (Krinsky et al, 1990). Unlike retinoids, high levels of carotenoids are nontoxic. Besides having provitamin A activity, carotenoids reportedly increase immunostimulation and protect against some types of cancer, heart disease, cataracts, age-related macular degeneration (AMD), and phototoxic reactions. Functions of retinol include stimulation of growth, differentiation of cells and bone, maintaining reproductive systems, resistance to infections, and ocular integrity.

The retina contains highly specialized photoreceptor cells.

The human retina is comprised of photoreceptor cells (differentiated by microstructure) known as rods and cones that encompass over 180 degrees of the eye's posterior globe (Figure 1). Each rod photoreceptor contains a stack of approximately 1000 disks. The membranes of these disks are packed with rhodopsin, consisting of the protein opsin and 11-*cis*-retinal. Rods, capable of responding to dim and bright light, are responsible for night vision. Cones are activated only by bright light and are responsible for colour vision. There are three kinds of colour receptors, blue,-, green-, and red-absorbing cones, responsible for colour vision. These photoreceptors also contain 11-*cis*-retinal bound to proteins other than opsin. The chromophore 11-*cis*-retinal is bound to rod and cone proteins by a Schiff-base linkage to an amino group. In humans, rods are more abundant than cones, with 100×10^6 rods compared to 3×10^6 cones per retina.

The focal point of the primate retina, known as the macula, is situated lateral to the exit for the optic nerve at the posterior of the globe. In the center of the macula is the fovea, which has as its central area the foveola, responsible for acute central vision (Figure 2). The human fovea has very little neural tissue, therefore incoming light hits the photoreceptors directly. Elsewhere in the retina, light must pass through the neural retina, cell bodies, ganglion, amacrine, bipolar, horizontal, nuclei, and Mueller cells (Handelman et al, 1992). The macula or *macula lutea*, (meaning yellow spot) derives its pigmentation from the yellow carotenoids lutein and zeaxanthin. The highest density of cone receptors are found in this area, and possibly the carotenoids function to protect these photoreceptors from damage. Protection may deteriorate with age, or damage may accumulate over many decades. Age-related macular degeneration affects 30% of humans

over 75 years of age, and is more prevalent in persons with light iris pigmentation, indicating a possible phototoxic etiology (Ferris, 1983).

Retinol is required for vision.

11-*cis*-Retinal is the photosensitive chromophore that acts as a light-harvesting antennae in all photoreceptors. Derived from all-*trans*-retinol, it absorbs a broad spectrum of visible light that matches the solar output and has a high absorption coefficient of 40,000 cm^{-1} M^{-1} at 500 nm in rod cells. Blue-, green-, and red-absorbing cone cell proteins (similar to opsin) shift the chromophoric properties of 11-*cis*-retinal over 100 nanometers from blue (440 nm) to red (625 nm) by changing the conformation of 11-*cis*-retinal's polyene chain. A deficiency of retinol or of carotenoids with vitamin A activity can result in night blindness (and possible deterioration of the rod outer segments), hence the adage that "carrots help you see in the dark".

The visual process begins with absorption of a photon by 11-cis-retinal.

The primary event in the visual process is the capture of a photon by 11-*cis*-retinal. Within a few picoseconds, light energy isomerizes 11-*cis*-retinal to all-*trans*-retinal causing a 5 Å increase in molecule length. The atomic motion of photoexcited rhodopsin initiates an enzymatic cascade that results in a decrease of cGMP (cyclic guanosine monophosphate) in the outer segment of rod photoreceptor surrounding the disks. The plasma membrane maintains an electrochemical gradient with Na^+ -K^+ ATPase pumps in the inner segment of the photoreceptors. In the dark-adapted state, the Na^+ channels are

open, allowing a steady flow of ions into the outer segment of the rod. Hydrolysis of cGMP closes the Na^+ channels in the plasma membrane, causing a negative ion gradient on the inside of the membrane. This light-induced hyperpolarization is transmitted to the synaptic body, leading to neurotransmitter release to the horizontal and bipolar cells. Signals from these cells then travel to ganglion cells (anterior retina) where the information is integrated and transmitted via the optic nerve to the visual cortex of the brain.

One photon closes hundreds of cation specific channels, blocking the flow of more than a million Na^+ ions. Interestingly, rod cells do not have all or none action potentials like other excitable cells, but display a graded response related to the intensity of light. After about a minute, the all-*trans* form diffuses away from the protein where its stereochemistry prohibits it from fitting into the binding site. All-*trans*-retinal is isomerized back to 11-*cis*-retinal (by retinal isomerase) in the dark, and is then available to reassociate with opsin to form rhodopsin.

The universal nature of 11-*cis*-retinal indicates that it is an excellent chromophore.

11-*cis*-Retinal is found in the visual pigments of mollusks, arthropods, and vertebrates. Retinal is also the chromophore in purple bacteria as bacteriorhodopsin and halorhodopsin. Its ubiquitous nature indicates that 11-*cis*-retinal is a very efficient chromophore, with features that make it particularly appropriate for light absorption. The polyene network of 6 double bonds allows electron transitions which provide an intense absorption band in the visible region of the spectrum, from 440 to 625 nm. It is easily isomerized, creating atomic motion of a sufficient magnitude to induce a cascade of events

leading to a nerve impulse. It is astonishingly photosensitive. One photon can excite a single chromophore molecule, while in contrast its rate of isomerization in the dark is estimated to be once in 1000 years.

The retinal pigment epithelium maintains the photoreceptors.

The retinal pigment epithelium (RPE) is a monolayer of cells situated anterior to the Bruch's membrane and posterior to the photoreceptor outer segments (Figure 3). The RPE cells are separated from each other by tight junctions, constituting a blood-retina barrier, and actually surround the tips of the outer segments. These cells are responsible for nourishment and maintenance of the photoreceptors. In addition, the RPE cells phagocytose and digest the aged disks that are constantly shed from the tip of the outer segments. The RPE is responsible for retinol storage (mainly esterified with palmitic acid), transport and isomerization of all-*trans*-retinal to 11-*cis*-retinal. The RPE also has high concentrations of endoplasmic reticulum, antioxidant enzymes, and melanin. Damage to the RPE results in damage or death of photoreceptor cells.

Section 2.

Oxidative damage to retinas and associated structures.

A range of studies suggest pathology of mammalian retinas and associated structures is mediated by oxidative damage.

- Vitamin E deficient dogs are susceptible to severe photoreceptor damage (Hayes et al, 1970; Riis, 1981).
- *In vitro*, rod outer segment (ROS) membranes from vitamin E-deficient cattle undergo increased lipid autoxidation compared with cattle on normal diets (Farnsworth, 1976).
- Autoxidation of cellular components (such as vitamin A) reportedly plays a significant role in lipofuscin accumulation. Vitamin A can undergo autoxidation and under specific conditions acts as a photosensitizer producing singlet oxygen. Vitamin A-deficient rats had a significant reduction in lipofuscin deposition when supplemented with vitamin E (Katz et al, 1978; Stone and Dratz, 1980).
- Vitamin E and selenium deficient rats had increased lipofuscin buildup in the RPE (Katz et al, 1978).
- Dogs exposed to elevated levels of oxygen (1 atm. for 48 hr.) display retinal detachment and hemorrhages, this effect is increased with the photosensitizers phenothiazine and chloroquine (Beehler and Roberts, 1968).
- In humans, an iron fragment in the vitreous chamber or near the retina will cause vision loss within 1-2 years (Grant, 1974). Fe^{2+} and H_2O_2 undergo a Fenton reaction to produce the highly damaging hydroxyl radical (OH•). Fe^{2+} also catalyzes a Haber-Weiss reaction between superoxide and H_2O_2 to produce hydroxyl radicals.

Oxidative stress increases lipofuscin buildup in the RPE.

The RPE is the main site of lipofuscin buildup. Lipofuscin is the indigestible residue that accumulates (as granules) after autoxidation of cellular components such as vitamin A and lipids, or possibly oxidation of protein. Age-related macular degeneration is characterized by a deterioration of the RPE and photoreceptors. The pathogenesis of age-related macular degeneration may include an increase in oxidative stress and/or a weakening of antioxidant defenses, leading to increased damage of the outer segments or the RPE. It is difficult to determine whether the primary event is caused by a malfunctioning RPE or if there is increased damage of the outer segments, overwhelming the RPE. In any event, one of the risk factors for neovascular age-related macular degeneration is low serum carotenoid levels (Eye Disease Case-Control Study Group, 1992). This finding, plus the observations of the above *in vivo* studies, provide convincing evidence that some diseases of the retina and associated structures have a free radical-based pathology, and that some antioxidant micronutrients (tocopherol, selenium, carotenoids) may protect. We turn now to consider the biochemical basis of antioxidant activity of the retina.

Antioxidants in the mammalian retina.

Antioxidant defense mechanisms in the retina were reviewed by Handelman and Dratz (1986) as follows:

- The mammalian retina contains scavengers that reduce active oxygen species such as glutathione (Hermann and Moses, 1945; Winkler and Giblin, 1983), selenium-dependent glutathione peroxidase (Reim, 1974; Singh, 1984), several enzymes of the

glutathione-S-transferase group (Stone and Dratz, 1980), Cu-Zn superoxide dismutase (SOD) (Bensinger et al, 1982), and catalase (Armstrong et al, 1981).

- Melanin is thought to function as a light-trap. Black people are found to have more choroidal and iris melanin and a lower incidence of AMD than people with light coloured irises (blue, green, and grey) (Ferris, 1983). Melanin is also found in the RPE, however, it appears to be independent of iris colour.
- Dietary antioxidants present in the retina include vitamin E, ascorbate, and carotenoids. The major carotenoids present are zeaxanthin and lutein.

A recent investigation of neovascular age-related macular degeneration in subjects over 55 years of age suggests that higher levels of serum antioxidant nutrients confer decreased risk of AMD. Subjects with medium and high serum (total) carotenoid levels had a one-third and one-half decreased risk of AMD respectively, compared to those with low serum levels (Eye Disease Case-Control Study Group, 1993). Photoxidative damage to the lens results in cataracts, while higher serum levels of the antioxidant vitamins (carotenoids, α-tocopherol, and ascorbate) are associated with decreased damage (Knekt et al, 1992; Taylor, 1993; Taylor, 1992). Protection against disease could be due to the higher levels of carotenoids, or possibly some other micronutrient associated with carotenoid-containing foods.

Possible roles of carotenoids in the retina.

Reportedly, only the retina (and especially the macula) accumulates lutein and zeaxanthin. These carotenoids are di-hydroxylated versions of β-carotene, with lutein having a double bond shifted from the 6' to 5' carbon in the β-ionone ring. The

accumulation of lutein and zeaxanthin, which give rise to the yellow colour of the *macula lutea*, may filter visible light reaching this highly specialized area. The concentration of carotenoids in the macula is sufficient to attenuate blue light by 80-90% (Handelman and Dratz, 1986) where the thickness of retina may be between 25 to 40 μm.

In vitro studies have found xanthophylls to be superior peroxyl radical scavengers compared to β-carotene (Kurashige et al, 1990; Lim et al, 1992; Terao, 1989; Bertram et al, 1991). β-Carotene antioxidant activity is reportedly increased at low partial pressures of oxygen (pO_2) (Burton and Ingold, 1984). Lutein and zeaxanthin may function as effective antioxidants at the low partial pressures of oxygen found in the neural layers of the human retina. Possibly, these carotenoids serve other lesser functions including:

- mechanical stability of lipid membranes;
- quenching of triplet states like singlet oxygen;
- sparing other endogenous antioxidants;

These mechanisms could serve to enhance the potential of carotenoids to prevent lipid peroxidation of retina membranes. It is important to compare carotenoids with different functional groups and structural changes, and determine how antioxidant potency changes. In addition, responses of different carotenoids to a range of free radical-generating systems will help define mechanisms of action.

Section 3.
The human retina as an experimental tool.

The use of human tissue gives us a more relevant model for extrapolation to the intact human than do experiments in organic solvent, micelles (an aggregate of bipolar molecules oriented with the polar heads facing out towards the aqueous environment), liposomes (multilamellar model membranes), or animal tissue systems. Human retinas from persons of various ages are available as a by-product of the post-mortem harvesting of corneas for transplant.

Human retina is a useful and available tissue for studies of antioxidants.

The following are features of this tissue that make it an appropriate tool for free-radical mediated research:

- The retina is constantly exposed to light, sometimes to intense light, which may lead to photooxidative damage. Blue light (440 nm) or green light (556 nm) reportedly damages the retinal pigment epithelium (Handelman and Dratz, 1986). Damage by light may therefore be due to a thermal mechanism (accidental exposure to intense light), or via a photosensitizing mechanism producing either singlet oxygen or superoxide.
- *In vitro*, vertebrate retinas exhibit a seven fold higher oxygen consumption/mg protein than any other tissue tested (Sickel, 1972).
- In vertebrates, rod membrane phospholipids contain about 50% docosahexaenoic acid (22:6ω3), the most unsaturated fatty acid found in nature, making them an excellent target for lipid peroxidation (Stone et al, 1979).

- The inner segments of the photoreceptor cells contain high concentrations of mitochondria which may leak active oxygen species from the electron transport chain (Handelman, 1986).
- For experiments that focus on photosensitized damage and protection by antioxidants, the human retina represents a tissue specialized for light reactions.
- The retina is readily homogenized, and homogenates are easily pipetted, and remains as an aqueous dispersion.
- The disrupted membranes respond reliably to oxygen-mediated stress.
- Tissues were made available for use by donation.
- Retinas stored at -80° C under argon are stable for up to 1 year after harvesting.
- Right and left retinas are similar in size, photoreceptor content, carotenoid content, and presumably antioxidant content. This allows for comparative investigations of different experimental conditions using retina pairs.
- Subject information and results from peroxidation experiments on retinas can be correlated with histological information related to changes in the associated structures posterior to the retina.

Oxygen-mediated stress is induced by photodynamic or chemical processes.

Photosensitizers are both endogenous and exogenous.

The light-absorbing characteristics of the retina are rapidly lost post-mortem as a result of the depletion of energy charge. To mimic the dye-sensitized processes of the living retina *in vitro*, it is therefore necessary to add exogenous photosensitizers. Endogenous photosensitizers include the blue-absorbing flavins, retinol, (Handelman, 1986) haems, and cytochromes (Kirschfeld, 1982). Xenobiotic photosensitizing drugs include the psychotropic drugs chlorpromazine and thioridazine, the antimalarial and antiarthritic chloroquine derivatives, some oral contraceptives, synthetic retinoids, tetracyline, and psoralens (Javitt and Taylor, 1991). These medications have the potential to increase sunburn to exposed skin or ocular damage. The question of whether they sensitize the retina to damage deserves attention.

Photodamage varies with the wavelength of light.

Ambient light is composed of a range of wavelengths or bands of energy, each associated with various biological effects. The retina is well protected against the most damaging wavelengths, namely ultraviolet radiation. Ultraviolet radiation is loosely categorized into 3 bands.

- UV-C (100-290 nm), is absorbed by the atmosphere's ozone layer. Any UV-C encountered is completely absorbed by the cornea. Exposure to UV-C as seen in "arc welder's flash" results in keratopathy or an ocular sunburn.

- UV-B (290-320 nm), causes sunburn, erythema, blistering, and possible melanoma. Nearly 100% of UV-B is absorbed by the lens.

- UV-A (320-400 nm), known as near UV is responsible for suntanning. The lens absorbs approximately 98% of UV-A.

The visible spectrum ranges from 400-700 nm, and of these wavelengths, blue light (400-500 nm) is reportedly the most phototoxic to the retina. Light of wavelengths greater than 400 nm cannot directly break covalent bonds. Nevertheless, visible light can induce damage to the retina through production of active oxygen species (singlet oxygen or superoxide anion) via a "dye-sensitized" photooxidation mechanism. The macular area contains very little neural tissue, and within it the fovea is the site of our most acute vision. Light directly focusing on this area could be particularly damaging to the photoreceptor membranes. A high flux of activated oxygen species might well increase peroxidation of the photoreceptors, overwhelming antioxidant defenses and degrading the RPE, as observed in AMD.

Light-induced damage to retina involves activated oxygen species, and is aggravated by a photosensitizer.

UV (small percentage of UV-A reaches the retina) and visible light induce lesions in the vertebrate retina. Although the primary events are not yet mechanistically characterized, chronic illumination *in vivo* results in lipid peroxidation of the retina with destruction of photoreceptor cells (Riis et al, 1981). The initial damage is seen at the tips

of the rod outer segments (ROS), in the form of dilations and vesiculations of the ROS disks. With continued illumination, the damage extends towards the inner segments (towards anterior of the eye) of the of the photoreceptor. Protection against light-induced photoreceptor lesions is provided by the test drug, WR-77913. This compound scavenges singlet oxygen, hydrated electrons and free radicals, indicating that the damage involves a photodynamic mechanism with the production of free radicals (Reme et al, 1991). In the current study, without a photosensitizer, retina homogenate resisted damage by fluorescent light, UV-C light, or a 300 watt quartz halogen incandescent bulb within a reasonable time period (1 hour). Nevertheless, substantial photodynamic damage (MDA equivalents) ensued when the photosensitizer rose Bengal was added. There are endogenous photosensitizers in the retina, thus their replacement with rose Bengal is an attempt to model processes that occur naturally in the retina.

Metal-catalyzed oxygen free radical injury.

In addition to the photosensitized reaction, an iron/ascorbate system can be used to generate different species of free radicals. Ferric iron, when added to porcine retina homogenate, increased lipid peroxidation as measured by the (thiobarbituric acid) TBA method (Hiramitsu and Armstrong, 1991). We have used a similar test system to assess the protective effects of dietary antioxidants. A common industrial injury encountered by ophthalmologists is the entry of a foreign metal particle into the eye. Therapeutic treatment of iron-induced retinal injury with metal chelators or dietary antioxidants could potentially allow more time to locate and remove the particle before it causes irreversible damage.

Future Research.

Functions of carotenoids (in the human retina) may include the following: acting as a mechanical filter for all wavelengths of light (especially attenuating phototoxic blue light), acting as singlet oxygen scavengers and antioxidants, acting to potentiate the actions of the more active antioxidants, sparing endogenous antioxidants, membrane stabilization, or serving as a pool for 11-*cis* retinal. Future studies could include looking at the ability of all serum carotenoids to cross the blood-retinal barrier. Possibly other carotenoids are able to cross the blood/retina barrier, but are preferentially are used up in biochemical and/or free radical processes. Resistance of photoreceptor membranes to lipid autoxidation as a function of carotenoid (or other antioxidant) level could be studied. This type of information would be valuable in histological research that determines actual physical damage to the photoreceptors. Such research could help define mechanisms of action of antioxidants and lipid autoxidation processes in diseases of the eye, as well as other diseases with a free-radical based pathology.

Summary.

Involvement of free radical-mediated processes in diseases of the eye, and especially the retina, is substantiated experimentally. To date, the roles of antioxidants are best defined in animal models. In humans, epidemiological studies imply correlations between serum β-carotene levels and incidence of cataracts and age-related macular degeneration. However, biochemical processes and mechanisms of action can only be determined by *in vitro* studies. *In vitro* studies with human retina give relevant information regarding responses to different types of oxygen-mediated stress and the effects of a range of antioxidants. This type of much-needed information will help in the development of dietary protocols and chemoprevention regimens for high risk and normal populations.

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Legends to Figures.

Figure 1. Diagram of a cross-section of the human eye. Reprinted by permission from: Handelman and Dratz. *Advances in Free Radical Biology and Medicine*. Vol. 2(1) page 5; 1986.

Figure 2. Light micrograph of the macular region of the macacque retina. Neural layers are thinnest at the foveola. Reprinted by permission from: Handelman and Dratz. *Advances in Free Radical Biology and Medicine*. Vol. 2(1) page 7; 1986.

Figure 3. Diagram of the cellular structure of the human eye. Reprinted by permission from: Handelman and Dratz. *Advances in Free Radical Biology and Medicine*. Vol. 2(1) page 6; 1986.

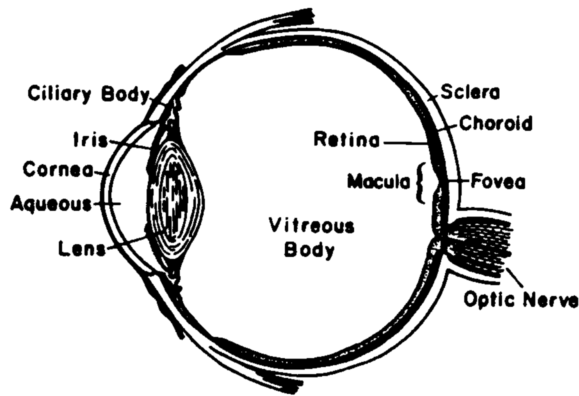

Diagram of cross-section of the human eye.

FIGURE 1

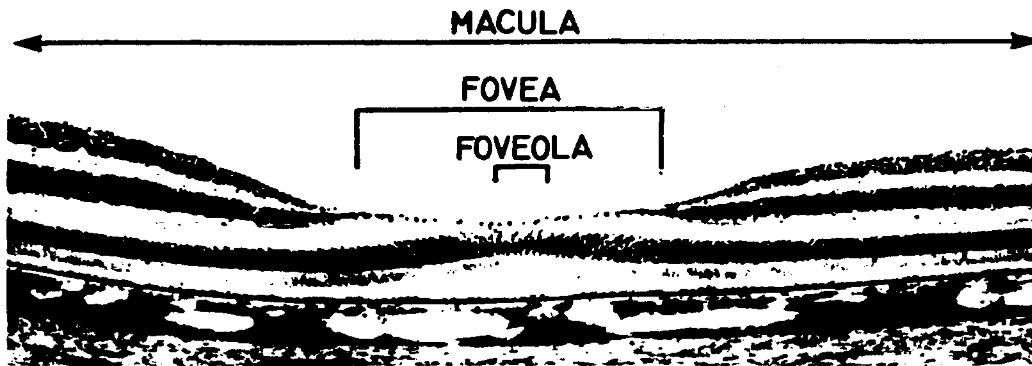

FIGURE 2

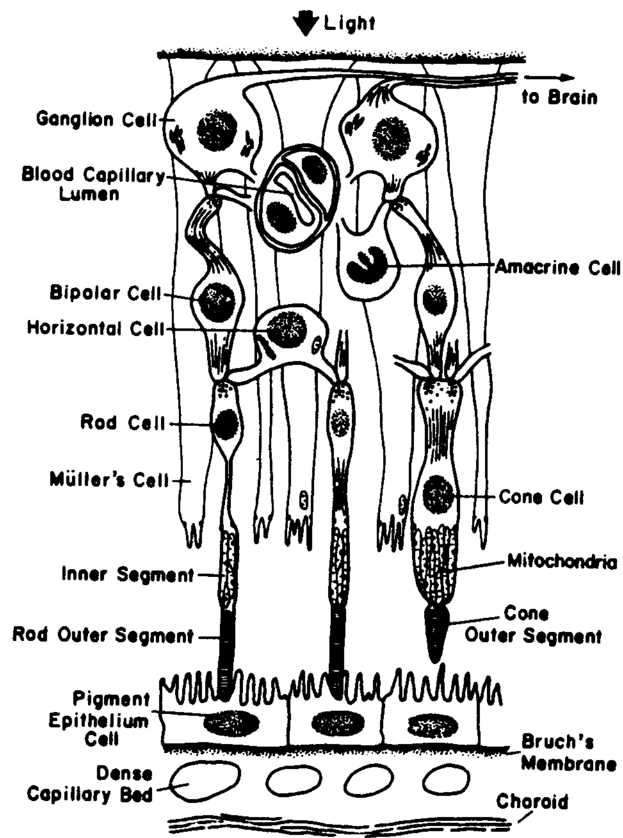

FIGURE 3

CHAPTER 6

Protection of human retina homogenate against light and Fenton-induced oxidative stress: A role for carotenoids and other dietary antioxidants.

by

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Abstract.

The human retina is an important tissue in free radical research, due to its anatomy, biochemistry, and physiologic function, and in particular its constant exposure to oxygen-mediated stress. Lipid peroxidation of human retina homogenate was induced by either photosensitization (rose Bengal and light) or metal-catalysis (iron and ascorbate). The concentration of lipid peroxides was determined by the TBA (thiobarbituric acid) assay. Lipid peroxidation in these systems is thought to mimic eye injuries, diseases, or the natural processes of aging. Ascorbate (1 mM) is a superior antioxidant in oxidative stress, protecting human retina homogenate against metal-catalyzed stress by 98% and against photosensitization by 68%. α-Tocopherol (1 mM) and β-carotene (.05 mM) protect against iron/ascorbate by 34 and 18 % and against photosensitization by 37% and 26%. Retinol (0.1 mM) protected only in the photosensitized system and then only by 23%. Potencies of the carotenoids varied widely, with α-carotene > canthaxanthin > astaxanthin > lycopene > β-carotene > lutein > zeaxanthin. Protection by lutein and zeaxanthin (the two retina carotenoids) did not reach significance at .05 mM, however they protected to significance at .01 mM ($p \leq 0.05$). α-Tocopherol was a better antioxidant at the higher partial pressures of oxygen. Shelf-aged (oxidized) β-carotene (determined to be < 5% pure), increased production of MDA at ambient pO_2. Reported accounts of the prooxidant activity of β-carotene at high pO_2 could be due to contamination with oxidation products. Concentrations of endogenous retina antioxidants as determined by HPLC were not significantly correlated with photochemical or Fenton-induced oxidative stress. Lutein is positively correlated with zeaxanthin. Similarly, total carotenoids are correlated with γ-tocopherol but not α-tocopherol or retinol . Our results indicate that, in

the human retina, a major candidate for protection against phototoxic reactions and free radical-mediated damage is the presence of ascorbate at 1 mM. α-Tocopherol, carotenoids, and retinol individually protect to a lesser degree. Nevertheless, synergistic effects *in vivo*, with each other or other antioxidants, may be important.

Introduction.

The retina of the eye is responsible for vision and has special features that make it an especially susceptible target for oxidative stress leading to formation of lipid peroxides. These processes have been linked to conditions such as retina deterioration, lipofuscin buildup in the retinal pigment epithelium (RPE), and the symptoms of age-related macular degeneration (AMD). In individuals 50 years and over, AMD is the leading cause of severe vision loss and in the USA occurs in 30% of Caucasians with grey, green, or blue irises, a higher percentage than those with dark choroidal and iris melanin (Ferris, 1983). Melanin presumably functions as a light trap to decrease photosensitized injury. The incidence of age-related macular degeneration (Eye Disease Case-Control Study Group, 1993) and cataracts (Knekt et al, 1992) is correlated with low serum carotenoid levels.

Photoreceptor membranes of the retina are particularly susceptible to lipid oxidation due to a high content of the most unsaturated fatty acid found in the human body, docosahexaenoic acid (22:6ω3). The retina is constantly exposed to light, leading to photooxidative damage. In addition, high concentrations of mitochondria found in the photoreceptor inner segments undoubtedly leak active oxygen species from their electron transport chains. If activated oxygen species are responsible for deterioration of either the photoreceptor outer segments or the RPE's maintenance capacity, a decrease in antioxidant levels may accelerate the processes seen in disease and aging. We therefore attempted to assess the impact of oxidative processes involved in natural aging or age-related macular degeneration, in terms of measures of lipid oxidation. Using this mode we sought to characterize and compare the actions of dietary antioxidants including carotenoids, α-tocopherol, retinol, and ascorbate.

The retina resists extensive damage by light, both visible and UV. However, addition of a photosensitizer enables light-induced damage. Rose Bengal in the presence of light is a known singlet oxygen-generating system. This system caused a dose-dependent breakage of chromosomes in cells with concomitant loss of biological activity (Di Mascio et al, 1989). Ferric iron has been used to induce lipid peroxidation in porcine retina tissue (Hiramitsu and Armstrong, 1991). In the metal-catalyzed system, we added ascorbate at prooxidant levels (0.1 mM) to redox cycle Fe^{3+} to Fe^{2+}. Descriptions of possible mechanisms of lipid peroxidation by these two systems are described. Extent of involvement of individual active oxygen species (endogenous or produced in the radical-generating system) can be determined by adding scavengers and metal-chelators.

Materials and Methods.

Chemicals.

β-Carotene, α-carotene, lycopene, lutein, retinyl acetate, α-tocopherol, retinol, crystalline bovine erythrocyte (copper-zinc) superoxide dismutase (SOD) 3100 U/mg protein), 1,4-diazabicyclo[2,2,2]octane (DABCO), bovine albumin, ferric chloride, sodium ascorbate, and tetrahydrofuran (THF) 99.9% with 0.03% butylated hydroxytoluene (BHT) were from Sigma Chemicals Co. (St. Louis, MO). Malonaldehyde-*bis(*diethylacetyl) (MDA) was from Aldrich Chemical Co., Milwaukee, WI. Catalase (bovine liver, 65,000 units/mg) was from Boehringer Mannheim (Germany). Desferrioxamine (desferal mesylate) was a gift from CIBA Geigy Pharmaceuticals (Switzerland). Lutein, zeaxanthin, astaxanthin, and canthaxanthin were kindly donated by Hoffmann-La Roche Inc., Basel, Switzerland and Nutley, NJ. Protein assays were performed using the Biorad Protein Assay Kit Standard I from Biorad, Richmond, California. All water used was double distilled, deionized, and autoclaved.

Test systems.

Source, harvesting and storage of retina tissue.

Eyes were donated by the Vancouver Eye Bank. Harvesting and dissection of the human retinas were performed according to techniques described by Handelman et al. (1992). Tissues were frozen at -80°C, under argon gas, in light-proof vials and used within 12 months as recommended by Handelman et al. (1988).

Preparation of the retina homogenate.

All experimental procedures were carried out under yellow or subdued light to prevent the degradation of antioxidants (especially carotenoids) and retina homogenate. In experiments requiring many trials, retinas were pooled. Retina samples in 1.0 ml cold normal saline (0.9%) were homogenized using a 2 ml Ten Broeck homogenizer for 2 minutes, then placed on ice in a light-proof vial. The amount of protein per ml of retina homogenate was determined using the Biorad Protein Assay®.

Phosphate Buffered Saline vs saline.

For these experiments saline was used instead of Phosphate Buffered Saline (PBS). The natural state of the retina is quite acidic, as up to 50% of all glucose is converted to pyruvate and then lactate, even in the fully oxygenated retina (Handelman, 1986). There was also a concern that the phosphate would affect the system, however, we found there is no difference between using saline or PBS. Sodium ascorbate rather than ascorbic acid was used to prevent the pH from decreasing where ascorbate was used as a prooxidant or antioxidant.

Organic solvents required as vehicles for the addition of lipid soluble antioxidants require careful controls.

Retina homogenate is an aqueous dispersion of membranes. Most of the chemical scavengers are water soluble and are added in 50 μl aliquots to generate the desired final

concentrations. α-Tocopherol dissolved in tetrahydrofuran (THF with .03% BHT) seems to lose its antioxidant capacity, in fact, in many trials MDA equivalents increased. α-Tocopherol dissolved in ethanol and added as 3% of the final reaction mixture volume (8.1 μl in 270 μl) retains its antioxidant capacities. Ethanol itself is a scavenger but gives insignificant protection when added up to 5% of the final volume.

One of the greatest obstacles in carotenoid research has been the problem of how to introduce carotenoids into aqueous dispersions. One approach is to use THF as a solvent to transfer the carotenoid to the aqueous medium. In this approach, all carotenoids and retinol are added in THF as 1% of the final reaction mixture volume (2.7 μl in 270 μl). The particularly polar THF is the preferred solvent for carotenoids, since it allows these non-polar compounds to be readily dissolved and then transferred to the aqueous medium, where they remain as a fine dispersion. THF can be obtained with or without the antioxidant butylated hydroxytoluene (BHT). Without BHT, however, THF is an extremely unstable solvent and peroxides are quickly formed in storage. These peroxides would interfere with oxidant sensitive experiments, therefore THF with BHT is preferable and its antioxidant activity is easily accounted for by using controls. THF must be stored under argon, in the dark, at -80 °C.

Argon protects.

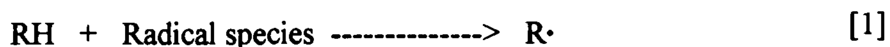
The partial pressure of oxygen (pO_2) is decreased by aerating all solutions with argon for a minimum of 15 minutes. Decreasing the pO_2 significantly reduced the amount of damage incurred in photosensitized reactions. It was necessary to increase the concentration of rose Bengal from 2.5 to 50 μM to maintain the same level of generated

MDA concentration. Increasing the partial pressure of oxygen by saturating all solutions with oxygen increased the amount of damage over that seen in air-saturated solutions.

What is the range of oxygen-derived active species candidate for the immediate agents of damage?

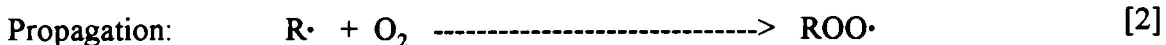
The process of lipid autoxidation ("peroxidation") occurs by the following steps:

Free radical initiation:

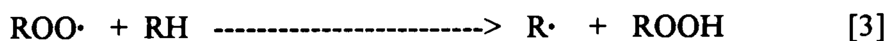


$$RH + \text{Radical species} \longrightarrow R\cdot \qquad [1]$$

Propagation:



$$R\cdot + O_2 \longrightarrow ROO\cdot \qquad [2]$$

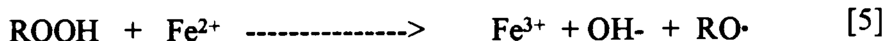


$$ROO\cdot + RH \longrightarrow R\cdot + ROOH \qquad [3]$$

An alternate initiator is singlet oxygen:

$$RH + {}^{1}O_2 \longrightarrow ROOH \qquad [4]$$

Metal-catalyzed re-initiation:



$$ROOH + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + RO\cdot \qquad [5]$$

$$RO\cdot + RH \longrightarrow ROH + R\cdot \qquad [6]$$

and

$$ROOH + Fe^{3+} \longrightarrow Fe^{2+} + H^+ + ROO\cdot \qquad [7]$$

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot \qquad [8]$$

Photochemically-induced lipid autoxidation of retina homogenate.

Photochemical damage was initiated using rose Bengal (2.5 μM) sensitization to 2 minutes of light from a quartz halogen incandescent bulb. Damaging species reportedly generated include singlet oxygen and some superoxide (Carballo et al, 1993). The major cause of lipid peroxidation is step [4] or initiation by singlet oxygen. Butylated hydroxytoluene (BHT), a powerful chain-breaking antioxidant, is present in the solvent tetrahydrofuran (THF) at .03%. THF as a solvent protects at concentrations higher than 3%. If this protection is due to BHT and not intrinsic to THF, this suggests that the propagation step [2] involving peroxyl radicals is also a contributing pathway in photosensitized lipid oxidation. Endogenous iron from the minute amounts of blood present in the retina homogenate could contribute to re-initiation. This involves a Fenton-type reaction, with alkyl peroxides in the presence of iron, creating alkyl (R·), alkoxyl (RO·), and peroxyl (ROO·) radicals [5-8]. In photosensitized damage the extent of this reaction is, however, limited by the low concentrations of iron present. Iron-mediated damage is clearly secondary to the damage mediated by singlet oxygen.

Iron/ascorbate-induced lipid autoxidation of retina homogenate.

Metal-catalyzed damage is initiated with iron (II) (50 μM) and ascorbate (100 μ M). THF with the antioxidant butylated hydroxytoluene (BHT) protects (see Figure 1). Therefore, in experiments where addition of THF was necessary to allow the introduction of carotenoids or retinol into the test system, the radical-generating system had to be adjusted. Iron (II) was increased to 1.5 mM and prooxidant levels of ascorbate were decreased to 10 μM. The initiation step in metal-catalyzed reactions begins with attack

on RH by hydroxyl and superoxide radicals, creating a delocalized pentadienyl radical [1]. In the propagation step, this pentadienyl radical combines with oxygen to form a peroxyl radical [2]. These peroxyl radicals can in turn, extract another hydrogen from RH, forming a peroxide (ROOH) [3]. BHT protects significantly at this propagation step, reflecting a strong role for peroxyl radicals. The re-initiation step here differs from photosensitized oxidations due to the much higher levels of iron (II) (50 μM in comparison to traces of endogenous iron). The higher levels of iron (II) catalyze the breakdown of peroxides (either endogenous or produced in the propagation step), to (R•), alkoxyl (RO•), and peroxyl (ROO•) radicals [5-8]. Ascorbate in low concentrations (<100 μM), acts as a prooxidant to reduce and recycle Fe^{3+} to Fe^{2+}. Maximal damage by the iron/ascorbate system produced up to 4 times more MDA equivalents than maximal damage by the rose Bengal/hv system. This reflects the extent of the contributions of amplification and its dependence on metals. In the presence of iron (II), the limiting factor is the availability of peroxides (created in propagation) to react with high levels of added iron (II) (50 μM). By using different methods of inducing lipid peroxidation, varying antioxidants, and changing the partial pressures of oxygen, researchers can begin to define the active oxygen species present and the processes occurring in these model systems.

Ascorbate is both a prooxidant and antioxidant.

In the photosensitized reaction, ascorbate (no THF/BHT) acts as an effective antioxidant at 1 mM (Figure 2). However, ascorbate at 1 mM in the presence of THF/BHT gives complete protection, masking the effects of other antioxidants. To study

the effects of ascorbate in combination with retinol or carotenoids (in THF), ascorbate was reduced from 1 mM to 0.2 mM (Figure 3).

In the metal-catalyzed system (no THF/BHT) ascorbate acts as a powerful antioxidant at 1 mM and a prooxidant at 0.1 mM. Ascorbate in combination with BHT gives such complete protection that all effects with other antioxidants are masked (Figure 4). Therefore, ascorbate in combination with retinol or carotenoids (in THF) has to be reduced from 1 mM to 0.2 mM. In addition, the radical-generating system had to be adjusted, with iron (II) increased from 50 μM to 1.5 mM and prooxidant levels of ascorbate decreased from 0.1 mM to 10 μM. Such fine-tuning allowed us to be within the acceptable range of spectrophotofluorometric readings.

Assays used.

The project uses three separate assays. Assay 1: The Protein Assay test determines the amount of protein per unit of retina tissue sample using the Biorad Protein Assay®. Assay 2: The TBA test examines the ability of added antioxidants (with a focus on carotenoids) to protect retina tissue *in vitro* against oxygen-mediated stress. Two radical-generating systems, (1) rose Bengal/light and (2) an iron/ascorbate (Fenton-radical generator) are used in all investigations. MDA equivalents as produced in the TBA test, were quantified by spectrophotofluorimetry. The MDA equivalents were converted to nmol of MDA. All final results are standardized to nmol MDA per mg protein, except when correlating MDA with HPLC values, where units were converted to nmol MDA per gram (wet weight) retina. Assay 3: HPLC analysis determines tissue levels of retina

carotenoids, α-tocopherol, and retinol using high pressure liquid chromatography (HPLC). All HPLC results are standardized to nmol per gram (wet weight) retina.

1. Test for total protein.

Protein was measured using the Biorad Protein Assay Kit I®. The Biorad Protein Assay contains an acidic solution of Coomassie Brilliant Blue G-250, a protein-binding dye, that changes its maximum absorbance from 465 nm to 595 nm when binding to protein occurs. The colour change of the dye responds to varying concentrations of protein. Readings are taken between 5 and 20 minutes when binding becomes constant. All final results were standardized as nmol MDA/per milligram of protein.

2. TBARS assay.

Determination of MDA with TBA: Biological Samples.

One of the most common assays for lipid peroxidation in biological materials is the thiobarbituric acid (TBA) method. It is based on the assumption that peroxidation of biological material results in the production of malondialdehyde (MDA), which under certain conditions (acid and heat), forms a pink MDA - TBA (1:2) product, which can be quantified spectrophotometrically at 532 nm. This chromophore also fluoresces, and we recorded the intensity by spectrophotofluorimetry at an excitation wavelength of 505 nm and an emission wavelength of 555 nm. Interference by other biological materials that absorb at similar wavelengths (such as bilirubin) are negligible when fluorometric rather than spectrophotometric methods are used (Ohkawa, 1979).

The TBA test is not an actual measure of lipid peroxidation per se, but an indicator of the extent of peroxidative tissue injury (Janero, 1990). There has been much debate on the validity of this assay as other aldehydes and tissue components can also combine with TBA to form the chromophore (Draper, 1993). Review of the data (Janero, 1990), however, indicates that sucrose (0.25 M) or DNA (deoxyribose) under physiological conditions did not increase TBARs (thiobarbituric acid reactive substances). Increased TBARs do result from the reaction of DNA with radiolysis products or exposure to hydroxyl radicals (OH•), however, our photosensitized reactions do not produce hydroxyl radicals. It is possible that the dramatic increase in MDA-like compounds seen in our iron/ascorbate system could be due to a hydroxyl radical-deoxyribose interaction, however this would be evident in our controls.

Human retina tissue contains the highest concentration of the most highly polyunsaturated fatty acid, docosahexaenoic acid (22:6ω3), which lends itself well to lipid peroxidation. Results also vary with the tissue preparation with a 50% decrease in MDA when tissue is minced as opposed to homogenized (Janero, 1990). Human retina tissue is easily homogenized to a consistent (and easily pipetted) mixture. For our reaction system, what the TBA test does indicate is the response of retina homogenate to an oxidative insult, with a quantitative measure of the resulting TBARs. Adding various antioxidants allows us to examine the change in MDA equivalents, therefore we can determine the effectiveness of each antioxidant. It does not indicate the origin or actual species creating the chromophore. Every type of experimental system will react differently with the TBA test, and we have had to customize our reaction and assay systems for maximum sensitivity.

The TBA assay proved to be very stable within our test system, and it provides a reproducible measure of oxygen-mediated stress to retina tissue. It has been suggested that the term "TBARs" may not be accurate, as the TBA procedure itself could create peroxides after the initial free radical insult has occurred (Draper, 1993), however this can be accounted for with controls. When making interpretations of lipid peroxidation, the endpoint spectrophotometric readings should be interpreted as MDA equivalents rather than TBARs.

3. The HPLC Assay.

Eyes were received from the Vancouver Eye Bank in pairs. Left and right retinas are similar in carotenoid concentration and distribution (Handelman, et al, 1988 and 1991). Information on levels of retinol and tocopherol in retinas was unavailable, therefore we have presumed that these levels would also be similar in concentration and distribution. All procedures involved with the preparation of tissue and HPLC analysis were carried out under yellow light. HPLC methods are as described in Handelman et al. (1988).

Retinas from the left eyes of 10 subjects were analyzed for lutein, zeaxanthin, retinol, γ-tocopherol, and α-tocopherol. All values were converted to nanomoles antioxidant per gram of retina (wet weight) (Table 1). Retinas from the right eyes were exposed to 2 minutes of light (as described for the photosensitization experiments), photochemical, or metal-induced oxidative stress. All values were corrected for baseline

and converted to nanomoles MDA per gram of retina (wet weight) (Table 2). The relationships between antioxidant content, resistance to stress, and age (Table 3) were investigated using multiple linear regression to determine slopes of the dose/response curves.

Experimental procedures.

Photosensitization with rose Bengal and light..

Light and the photosensitizer rose Bengal were used to induce photochemical oxygen-mediated stress. Reactions were carried out in Lab-Tek Products® tissue culture chambers. Individual cells of the plastic microtray measured 1 x 1 cm, with four chambers (forming a square) measuring 2.3 x 2.3 cm. The light source was a 300 watt quartz halogen incandescent bulb in a Kodak Carousel auto-focus 7604 slide projector with a Kodak Ektanar C 102 mm (f 2.8) projector lens. The projector was secured in an upright position, with the light source pointing down, and the lens adjusted flush with the edge of the projector casing. The microtray was placed under the light source, and raised flush with the lens. The top of the microtray was 0.5 cm from the actual lens. The 4 chambers (2.3 cm total width) were within a 4 cm diameter area of even light-intensity distribution. A mirror was placed under the microtray to increase light intensity.

Pre-determined volumes of saline were added to the microtray cells, making the final volume (after all the reactants and antioxidants were added) equal to 270 μl. To the saline was added 50 μl of retina homogenate plus one of the following: The carotenoids

β-carotene, α-carotene, lycopene, lutein, zeaxanthin, canthaxanthin, astaxanthin; the vitamins A (retinol), C (ascorbate), and E (α-tocopherol); the scavengers mannitol, catalase, superoxide dismutase (SOD); the singlet oxygen quencher DABCO; the metal chelator desferrioxamin, and albumin. The carotenoids or retinol were dissolved in tetrahydrofuran (THF with .03% BHT) and added as 2.7 μl or 1% of the final reaction mixture. α-Tocopherol dissolved in ethanol was added as 8.1 μl or 3% of the final reaction mixture. The antioxidants were injected quickly with rapid stirring (with the pipette tip) and allowed to incubate with the retina homogenate a minimum of 3 minutes. All water-soluble antioxidants and scavengers were dissolved in distilled water and added as 50 μl aliquots to various final concentrations. Catalase, superoxide dismutase, and albumin were made up in 150 mM phosphate buffered saline (PBS) pH 7.4. Photosensitization was induced with 50 μl rose Bengal (giving a final concentration of 2.5 μM in 270 μl), and illumination with a 300 watt quartz halogen incandescent bulb for 2 minutes.

Free radical generation with iron and ascorbate.

The Fenton-type free radical generating system, iron/ascorbate, was used to induce a different kind of oxidative stress. Reactions were carried out in polypropylene 1.5 ml centrifuge tubes. Saline, retina homogenate, and antioxidants were added as in system 1. The generation of free radicals was initiated by adding iron (II) and then ascorbate to final concentrations of .05 mM and 0.1 mM. The system was then allowed to incubate 1 hour in the dark. Systems with carotenoids or retinol contained THF (with .03 % BHT) at 1 % of the final volume, which reduced MDA equivalents below acceptable levels for experiments in which it was desired to examine the effect of

protective agents. After some experimentation, we optimized iron (II) and ascorbate to 1.5 mM and 10 μM. The THF still protected by 88%, however, total damage was so great that MDA equivalents reached acceptable levels.

Thiobarbituric acid assay.

Immediately after light exposure or incubation, 0.2 ml of the homogenate and reactants was added to a 15 ml polypropylene tube with 0.2 ml of 8.1 % sodium dodecyl sulfate, 1.5 ml of 20 % acetic acid solution (pH 3.5), 1.5 ml 0.8 % TBA, and 0.6 ml H_2O (Hiramitsu et al, 1991). The mixture was incubated at 95°C for 60 minutes and then placed on ice for 30 minutes. To this was added 1 ml H_2O and 5 ml butanol:pyridine (15:1). Each tube was vortexed for 30 seconds and then centrifuged for 10 minutes at 1500 rpm, extracting the TBA-MDA fluorescent chromagen into the upper phase. This upper phase was pipetted into a quartz cuvette, with a 1 cm path. The concentration of MDA was quantified by spectrophotofluorimetry using a Turner Model 430 Spectro Fluorometer at an excitation wavelength of 505 nm and an emission wavelength of 555 nm.

Preparation of the retina homogenate for HPLC analysis.

One pre-weighed retina sample was placed into a glass Ten Broeck homogenizer and to this was added 0.5 ml buffer (10 mM HEPES, pH 7.4, 0.1 M NaCl, 1 mM Na_2EDTA) and 0.5 ml ethanol solution (50 μg/ml butylated hydroxytoluene in ethanol). Samples were then homogenized for 60 seconds, and transferred to a polypropylene test

tube. The homogenizer is rinsed by homogenizing with another 0.5 ml buffer and 0.5 ml ethanol solution, and the remainder added to the sample. Internal standard solution (0.2 ml retinyl acetate in methanol) was added to 0.8 ml hexane, followed by 3 ml hexane. This preparation was vortexed for 2 minutes, and centrifuged (800 *g* for 30 seconds) (Handelman et al, 1988). The upper phase was then pipetted, transferred to a vial, and evaporated under argon at 40°C. The residue was dissolved in 0.2 ml methanol and centrifuged for 30 minutes at 12,000 rpm on an Eppendorf Silencer H25 F1 Tabletop Microcentrifuge. A 50 µl portion of the supernatent was analyzed by HPLC for tocopherol (285 nm), retinol (325 nm), and carotenoids (450 nm).

HPLC Assay Chromatography.

The instrumentation consisted of a SP8700 solvent delivery system (Spectra Physics, San Jose, CA) a Spectraflow 757 absorbance detector (Kratos Analytical Instruments, Ramsey NJ), a Beckman 165 variable wavelength detector (Beckman Instruments, Altex Division, San Ramon CA) and a Spectra Physics SP4500 integrator run under Winner on Windows integration software (Thermo Separation Products, Spectra Physics Analytical, Inc., Fremont, CA). The column was a Merck LiChrosphere (250 X 4 mm) 100 RP 18, 5 µm packing with guard column (EM Separations, Gibbstown, NJ).

A mobile phase at a flow rate of 1.0 ml/min using a mixture of methanol (BDH, HPLC grade), double distilled water and acetonitrile (Fisher, HPLC grade) (46:3:1 v:v:v), buffered with 1 mM *bis-tris* propane (Sigma, SigmaUltra) at pH 6.8 was run for 15 minutes. A mixture of methylene chloride (Fisher HPLC grade) and methanol

(35:65 v:v) buffered with 1 mM *bis- tris* propane (pH 6.8) was introduced at 15.1 minutes to create a gradient ramp. At 30 minutes, the latter mixture comprised 35% of the mobile phase. Column elutants were monitored at 325 nm and 450 nm using the Spectraflow 757 (detection wavelength was manually switched after the retinyl acetate peak passed). The Beckman detector monitored the elutants at 285 nm.

To determine the state of oxidation of the β-carotene, a VYDAC 150 X 2.1 mm RP18 5 µm packing was employed (Mandel Scientific, Guelph, Ont. Sep/a/ra/tions Group, Hesperia, CA). The mobile phase was methanol with 1mM *bis-tris* propane added as a buffer, at a flow of 0.1 ml/min. Detection was at 450 nm using a Spectraflow 757 absorbance detector.

We were unable to obtain β-apo-8'-carotenal as a source of the internal standard carotenal-ethyl-oxime in the method of van Kuijk et al. (1985). Accordingly, retinyl acetate was substituted as the internal standard.

Experimental controls.

Controls for each experiment included retina homogenate and retina homogenate under oxidative stress. Temperature of the reaction chambers under quartz-halogen illumination reached 32 °C by the end of two minutes. Controls incubated at 32 °C showed no difference in MDA values within this time period. Where nonpolar antioxidants were added, solvent controls were included both with and without oxidative stress. In addition, the addition of antioxidants to retina homogenate often gave an effect, therefore each test condition was run both with and without oxidative stress. All test

values were adjusted to the appropriate baseline control values to determine percent protection.

Statistical analysis.

Standard deviations (SD) of triplicate samples of the dose/response data were greater at the higher MDA values. A plot of the standard deviations vs the means of the dose/response data revealed a constant relative standard deviation; estimated as 3% of the mean value. The standard error of the mean (SEM) was calculated as usual based on the sample size. In estimates of percent protection we obtained a pooled estimate of the variance. For the current data regarding % protection, the SD was estimated as an absolute value of 10.58 % based on a plot of SD as a function of % protection. The criterion for significance at $p < .05$ is 2 x SEM (13% where $n = 3$, 16% where $n = 2$).

Results.

Photosensitization with rose Bengal and light.

1. Scavengers and metal chelators.

We have compared metal-binding and chain-breaking antioxidants in light-initiated damage (Figure 5 and Table 4). DABCO (10 mM) a singlet oxygen quencher and desferrioxamine (10 mM) a metal chelator, were the only compounds to protect significantly at 45% and 13 %. Catalase (20 units/ml), superoxide dismutase (20 units/ml), mannitol (10 mM), and albumin (20 units/ml) failed to protect ($p > .05$).

2. Dietary antioxidants.

Ascorbate at 0.1 and 1 mM protects by 29 and 68% and α-tocopherol (3% of final volume) at 0.1 and 1 mM protects by 25% and 37%. Retinol at 0.1 mM (1% of final volume) protects by 23%. Over several trials, β-carotene gave variable results ranging from 0 to 28% protection at .05 mM (Figure 6). Because of this unreliable response, in general we can say that β-carotene did not provide significant protection. The other carotenoids α-carotene, canthaxanthin, astaxanthin, lutein, zeaxanthin, and lycopene failed to protect at $p < .05$ significance, or aggravated damage (Figure 7) (Table 5).

Unless otherwise stated, "β-carotene" will refer to Batch #1 β-carotene, determined at the beginning of these experiments to be 98% pure as determined by spectrophotometry at 450 nm. For experimental consistency we maintain the same source

of β-carotene throughout this experimental work. However, even with rigorous storage controls, a certain amount oxidized throughout the course of the experiments. By the end of the set of experiments (12 months) nearly 25% of the β-carotene had oxidized. The inconsistency of the β-carotene data may be due to the presence of variable amounts of oxidation products.

3. *Synergistic effects.*

Figure 8 and Table 6 indicate no significant synergistic activity or additive effects between any of the dietary antioxidants.

4. *PO_2 effects.*

α-Tocopherol at 1 mM protected under air (ambient pO_2) and oxygen (high pO_2) by 37 and 34% and protected by 17% under argon (low pO_2). β-Carotene in this system did not protect significantly under high, ambient, or low pO_2 (Figure 9 and Table 7).

5. *Effects of oxidized β-carotene.*

Figure 10 shows the effect of oxidized β-carotene in the TBARS assay. Batch #2 of shelf-aged β-carotene (6 years) was determined spectrophotometrically (450 nm) to be > 95% oxidation products. There is no significant difference between retina homogenate /THF under photosensitized stress and β-carotene Batch #2 (delivered in THF to water) with and without photosensitization. In the presence of retina homogenate, this oxidized β-carotene increased MDA production over 300%. In the presence of oxidized

β-carotene, there was no difference between controls and trials under photosensitized stress.

Iron/ascorbate-induced damage.

1. Scavengers and metal chelators.

We have compared metal-binding and chain-breaking antioxidants in protection against iron/ascorbate oxidative stress. Figure 11 and Table 4 show protection by scavengers and metal chelators. Desferrioxamine (1mM) protected completely, DABCO, catalase, mannitol, and SOD protected by 77, 44, 39, and 32%. There was no significant difference between control and albumin.

2. Dietary antioxidants.

Ascorbate at 1 mM protected by 98%, but at 0.1 mM ascorbate is prooxidant. α-Tocopherol at 0.1 mM and 1 mM protected by 21% and 34%. Retinol failed to protect. Over several trials β-carotene gave variable results ranging from 0 to 18% protection. Because of this unreliable response, in general we can say that β-carotene did not provide significant protection (Figure 12). The carotenoids α-carotene, canthaxanthin, astaxanthin, and lycopene (.05 mM) protected by 49, 48, 46, and 39%, respectively. Lutein and zeaxanthin (.05 mM) failed to protect significantly, however at .01 mM they protected modestly by 21 and 17% at $p < .05$ (Figure 13 and Table 5).

3. Synergistic effects.

Figure 14 indicates no significant synergistic activity between any of the dietary antioxidants. Table 6 shows α-tocopherol (in ethanol) in combination with ascorbate (water-soluble). Percent protection of α-tocopherol and ascorbate in combination is greater than either of the separate values, but in combination protect no more than an additive effect.

4. Effect of different pO_2 and different batches of β-carotene.

Figure 15 and Table 7 show the effects of α-tocopherol and β-carotene (Batch #1, 25% oxidation products) on MDA generation by metal-catalyzed autoxidation of retina homogenate. α-Tocopherol (1 mM) protected by 54 and 26% under low and high pO_2, but did not protect significantly under ambient pO_2. β-Carotene (Batch #1) did not protect significantly under low or ambient pO_2 conditions, and was highly prooxidant under high pO_2 conditions.

As noted previously, Batch #2 of β-carotene (95% oxidation products) was highly prooxidant under ambient pO_2 conditions (Figure 10), where it induced lipid autoxidation of retina homogenate even in the absence of photochemical. For this reason, we obtained a new batch of β-carotene for comparative purposes. Batch #3 of β-carotene was assayed to be 98% pure. Batch # 1 (25% oxidation products) was then compared with Batch #3 (less than 2% oxidation products) for its effect on lipid autoxidation induced by photochemical and iron/ascorbate systems. The results indicate that neither batch of β-carotene was able to offer significant protection against photochemical (Figure 16) or iron/ascorbate-induced (Figure 17) autoxidation under low, ambient, or high pO_2

conditions (Table 8). Under metal-catalyzed stress, β-Carotene (Batch #1) was not prooxidant under low or ambient pO_2 conditions, but was highly prooxidant under high pO_2 conditions.

5. HPLC analysis of human retina.

There were no significant correlations between the levels of antioxidants and levels of MDA produced by baseline, light, photochemical, or metal/ascorbate-induced stress. However, there was a significant positive correlation between lutein and zeaxanthin (Figure 18) and between total carotenoids and γ-tocopherol ($p < .05$ (Figure 19). Age and antioxidant levels were not significantly correlated, in concurrence with the findings of Handelman et al. (1988). There was also no significant correlation between age and resistance to photochemical or Fenton-induced oxidative stress (Table 3).

Discussion.

Activated oxygen species generated.

Photosensitization reaction.

In the light/rose Bengal system, singlet oxygen is a significant damaging species. DABCO, a known singlet oxygen quencher protects significantly at 43%. Catalase, superoxide dismutase, and mannitol did not protect. Desferrioxamine protected by 13% (just significant at $p < .05$), an indication that traces of metals could be present, in part as minute quantities of blood from the retina blood vessels. Lack of protection by albumin, capable of metal chelating and antioxidant chain-breaking, (Wayner et al. 1987) may indicate that metal-catalyzed reactions are not crucial to the photosensitized damage.

THF containing .03% BHT was used as a solvent for non-polar compounds. THF at concentrations higher than 3% (final concentration of BHT = .017 μM) protected against the photosensitized damage, and therefore we chose to add it at 1% of the final volume. THF without BHT forms peroxides, and without the BHT would undoubtedly increase MDA equivalents in our systems. BHT is a potent antioxidant, and may be able to scavenge peroxyl radical in this system at .017 μM. Therefore, the initial damage is caused by singlet oxygen, but there may be some subsequent contribution by chain reactions propagated in the lipid membranes by peroxyl radicals. Hydrogen peroxide, superoxide, and hydroxyl radical species, endogenous or generated, are negligible in relation to singlet oxygen and propagated species.

Iron/ascorbate-induced reactions.

Peroxidation of retina homogenate lipids was increased by the presence of iron (II) and decreased by the metal chelator, desferrioxamine, both in a dose dependent manner. Desferrioxamine at 1 mM protected completely. Catalase protected by 44 % indicating damage by hydrogen peroxide (H_2O_2). Apparently, a site-specific (generated at the target) Fenton-type reaction mediates damage.

Mediation by site-specific hydroxyl radicals is consistent with the observation that 10 mM mannitol protects by 39%. Hydroxyl radicals are so highly reactive and therefore relatively non-selective, that they have a very short lifetime and are unable to diffuse extensively into biological systems. Site-specific radicals generated at the target site are therefore more important in iron/ascorbate initiated damage to biological systems.

Superoxide dismutase protects by 44% implicating superoxide radical ions in the damage. Albumin does not protect, suggesting that protection by catalase and superoxide dismutase is not by virtue of the metal-chelating or radical scavenging properties of their protein component. Interestingly, DABCO, the singlet oxygen quencher, protected against iron/ascorbate by 77 %. Possibly, DABCO possesses metal-chelating or antioxidant properties not mentioned in the literature, or that singlet oxygen is indeed present. In iron/ascorbate-induced damage, even at 1% final concentration, THF containing .03% BHT protected so strongly that any systems with this solvent had to have iron (II) increased from 50 μM to 1.5 mM and ascorbate (which at higher concentrations acted as an antioxidant) decreased from 100 to 10 μM. This indicates a possible role for peroxyl radicals as propagators in the lipid peroxidation chain reaction. We conclude that

superoxide, hydroxyl radicals, and possibly singlet oxygen play important roles in the initiation and propagation of iron/ascorbate-induced peroxyl radical production.

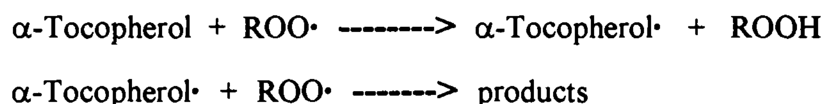
Dietary antioxidants.

Concentrations of antioxidants were a compromise between literature information, relevance to *in vivo* levels, and the actual dose/responses in preliminary studies. Serum retinol and α-tocopherol concentrations of healthy persons reportedly average 2.56 μM and 23.46 μM (Ito et al, 1990). Carotenoid levels can vary with diet and supplementation (Constantino et al, 1988; Gilbert et al, 1990). Other studies report serum β-carotene (.63 ± .328 μM), total serum carotenes 1.187 ± .617 μM, and total xanthophylls .812 ± .275 μM (Krinsky et al, 1990). Ascorbate levels in human blood plasma (27-51 μM) (Letner, 1984) are lower than tissue levels, where levels may reach up to 1.5 mM in the cornea, lens, and aqueous humor of the eye (Hornig, 1975). Retina levels have not been reported. Experimental values were determined by dose/response trials and set at concentrations which gave an antioxidant effect. Comparisons with our experimental data are as follows:

Compound	**Serum values**	**Experimental values**
Retinol	2.56 μM	10 and 100 μM
α-Tocopherol	23.46 μM	100 and 1000 μM
β-Carotene	.63 ± .32 μM	10 and 50 μM
Carotene	1.187 ± .617	10 and 50 μM
Xanthophylls	.812 ± .275 μM	10 and 50 μM
Ascorbate	27-51 μM	100 and 1000 μM

α-Tocopherol.

The substantial protection by α-tocopherol in the photosensitized reaction and metal-catalyzed system can be accounted for as singlet quenching or chain-breaking activities. α-Tocopherol has about 50% of the singlet oxygen quenching ability of β-carotene (Kaiser, et al, 1990). α-Tocopherol is also an effective chain-breaking antioxidant, with the theoretical capacity to scavenge two peroxyl radicals (ROO•), as follows:



α-Tocopherol + ROO• --------> α-Tocopherol• + ROOH

α-Tocopherol• + ROO• -------> products

In the current studies, α-tocopherol was only half as effective as ascorbate (equimolar concentrations) in the photosensitized reaction, and one third as effective in the metal-catalyzed system. Rose Bengal and light generate singlet oxygen in the aqueous phase, whereas α-tocopherol (like β-carotene) is a lipid-soluble antioxidant with and affinity for membranes. α-Tocopherol is most effective in organic solution where it is free to react with radicals. In retina homogenate preparations, the molecule has restricted mobility within the membranes limiting its interactions with peroxyl radicals. Its role as a single oxygen quencher is likely less important than its chain-breaking activity, since lipid peroxidation occurs primarily within the membranes.

Ascorbate.

The current finding that ascorbate is the most effective antioxidant in human retina, is consistent with earlier results in human blood plasma (Frei et al, 1989; Wayner et al,

1987). Ascorbate was the most effective antioxidant, protecting by over 2/3 in the photosensitized reaction and by 98% in the metal-catalyzed system. In the metal-catalyzed system, ascorbate has a biphasic response, with high concentrations (1 mM) offering complete protection and lower concentrations (< 0.1 mM) acting as a prooxidant, presumably acting to allow redox cycling of the ferric (Fe^{3+}) iron. Ascorbate is a versatile antioxidant, protecting against superoxide and hydroxyl radicals and singlet oxygen, however it's most important role in retina homogenate and *in vivo* may be its superior peroxyl radical scavenging ability.

Retinol.

Retinol is not generally included in *in vitro* experiments looking at oxygen-mediated stress. Although retinol protected modestly in the photosensitized reaction it was ineffective in the metal-catalyzed system. Its 5 double bonds make retinol a relatively weak singlet oxygen quencher and antioxidant, compared with ascorbate, the tocopherols, and carotenoids. Retinol is often included in epidemiological cancer studies such as the Carotene and Retinol Efficacy Trial (CARET) (Thornquist et al, 1993), and its purported ability to offer protection against some forms of tumor production, lipid peroxidation, and its importance in the human retina prompted its inclusion in these experiments. Adriamycin, the redox-active anticancer drug, is reduced by cellular enzymes and increases lipid oxidation of membranes. Our finding of 23 % protection in the photosensitized assay is consistent with results of Vile and Winterbourne (1988) who found retinol (100 nmol/mg microsomal protein) protects by 39 % against adriamycin-induced microsomal lipid peroxidation.

Carotenoids.

Photochemically-induced oxidative stress.

Surprisingly, none of the carotenoids protected dramatically against the photosensitized reaction, not even the known singlet oxygen quenchers lycopene and β-carotene. Lycopene is a superior singlet oxygen scavenger compared to β-carotene (Di Mascio, 1989). Where other scavengers and dietary antioxidants gave consistent results between subjects, β-carotene gave variable results (from 0-26 %), suggesting that its potency may depend on the percentage purity of the β-carotene, the natural antioxidant state of the retina, or other factors.

Iron/ascorbate-induced damage.

Under metal-catalyzed oxidative stress, the carotenoids (.05 mM) α-carotene, canthaxanthin, astaxanthin, and lycopene protected, where lutein and zeaxanthin failed to protect significantly ($p < .05$). These results are in agreement with the findings of Miki (1991) and Kurashigi et al. (1990) who found astaxanthin to be an antioxidant and singlet oxygen quencher. Palozza and Krinsky (1992) report astaxanthin and canthaxanthin to be as effective as α-tocopherol (~30 % protection at 10 nmol/mg protein) in protecting rat liver microsomes against lipid peroxidation. Canthaxanthin and astaxanthin also suppressed the production of methyl linoleate hydroperoxides more effectively than β-carotene and zeaxanthin (Terao, 1989).

Reportedly, the conjugated oxo-groups in canthaxanthin and astaxanthin increased their peroxyl radical trapping activity (Terao, 1989) by stabilizing their architectural location in the membrane through hydrophobic bonding of the polyene chain and hydrogen bonding of the hydroxy and keto groups on the cyclohexane ring (Miki, 1991). α-Carotene and lycopene, however protected as effectively, yet they possess no oxo groups. α-Carotene has a similar structure to β-carotene, with a bond shift from the 6' to the 5' position. Lycopene is clearly a quencher, and we found one report of antioxidant activity in the literature (Bertram et al, 1991), perhaps due to its prohibitive cost. The finding that lycopene protected by about 40% in the current study, is therefore novel.

Lutein and zeaxanthin are the only two carotenoids that accumulate in the human retina, concentrating in the macula and fovea, with similar values within right and left eyes of the same subject. Microdensiometric analysis of the fovea by Handelman et al. (1991) indicated that these macular pigments are non-randomly distributed among the tissues. These facts, plus the finding that the ratio of zeaxanthin to lutein is low in plasma but high in the macula, has led to speculations of a biological control mechanism for macular carotenoids with specific binding proteins and receptors (Bone et al. 1988). Patterns of accumulation are reportedly determined by genetic factors (Handelman et al, 1991).

A surprising finding was the lack of protection by lutein and zeaxanthin at high concentrations and significant protection ($p < .05$) at lower concentrations. Possibly, accumulation in high concentrations (as in the macula) protects individual carotenoid molecules from oxidation processes. It is tempting to speculate that lutein and zeaxanthin are spared in oxygen-mediated processes and conserved to attenuate light, in the phototoxic short (blue) wavelengths, that reaches the retina. These filtering properties would be especially important at the center of our visual acuity, the *macula lutea* (yellow

spot), where these carotenoids concentrate. Other carotenoids, may be sacrificed in the functioning retina in free radical scavenging, and so they are not detected by HPLC. Lutein and zeaxanthin may dominate through elimination of other carotenoids, rather than selective uptake by the retina (Bone et al, 1988; Handelman et al, 1991.

Absence of additive or synergistic effects.

We looked for synergism between ascorbate, α-tocopherol, and β-carotene and found none in either the photosensitized or metal-catalyzed systems. Protection by ascorbate and α-tocopherol exceeded that of either antioxidant, but not sufficiently to show even additive significance. Although the regeneration of tocopherol from the tocopheroxyl radical by ascorbate is generally accepted, the only evidence in the literature of a synergistic effect between ascorbate and α-tocopherol is a study by Palozza and Krinsky (1992). The experimental difficulties of using two solvents, membrane preparations, and polar and nonpolar antioxidants would make even significant positive results questionable.

Effects of partial pressure of oxygen.

β-carotene is reportedly a better antioxidant at low pO_2 (Burton, 1984), but pO_2 did not significantly alter the small protective action of β-carotene in either photodamage nor iron/ascorbate. In the photosensitization reaction, α-tocopherol under low, ambient, and high pO_2 conditions protected by 17, 37, and 34 %, respectively. Evidently, under normal oxygen conditions, there is ample O_2 present for production of singlet oxygen and

peroxyl radicals. Although percent protection remains constant, the decrease in total damage to the system under low pO_2, may be due to a decreased production of singlet oxygen or peroxyl radicals.

Partially oxidized β-carotene dramatically increases MDA production at high pO_2.

Shelf-aged β-carotene (Batch # 1 stored under argon at -80 °C for 12 months) was determined to be 25 % oxidation products. Under metal-induced stress, this partially-oxidized β-carotene protected insignificantly by 6 % at ambient pO_2, however, it dramatically increased damage (by 356%) at high pO_2. In comparison, fresh β-carotene (Batch # 3, 98% purity) at high pO_2 did not increase damage or protect significantly (Figure 17 and Table 8). Possibly, the increase in oxygen pressure caused the oxidation products (25 %) in the sample to autoxidize and accelerate lipid oxidation.

Oxidized β-carotene dramatically increases MDA production at ambient pO_2.

All batches β-carotene are as described in the above sections. The evidence suggests that β-carotene oxidation products are TBA reactive substances themselves, but their most important effect is to accelerate lipid oxidation of membranes. Partially oxidized β-carotene does not accelerate lipid oxidation of membranes until exposed to high partial pressures of oxygen, however highly oxidized β-carotene will increase damage at ambient conditions (Figure 10).

Shelf-aged β-carotene may contain high levels of oxidation products. Even under laboratory storage conditions, β-carotene oxidized 25 % in 12 months. This is of particular importance to people who take pharmacological doses of β-carotene, such as cancer patients, those participating in chemoprevention trials, or the general population supplementing their diet with vitamins containing β-carotene. β-carotene capsules kept at room temperature for many months may do as much harm as good.

Concentrations of endogenous carotenoids, retinol, and tocopherols in human retina.

Concentrations of antioxidants (nmols per gram of wet weight retina) are given in Table 1. Retina concentrations of retinol and tocopherols could not be found in the literature, but several studies have looked at retina carotenoids (Handelman et al 1988, 1991, and 1992). Zeaxanthin accumulates in the macula and lutein is distributed throughout the retina (Handelman et al, 1988 and 1991). In the current study, zeaxanthin concentrations were about 50% of lutein. Average values of lutein (.411 ± .26) and zeaxanthin (.214 ± .14) in units of nmol/gram retina were 54.2 and 28.2 nanograms per retina (formula weight of carotenoids = 568.85 and each retina averages .232 grams). These data agree with the findings of Handelman et al (1988) who report lutein and zeaxanthin values averaging 37.9 and 20.4 nanograms per retina, with zeaxanthin averaging 44% of lutein.

Our results concur with previous findings that report no significant correlation between age and antioxidant levels (Handelman et al, 1988). In addition, no significant correlations were found between endogenous antioxidant levels and photosensitized or Fenton-induced oxidative stress. Interestingly, lutein and zeaxanthin ($r = .95$, $p < .05$), as

well as total carotenoids and γ-tocopherol levels ($r = .822$, $p < .05$) were positively correlated. Total carotenoid values were not correlated to α-tocopherol levels. Carotenoid accumulation in human tissues depends on diet and individual absorptive capacities. Some common dietary sources of lutein include citrus fruits, spinach, asparagus, eggs, and zeaxanthin is rich in navel oranges and eggs. Possibly, the macular carotenoids, lutein and zeaxanthin, attenuate light, but do not protect against singlet oxygen, free radicals, or the propagated species created in lipid autoxidation.

Ascorbate is reportedly the most important antioxidant in human plasma, and reaches concentrations of 1.5 mM in the cornea, lens, and aqueous humor of the eye (Horning, 1975). High concentrations in these tissues would protect eye structures from peroxyl radical damage. Unfortunately, no data is available on ascorbate concentrations in the human retina. It would be interesting to correlate endogenous ascorbate levels with resistance to oxygen-mediated stress. Unlike the fat-soluble vitamins, ascorbate must be analyzed immediately to minimize vitamin destruction. In the current study, all tissues were frozen and stored for a minimum of several days, prohibiting ascorbate analysis.

Future studies should address the relationship between tissue levels of carotenoids or other compounds, and the physical changes associated with aging and diseases of the retina and associated structures. The posterior poles of the eyes from this study, with the retinal pigment epithelium and Bruch's membrane, have been preserved in formaldehyde. Histologic sections could be prepared and the damage determined by microscopy. Lipofuscins or aging pigments are fluorescent and can be determined by fluorescence microscopy. Such data can be correlated with clinical information and dietary analysis to delineate the role of free radical processes in ocular pathology.

Summary.

It may be years before contributions of free radical-mediated processes to conditions such as retina damage, lipofuscin (aging pigment) buildup in the retinal pigment epithelium, or the age-related macular degeneration are precisely known. Clearly, however, retina photoreceptor membranes, by virtue of their high levels of polyunsaturated fatty acids, are extremely susceptible to induced oxidation under photosensitization or free radical stress. Ascorbate, and to a lesser extent tocopherol, retinol, and carotenoids protect. No correlations were found between endogenous levels of antioxidants and oxidative damage to human retina. Positive correlations between lutein, zeaxanthin and γ-tocopherol may reflect similar food sources. The macular pigments appear to function as light traps, rather than scavengers and quenchers of activated oxygen species. Carotenoid oxidation products have the potential to increase lipid autoxidation of membranes, and this damage is potentiated by high partial pressures of oxygen. If activated oxygen species are responsible for damage either to the photoreceptor outer segments or the RPE's maintenance capacity, a decrease in antioxidant levels may be responsible for the processes present in eye disease and aging processes.

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Chapter 6

Legends to Tables.

Table 1. HPLC values of endogenous antioxidants for 10 subjects. Antioxidant values were determined by HPLC at the following wavelengths: tocopherol (285 nm), carotenoids (450 nm), and retinol (325 nm).

Table 2. Spectrophotofluorometric (MDA) values of baseline, light, photochemical, and metal-induced lipid autoxidation of human retina homogenate for 10 subjects.

Table 3. Regression analysis of MDA values of baseline, photochemical, and metal-induced lipid autoxidation of human retina homogenate as a function of HPLC antioxidant concentration or age.

Table 4. Effects of scavengers on human retina homogenate against oxygen-mediated stress. Lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light (RB/Hv) or iron (II) (50 μM) and ascorbate (100 μM) (Fe/A).

Table 5. Effects of dietary antioxidants on human retina homogenate against oxygen-mediated stress. Lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light (RB/Hv). Metal-catalyzed stress (Fe/A) with THF (carotenoids and retinol) was induced by iron (II) (1.5 mM) and ascorbate (10 μM) and without THF (ascorbate and tocopherol) by iron (II) (50 μM) and ascorbate (100 μM).

Table 6. Effects of dietary antioxidants in human retina homogenate against oxygen-mediated stress: Effects in combination. All conditions are as described above.

Table 7. Protection by dietary antioxidants in human retina homogenate against oxygen-mediated stress: Effects of partial pressures of oxygen.

Table 8. Protection by β-carotene and partially oxidized β-carotene in human retina homogenate against oxygen-mediated stress: Effects of partial pressures of oxygen.

Chapter 6

Legends to Figures.

Figure 1. Concentration of iron (II) as a function of TBARS assay-generated MDA equivalents: Effect of 0% (□) and 1% (■) THF. MDA equivalents were read on a specrophotofluorimeter at an excitation hν of 505 nm and an emission hν of 555 nm.

Figure 2. Concentration of ascorbate as a function of TBARS assay-generated MDA equivalents: Effect of rose Bengal (2.5 μM) and 2 minutes light (▩) compared to controls with no rose Bengal or light (□)

Figure 3. Concentration of ascorbate as a function of TBARS assay-generated MDA equivalents: Effect of 0% (■), 1% (▩), and 2% (□) THF in human retina homogenate under rose Bengal (2.5 μM) and 2 minutes light.

Figure 4. Concentration of ascorbate as a function of TBARS assay-generated MDA equivalents: Effect of 0% (□) THF under iron (II) (50 μM) and 1% (■) THF under iron (II) (1.5 mM) in human retina homogenate.

Figure 5. Effects of various water-soluble scavengers, singlet oxygen quenchers, and metal chelators in human retina homogenate. Ascorbate (1 mM), DABCO (10 mM), desferrioxamine (Desfer) (10 mM), catalase (20 units/ml), superoxide dismutase (SOD) (20 units/ml),albumin (20 units/ml), and mannitol (10 mM) were added to the homogenate and lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light. TBARS assay-generated MDA equivalents were read on a specrophotofluorimeter at an

excitation hν of 505 nm and an emission hν of 555 nm. All antioxidant baselines were adjusted to control baseline values, and antioxidant values were similarly adjusted. Percent protection was calculated from control values. Error bars represent 1 SEM.

Figure 6. Effects of various dietary antioxidants in human retina homogenate. Ascorbate (1, 0.1 mM) in H_2O, α-tocopherol (1, 0.1 mM) in ethanol (3% of final volume), and β-carotene (.05, .01 mM) and retinol (.1, .01 mM) both in THF (1% of final volume) were added to human retina homogenate. Lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 7. Effects of various carotenoids in human retina homogenate. Astaxanthin (Asta), lycopene (Lyc), α-carotene (αcar), β-carotene (βcar), canthaxanthin (Canx), lutein (Lut), and zeaxanthin (Zeax) all at .05 and .01 mM, were added in THF (1% of final volume) to human retina homogenate. Lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 8. Effects of dietary antioxidants in combination. Ascorbate (Asc) added as 1 mM in H_2O, α-tocopherol (Toc) added as 1 mM in ethanol (3% of final volume), and β-carotene (βcar) added as .05 mM in THF (1% of final volume) were added to human retina homogenate. Lipid autoxidation was induced with rose Bengal (2.5 μM) and 2 minutes light. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 9. Protection by β-carotene and α-tocopherol against photochemically-induced lipid autoxidation: Effects of partial pressures of oxygen. All reagents were left at ambient pO_2 (air) or saturated with etiher argon or oxygen. β-carotene (□) (.05 mM in THF, 1% of final volume) and α-tocopherol (■) (1 mM in ethanol , 3% of final volume) were added to human retina homogenate. Lipid autoxidation was induced with rose Bengal (50 μM) and 2 minutes light. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 10. Oxidized β-carotene increases MDA equivalents: Effects of lipid membranes. Retina homogenate with 1% THF (RH/THF), oxidized β-carotene (oxβcar) in THF (no retina homogenate), and oxidized β-carotene added to retinal homogenate (RH/oxβcar) were sensitized with rose Bengal (2.5 μM) and 2 minutes light (photodamage). β-carotene (batch #2, 6 years old) was determined spectrophotometrically to be > 95% oxidation products. THF or oxidized β-carotene were added as 1% of final volume. MDA equivalents were read on a specrophotofluorimeter at an excitation hν of 505 nm and an emission hν of 555 nm. Error bars represent 1 SEM.

Figure 11. Effects of various water-soluble scavengers, singlet oxygen quenchers, and metal chelators in human retina homogenate. Ascorbate (1 mM), DABCO (10 mM), desferrioxamine (Desfer) (10 mM), catalase (20 units/ml), superoxide dismutase (SOD) (20 units/ml), albumin (20 units/ml), nd mannitol (10 mM) were added to retina homogenate. Lipid autoxidation was induced with iron (II) (50 μM) and ascorbate (0.1 mM). TBARS assay-generated MDA equivalents were read on a specrophotofluorimeter at an excitation hν of 505 nm and an emission hν of 555 nm. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 1. Error bars represent 1 SEM.

Figure 12. Effects of various dietary antioxidants in human retina homogenate. Ascorbate (1, .1 mM) in H_2O, α-tocopherol (1, .1 mM) in ethanol (3% of final volume), and β-carotene (.05, .01 mM) and retinol (.1, .01 mM) both in THF (1% of final volume) were added to human retina homogenate. Lipid autoxidation in water and ethanol solvent conditions were induced with iron (II) (50 μM) and ascorbate (0.1 mM). Lipid autoxidation in THF solvent conditions were induced with iron (II) (1.5 mM) and ascorbate (10 μM). TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 13. Effects of various carotenoids in human retina homogenate. α-Carotene (αcar), canthaxanthin (Canx), Astaxanthin (Asta), lycopene (Lyc), β-carotene (βcar), zeaxanthin (Zeax), and lutein (Lut), all at .05 and .01 mM, were added in THF (1% of final volume) to human retina homogenate. Lipid autoxidation was induced with iron (II) (1.5 mM) and ascorbate (10 μM). TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 14. Effects of dietary antioxidants in combination. Ascorbate (Asc) added as 1 mM in H_2O, α-tocopherol (Toc) added as 1 mM in ethanol (3% of final volume), and β-carotene (βcar) added as .05 mM in THF (1% of final volume) were added to human retina homogenate. Lipid autoxidation in water and ethanol solvent conditions were induced with iron (II) (50 μM) and ascorbate (0.1 mM). Lipid autoxidation in THF solvent conditions were induced with iron (II) (1.5 mM) and ascorbate (10 μM). TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 15. Protection by β-carotene and α-tocopherol against chemically-induced lipid autoxidation: Effects of partial pressures of oxygen. All reagents were left at ambient PO_2 (air), or saturated with argon or oxygen for a minimum of 15 minutes to create low PO_2 or high PO_2 conditions. (β-carotene (□) (.05 mM in THF, 1% of final volume) and α-tocopherol (■) (1 mM in ethanol , 3% of final volume) were added to human retina homogenate. Lipid autoxidation in ethanol solvent conditions were induced with ferric iron (50 μM) and ascorbate (0.1 mM). Lipid autoxidation in THF solvent conditions were induced with ferric iron (1.5 mM) and ascorbate (10 μM). β-carotene results under high PO_2 conditions are also included in Figure 13. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 16. Protection by β-carotene and partially oxidized β-carotene: Effects of partial pressures of oxygen. All PO_2 conditions are as in Figure 11 Lipid autoxidation was induced with rose Bengal (50 μM) and 2 minutes light. TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 17. Protection by fresh β-carotene (Batch #3) and partially oxidized β-carotene (Batch #1): Effects of partial pressures of oxygen. All PO_2 conditions are as in Figure 11 Lipid autoxidation was induced with ferric iron (1.5 mM) and ascorbate (10 μM) TBARS assay-generated MDA equivalents and percent protection were determined as in Figure 5. Error bars represent 1 SEM.

Figure 18. Zeaxanthin concentration as a function of lutein concentration. Endogenous levels of lutein and zeaxanthin were determined by HPLC at 450 nm.

Figure 19. Gamma-tocopherol as a function of total carotenoids. Endogenous levels of carotenoids and gamma-tocopherol were determined by HPLC at 450 and 285 nm.

HPLC values Antioxidant (nmol/gram retina)								
Subject	**wt retina**	**Lutein**	**Zeaxanthin**	**Retinol**	**a-Tocopherol**	**g-Tocopherol**	**Tot Car**	**Tot Toc**
x1	0.214	0.379	0.157	18.785	58.766	0.896	0.536	59.663
x11	0.235	0.360	0.164	18.918	70.049	3.980	0.524	74.029
x13	0.288	0.460	0.188	4.513	60.049	7.633	0.647	67.683
x16	0.206	0.138	0.175	12.299	41.923	1.231	0.313	43.154
x18	0.242	0.360	0.179	7.385	30.712	3.264	0.538	33.977
x29	0.272	0.893	0.482	7.513	58.992	8.059	1.375	67.051
x31	0.23	0.117	0.070	11.365	22.736	0.000	0.186	22.736
x35	0.178	0.812	0.456	9.049	53.201	6.978	1.269	60.179
x43	0.267	0.273	0.114	4.358	56.620	2.198	0.387	58.818
x44	0.184	0.322	0.159	2.285	36.485	4.407	0.480	40.891
mean	0.232	0.411	0.214	9.647	48.953	3.865	0.625	52.818
sd	0.037	0.256	0.139	5.754	15.155	2.899	0.390	16.687

car = carotenoid, a-toc = alpha-tocopherol, g-toc = gamma-tocopherol

TABLE 1

		Spectrophotofluorometic readings MDA (nmol/gram retina)						
Subject	Age	Base	hv	RB/hv	Fe	hv-Base	RB/hv-Base	Fe-Base
x1	80	5.63	5.89	12.58	31.14	0.26	6.95	25.51
x11	15	2.97	2.98	10.57	24.29	0.01	7.60	21.32
x13	74	2.81	2.69	8.47	22.34	-0.11	5.66	19.53
x16	84	1.96	2.07	8.37	21.18	0.11	6.41	19.22
x18	23	2.62	2.63	9.49	3.36	0.01	6.87	0.74
x29	53	2.60	2.40	8.08	22.44	-0.19	5.48	19.84
x31	88	4.67	4.82	12.28	28.69	0.16	7.62	24.02
x35	41	5.51	5.53	15.68	27.24	0.02	10.17	21.73
x43	22	4.55	4.59	11.61	19.77	0.04	7.06	15.22
x44	62	4.34	4.41	12.48	24.56	0.07	8.14	20.22
mean	54.2	3.76	3.80	10.96	22.50	0.04	7.20	18.74
sd	27.6	1.32	1.40	2.43	7.59	0.13	1.34	6.91

hv = light only, RB/hv = photochemical damage with rose Bengal and light, Fe = iron (50 uM and ascorbate 100 uM)

Values adjusted from nmol MDA/mg protein to
nnmol MDA per gram (wet weight) retina

TABLE 2

HPLC values	Baseline, Light, Photochemical, and Metal-induced Stress						
	Base		**RB-Base**		**Fe-Base**		
Antioxidant	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	
Lutein	0.0649	0.86	0.1041	0.77	0.0636	0.86	
Zeaxanthin	0.0364	0.92	0.1432	0.69	0.0709	0.85	
Retinol	0.1009	0.78	0.0644	0.86	0.3811	0.28	
a-Tocopherol	0.0258	0.94	0.1517	0.68	0.3123	0.38	
g-Tocopherol	0.2236	0.53	0.0446	0.9	0.0394	0.91	
Total carotenoids	0.0297	0.94	0.1193	0.74	0.067	0.85	
Total tocopherol	0.0623	0.86	0.1455	0.69	0.2768	0.44	
Age vs Stress	0.0924	0.8	0.2311	0.52	0.5397	0.11	
	Lutein	Zeaxanthin	Retinol	a-Toc	g-Toc	Total Car	Total Toc
Age vs:							
R	0.2586	0.1655	0.0711	0.343	0.2568	0.2287	0.3561
P-value	0.47	0.65	0.85	0.33	0.47	0.53	0.31
Lutein	Zeaxanthin			Total Car	g-Toc	a-Toc	
R	0.9473			R	0.822	0.427	
P-value	0.000032*			p - value	0.0034*	0.2173	

* = significance at p < .05, a-toc = alpha-tocopherol, g-Toc = gamma-tocopherol, car = carotenoids

Regressions of HPLC data:

HPLC : All antioxidant concentrations are in nmol per gram of retina.

Stress test : All MDA values are in nmol per gram of retina.

TABLE 3

Effects of scavengers and metal chelators on human retina homogenate against oxygen-mediated stress.

Compound	Concentration (mM)	% Protection (Rb/Hv)	% Protection (Fe/A)
DABCO	10	45*	77*
Desferrioxamine	1(Fe), 10(RB)	13*	101*
Catalase	20 units/ml	-12	44*
Mannitol	10	4	39*
SOD	20 units/ml	-14	32*
Albumin	20 units/ml	7	3

Rb/Hv = Rose Bengal and light photosensitization reaction
Fe/A = Iron and ascorbate metal-catalyzed reaction
* = significance at $p < 0.05$ level

TABLE 4

Effects of dietary antioxidants on human retina homogenate against oxygen-mediated stress.

Compound	Concentration (mM)	% Protection (Rb/Hv)	% Protection (Fe/A)
Ascorbate	1	68*	98*
	.1	29*	-24
α-Tocopherol	1	37*	34*
	.1	25*	21*
Retinol	.1	23*	-1
	.01	10	-13
α-Carotene	.05	2	49*
	.01	0	34*
Canthaxanthin	.05	3	48*
	.01	-4	12
Astaxanthin	.05	9	46*
	.01	5	24*
Lycopene	.05	4	39*
	.01	5	31*
β-Carotene	.05	3	18*
	.01	-1	18*
Zeaxanthin	.05	-13	6
	.01	-9	17*
Lutein	.05	-12	-6
	.01	-7	21*

Rb/Hv = Rose Bengal and light photosensitization reaction
Fe/A = Iron and ascorbate metal-catalyzed reaction
* = significance at $p < 0.05$ level

TABLE 5

Protection by dietary antioxidants in human retina homogenate against oxygen-mediated stress: Effects in combination.

Compound	Concentration (mM)	% Protection (Rb/Hv)	% Protection (Fe/A)
Ascorbate	1	62*	72*
α-Tocopherol	1	25*	18*
Retinol	.1	23*	-1
β-Carotene	.05	26*	-25
β-Car/α-Toc	as above	25*	n/a
β-Car/Asc	as above	13*	21*
α-Toc/Asc	as above	13*	80*
β-Car/α-Toc/Asc	as above	27*	12

Rb/Hv = Rose Bengal and light photosensitization reaction
Fe/A = Iron and ascorbate metal-catalyzed reaction
* = significance at $p < 0.05$ level

TABLE 6

Protection by dietary antioxidants in human retina homogenate against oxygen-mediated stress: Effects of partial pressures of oxygen.

PO_2	Antioxidant	% Protection (Rb/Hv)	% Protection (Fe/A)
Low	α-Tocopherol	17*	54*
	β-Carotene	9	11
Ambient	α-Tocopherol	37*	10
	β-Carotene	6	7
High	α-Tocopherol	34*	26*
	β-Carotene	6	-356

Rb/Hv = Rose Bengal and light photosensitization reaction
Fe/A = Iron and ascorbate metal-catalyzed reaction
* = significance at $p < 0.05$ level
β-Carotene is from Batch #1, determined to be 75% pure.

TABLE 7

Protection by β-Carotene and partially oxidized β-Carotene in human retina homogenate against oxygen-mediated stress: Effects of partial pressures of oxygen.

PO_2	Antioxidant	% Protection (Rb/Hv)	% Protection (Fe/A)
Low	β-Carotene (#1)	9	11
	β-Carotene (#3)	-29	-6
Ambient	β-Carotene (#1)	6	7
	β-Carotene (#3)	-3	-5
High	β-Carotene (#1)	6	-356
	β-Carotene (#3)	1	9

Rb/Hν = Rose Bengal and light photosensitization reaction
Fe/A = Iron and ascorbate metal-catalyzed reaction
β-Carotene from Batch #1 determined to be 75 % pure.
β-Carotene from Batch #3 determined to be 98 % pure.
* = significance at $p < 0.05$ level

TABLE 8

Metal-induced Lipid Autoxidation in Retina Homogenate: Effects of THF (1%) at Varying Concentrations of Iron

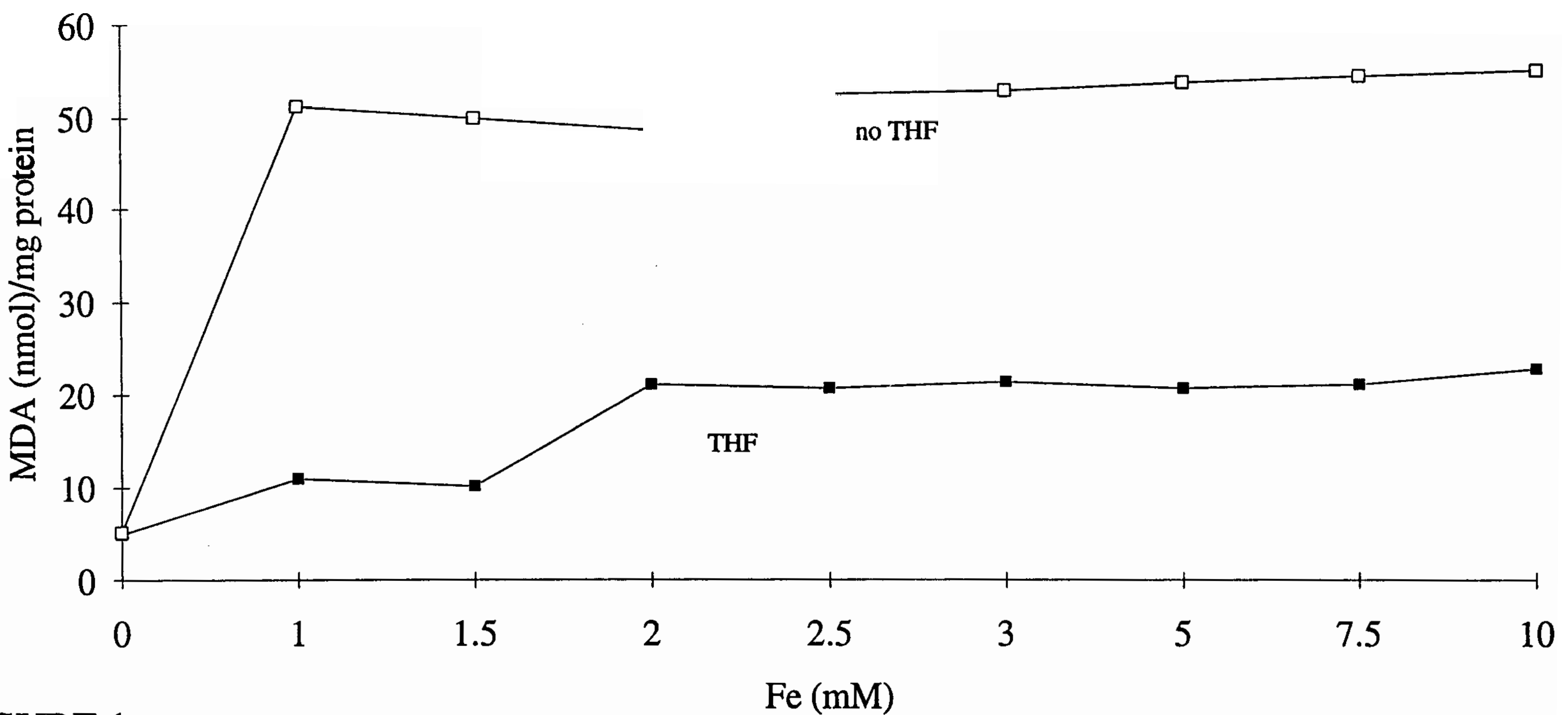


FIGURE 1

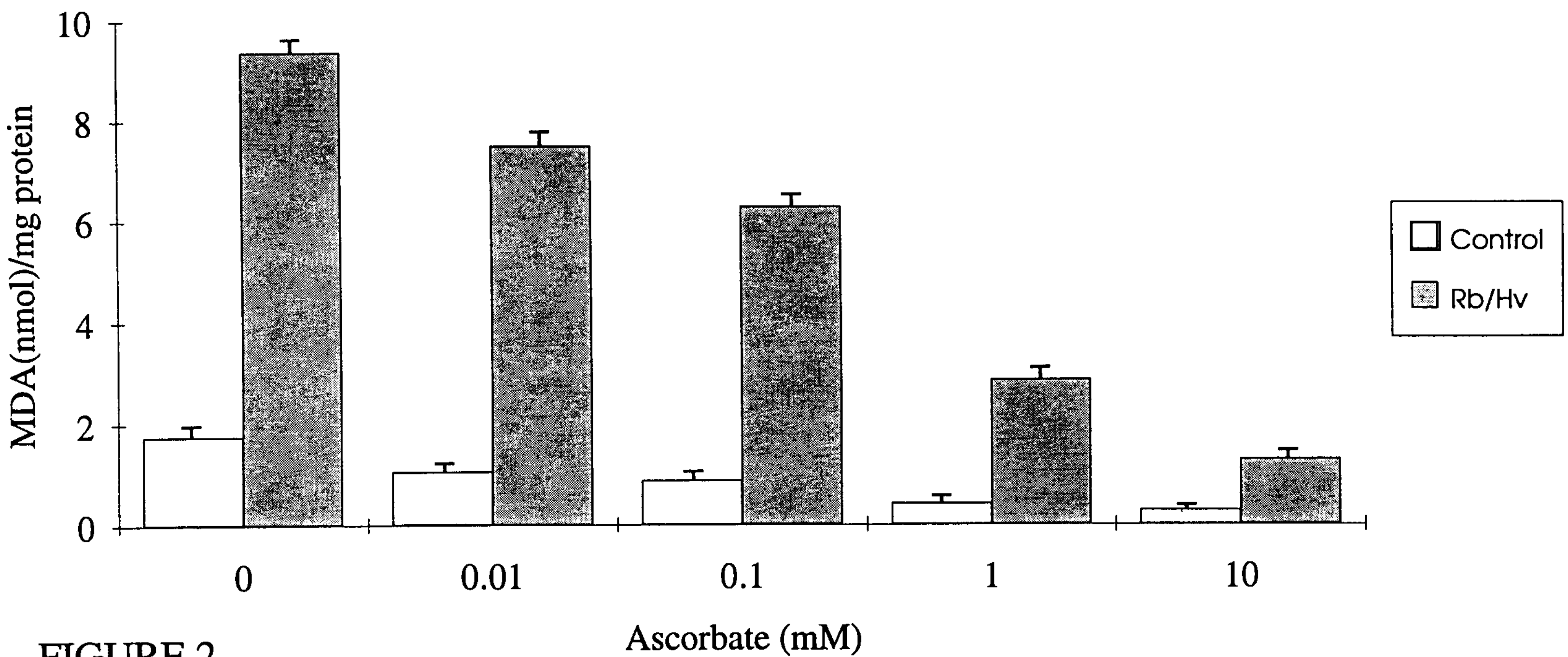


FIGURE 2

Photochemically-induced Lipid Autoxidation in Retina Homogenate: Protective Actions of Ascorbate With and Without THF

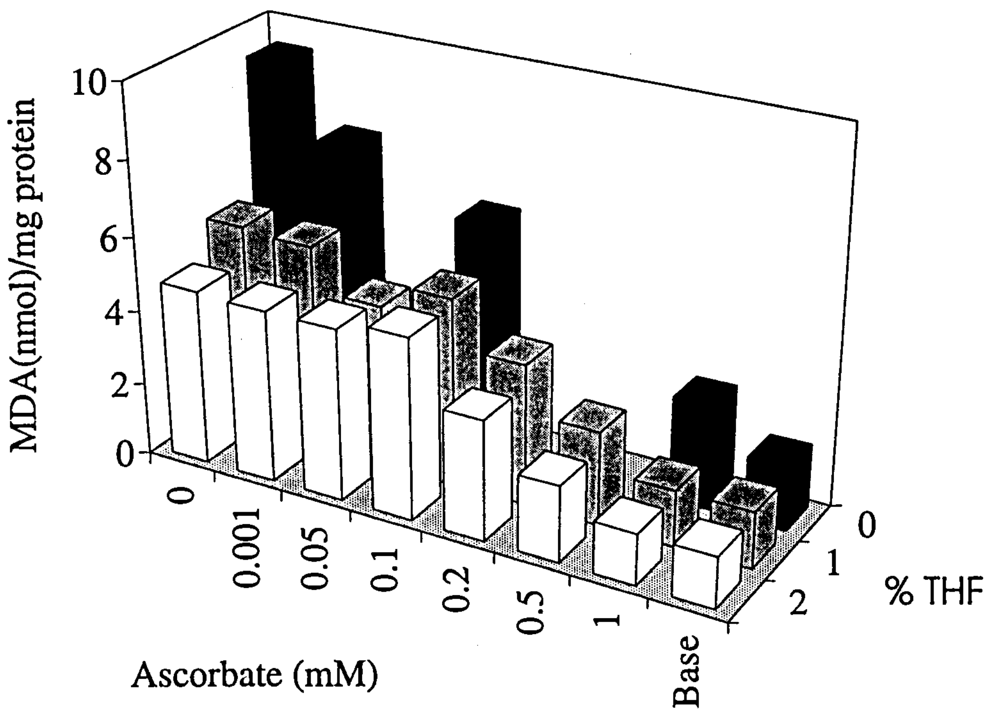


FIGURE 3

Metal-induced Lipid Autoxidation in Retina Homogenate: Effects of Ascorbate at 0 and 1 % THF

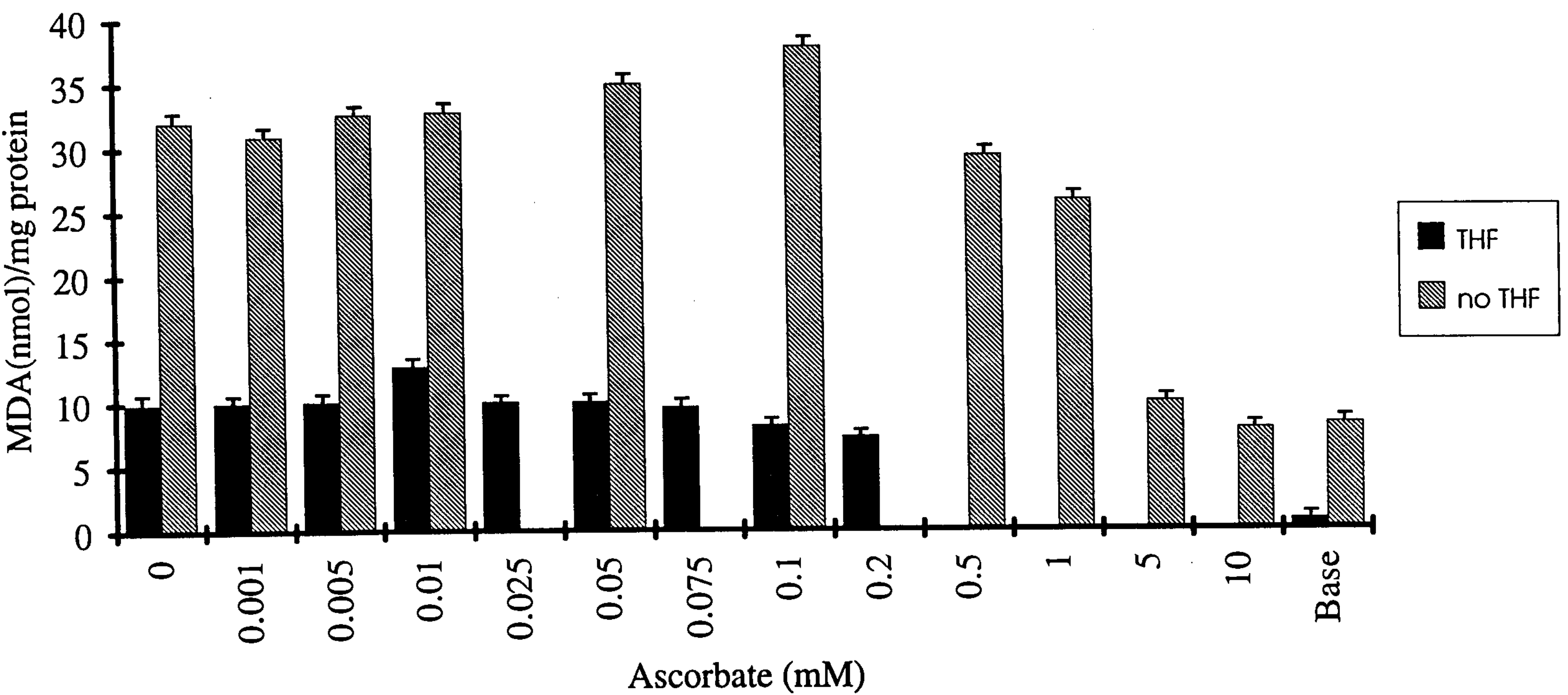


FIGURE 4

Photochemically-induced Lipid Autoxidation in Retina Homogenate: The Effects of Scavengers and Metal Chelators

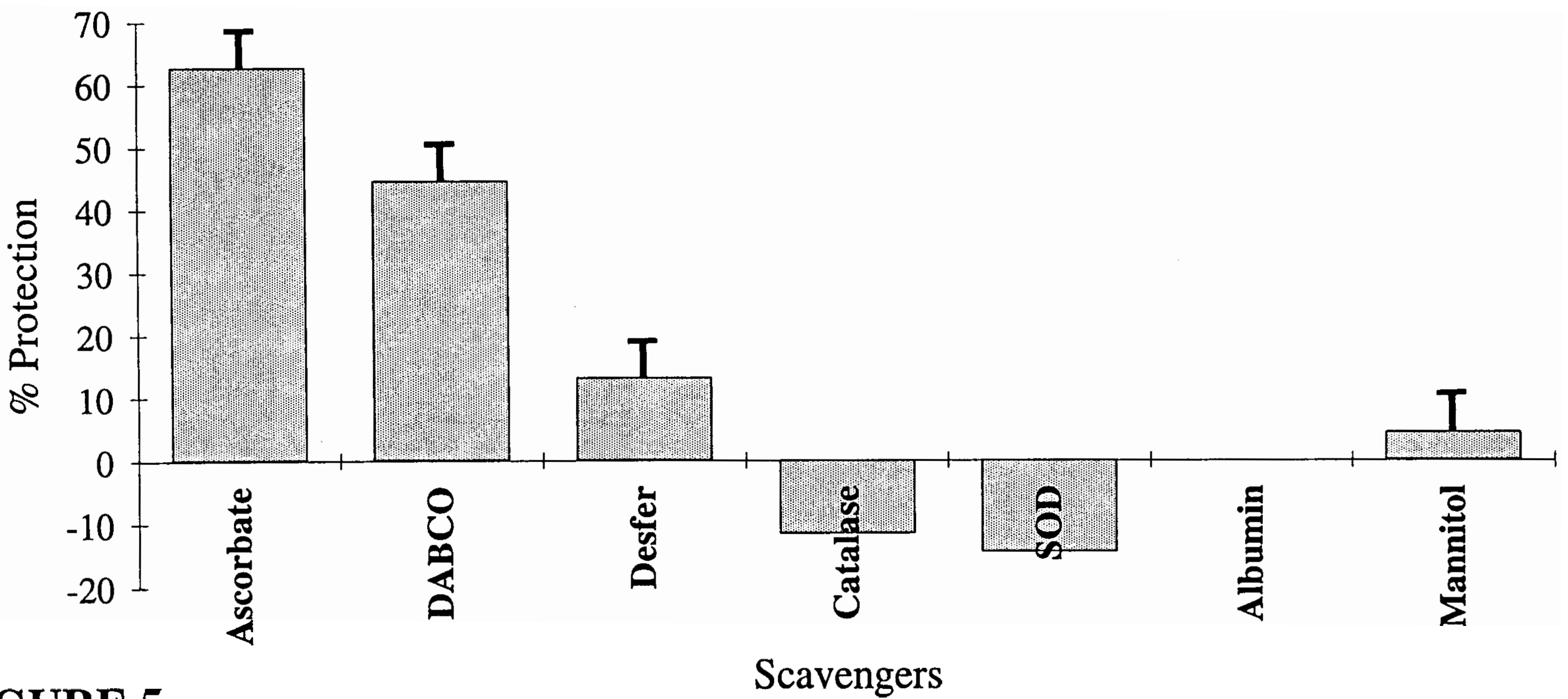


FIGURE 5

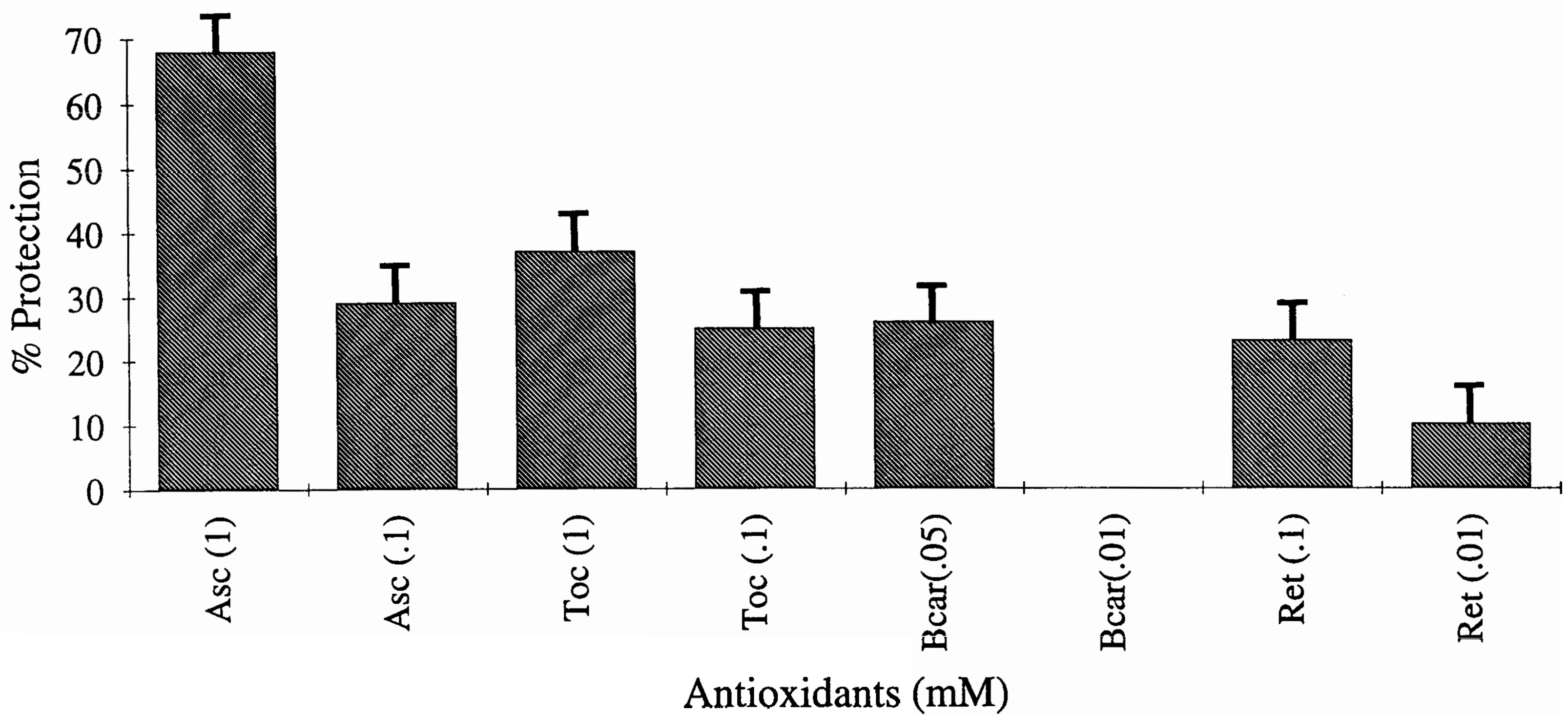


FIGURE 6

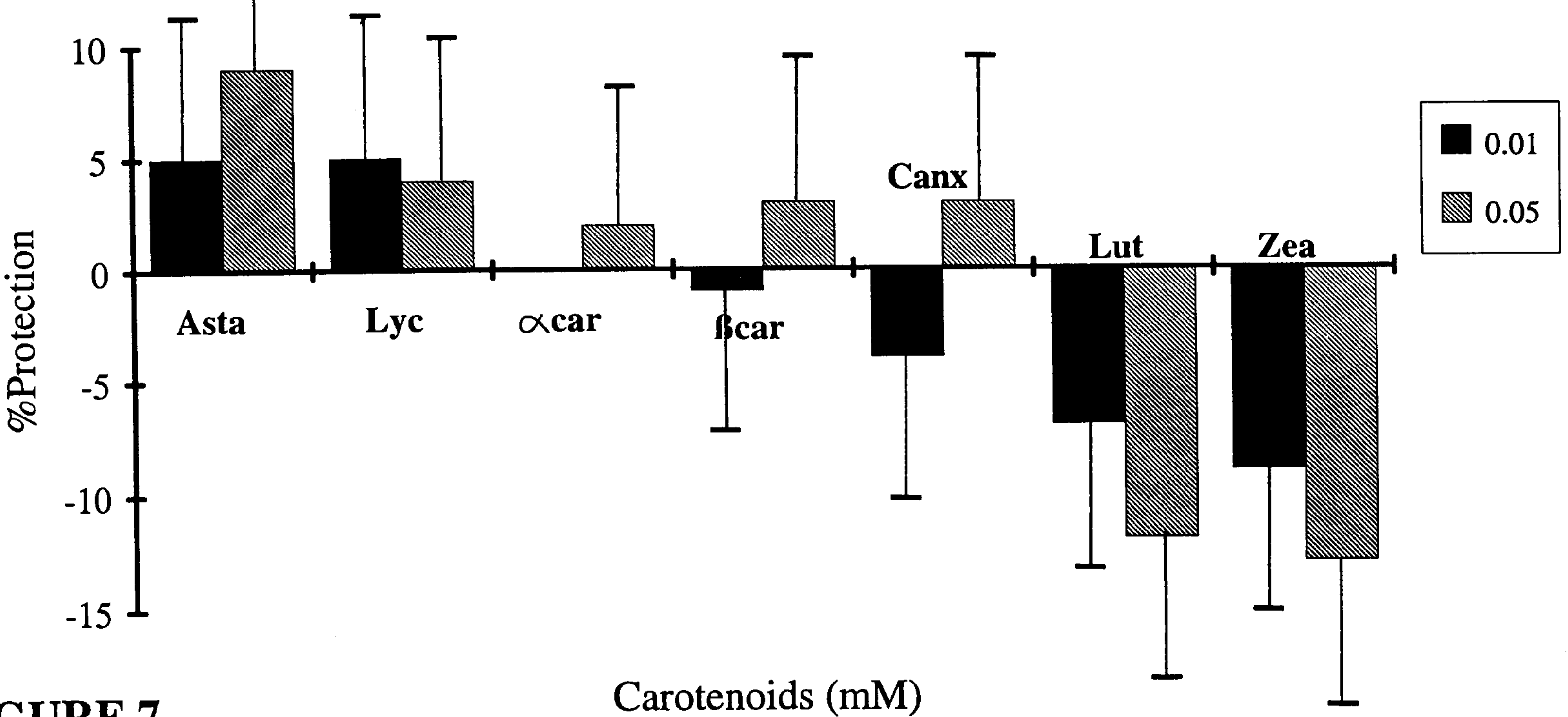


FIGURE 7

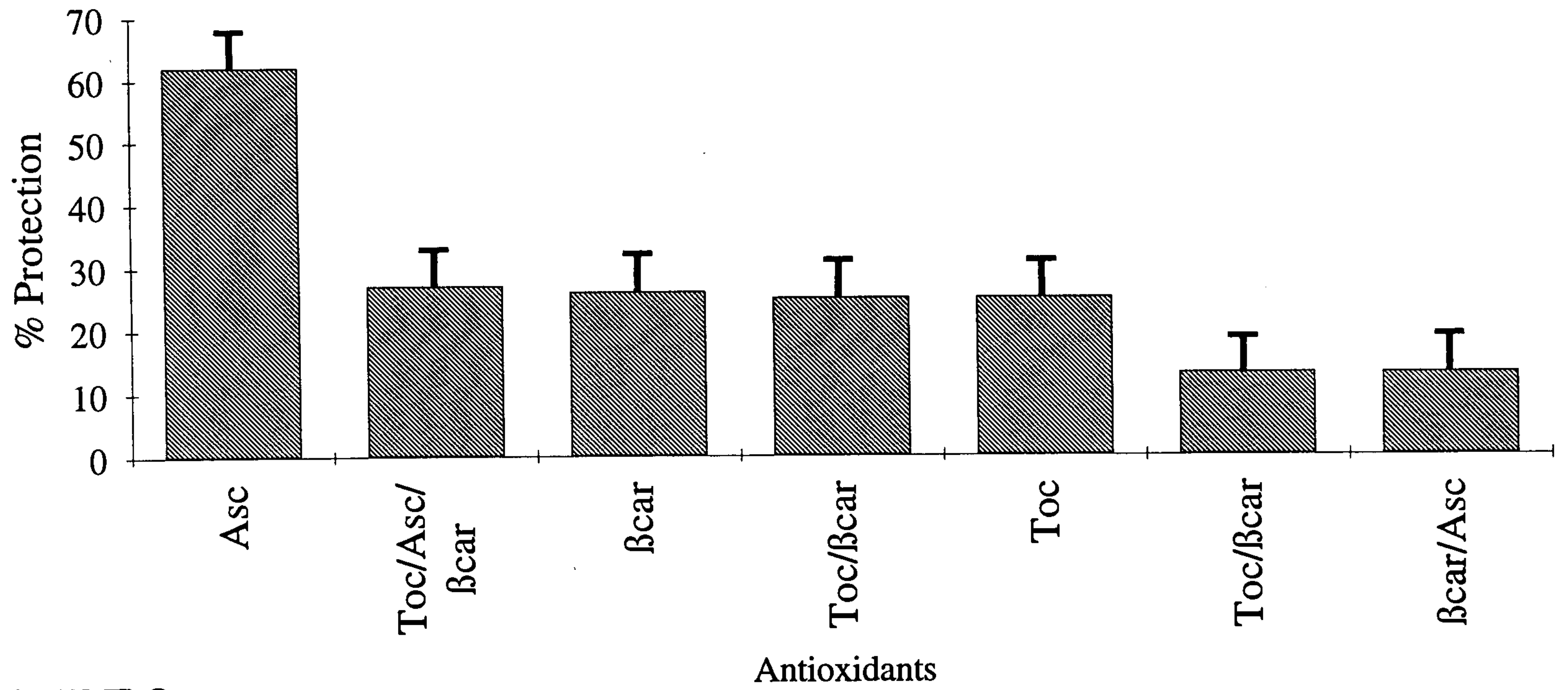


FIGURE 8

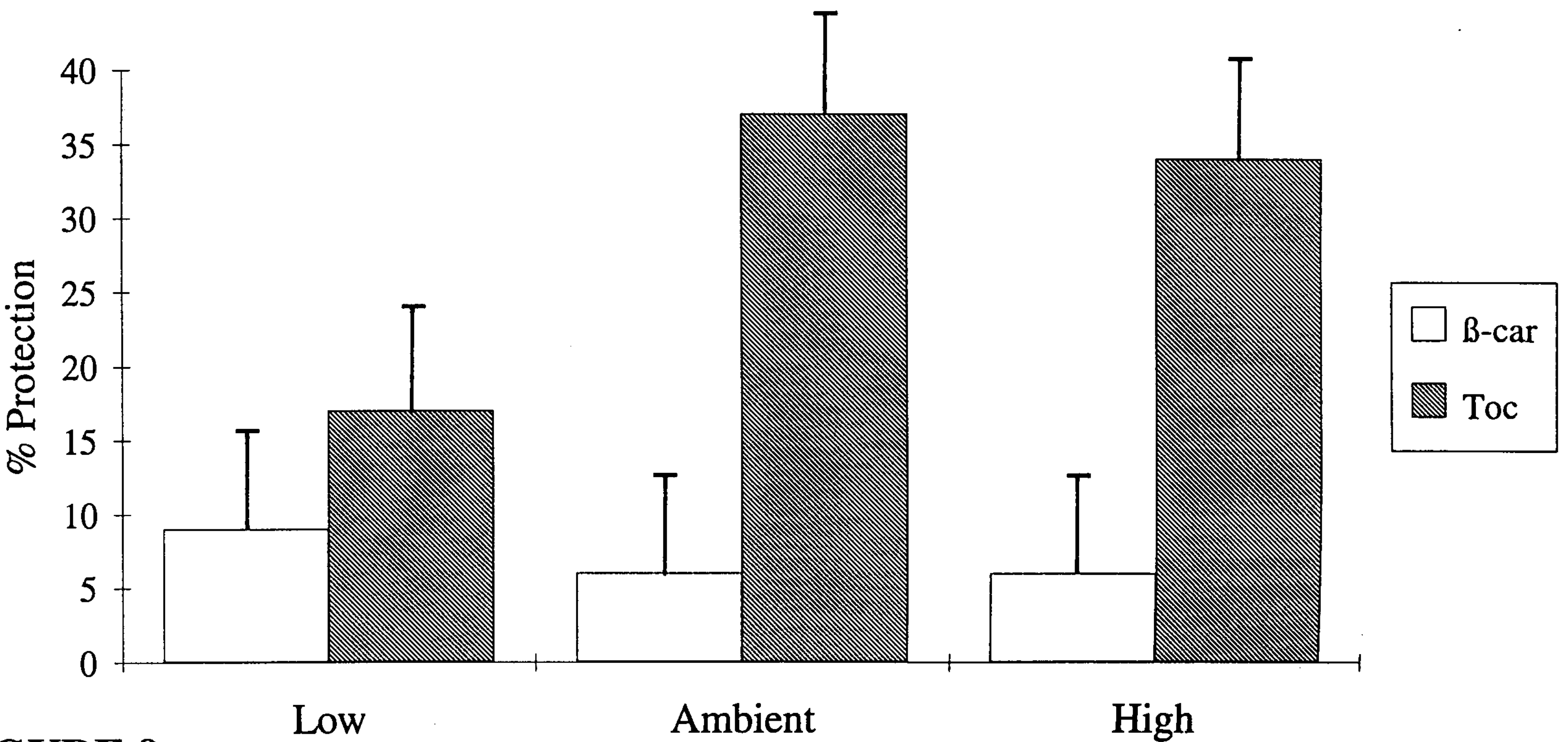


FIGURE 9

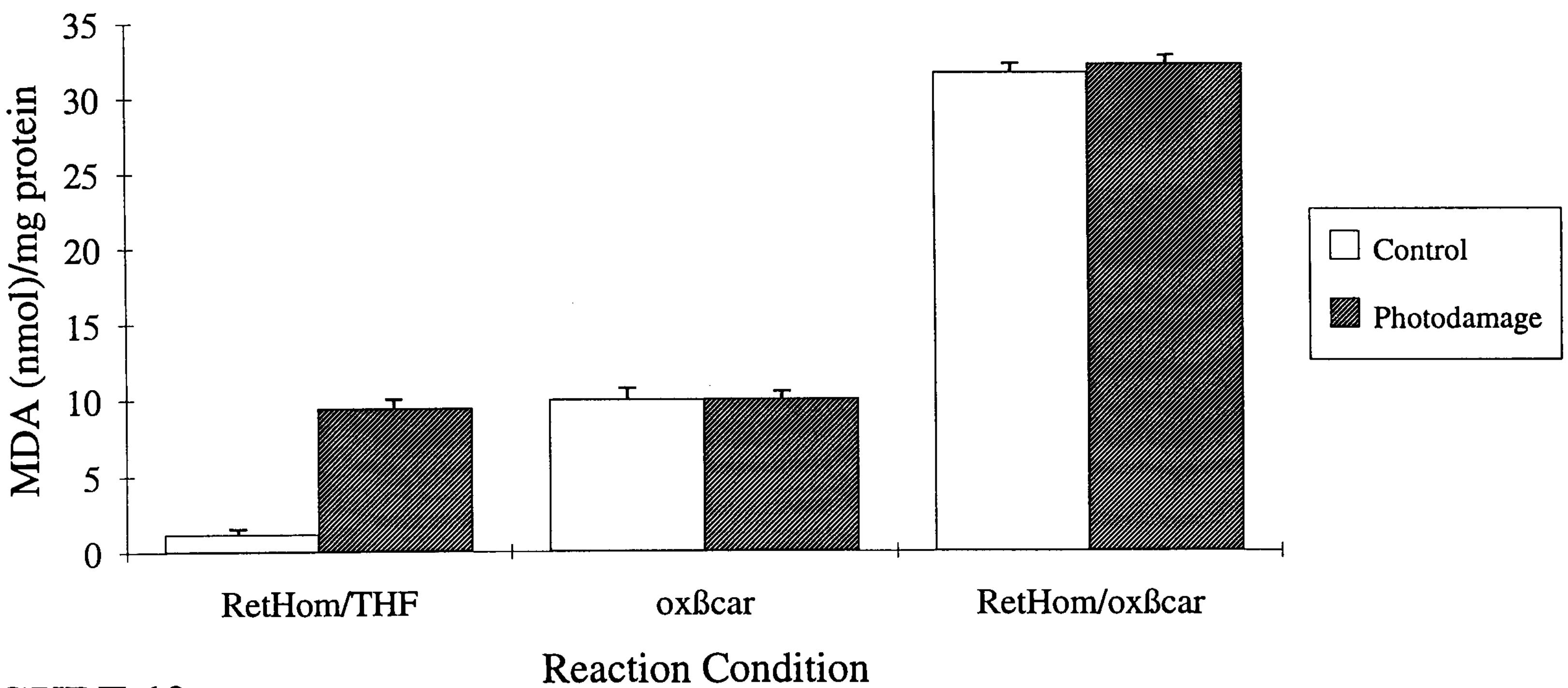


FIGURE 10

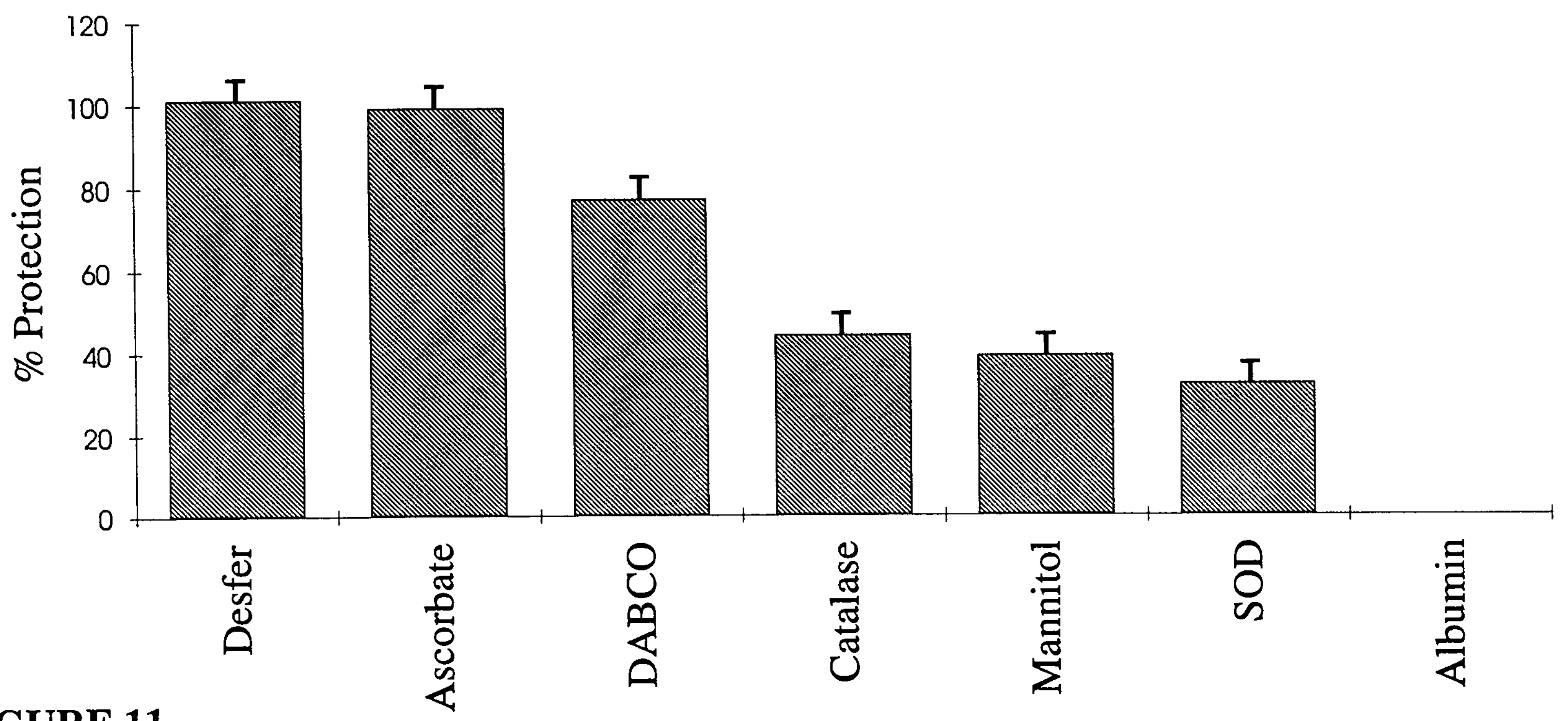


FIGURE 11

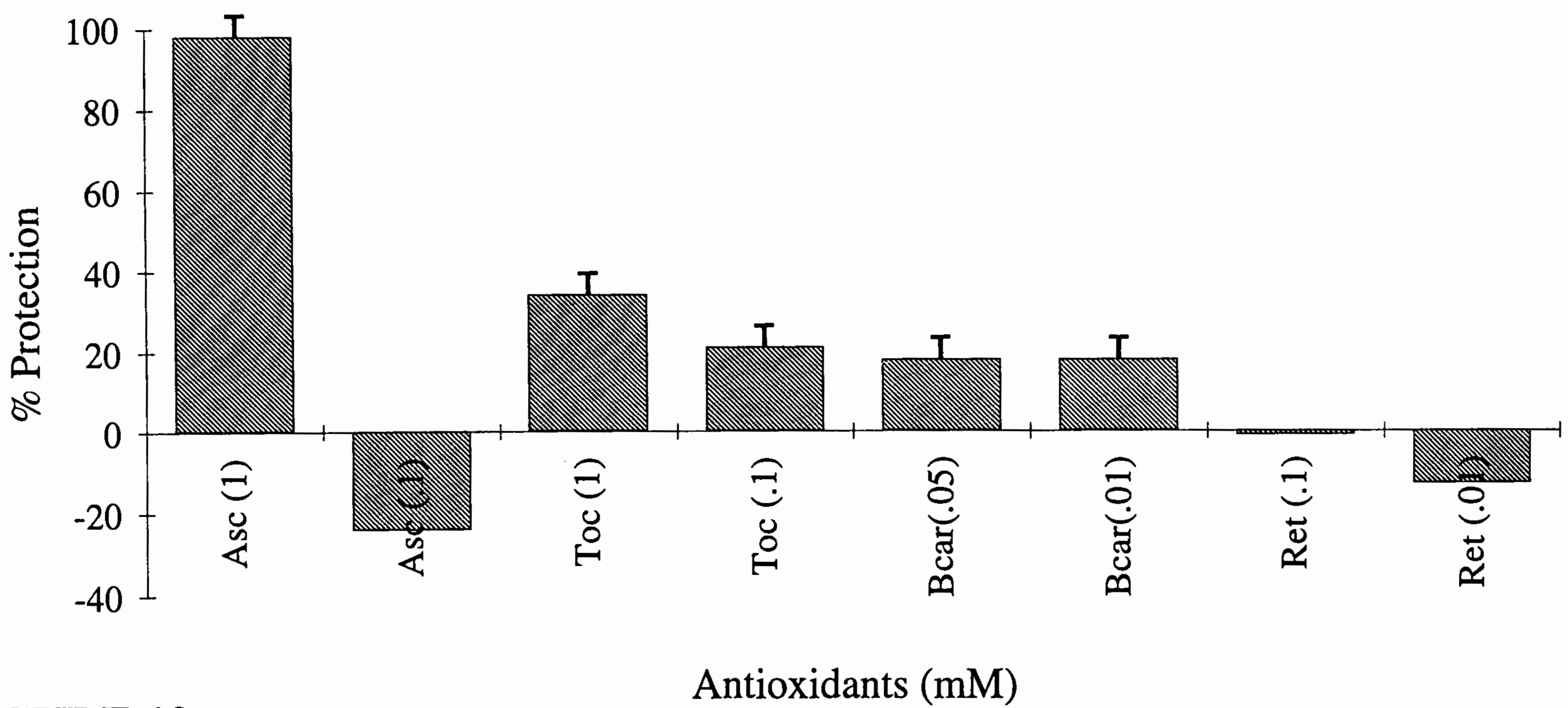


FIGURE 12

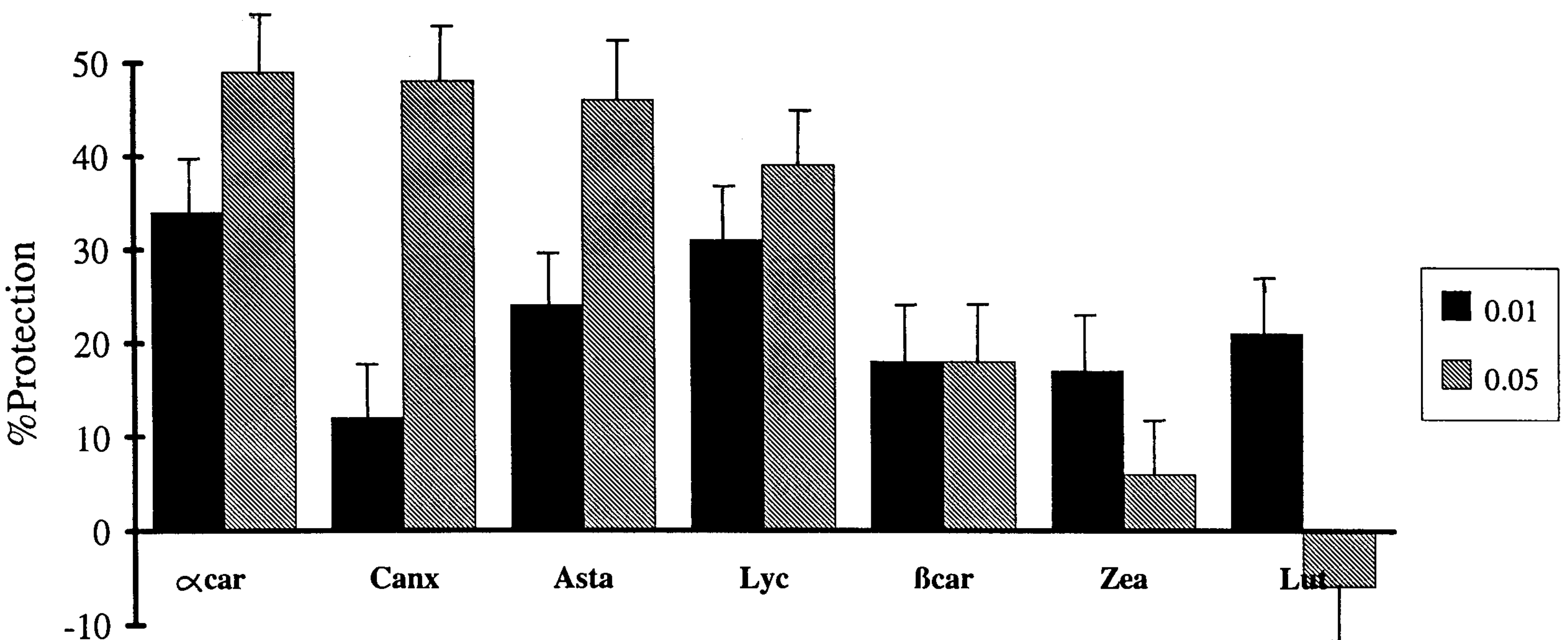


FIGURE 13

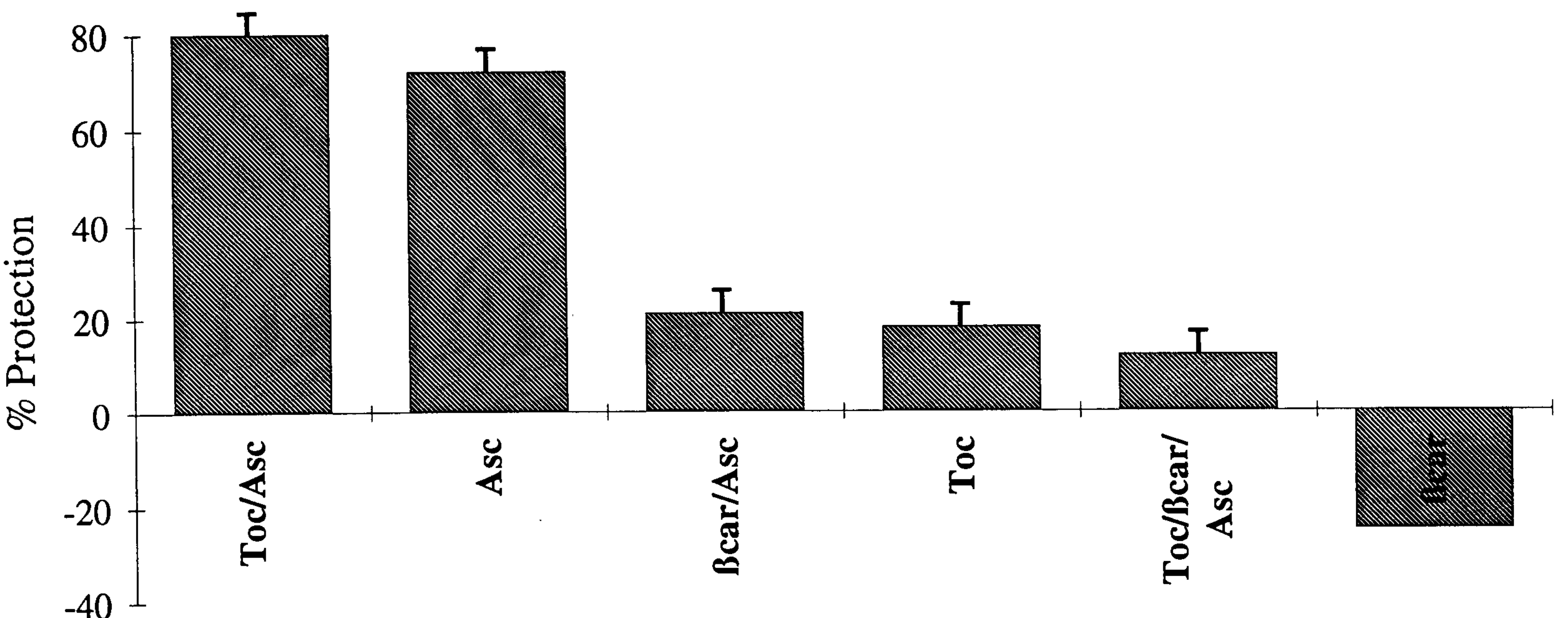


FIGURE 14

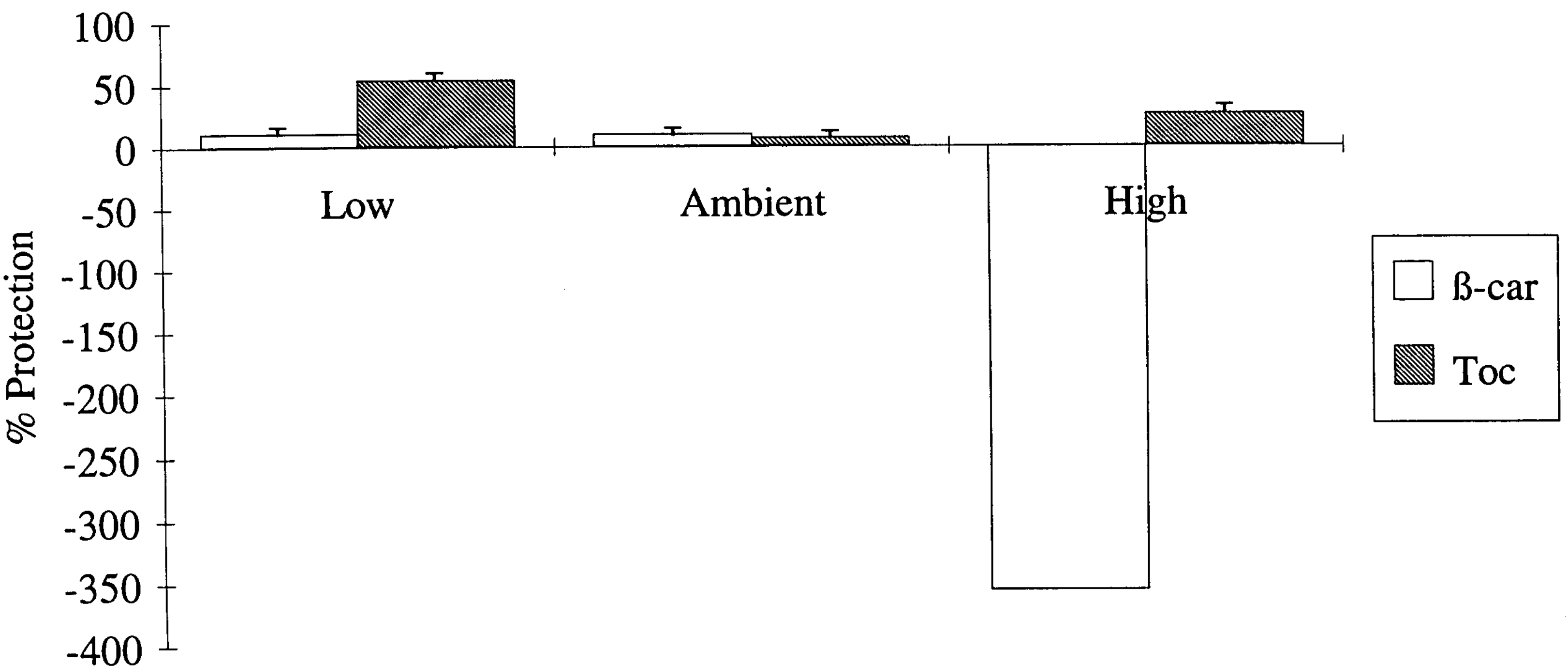


FIGURE 15

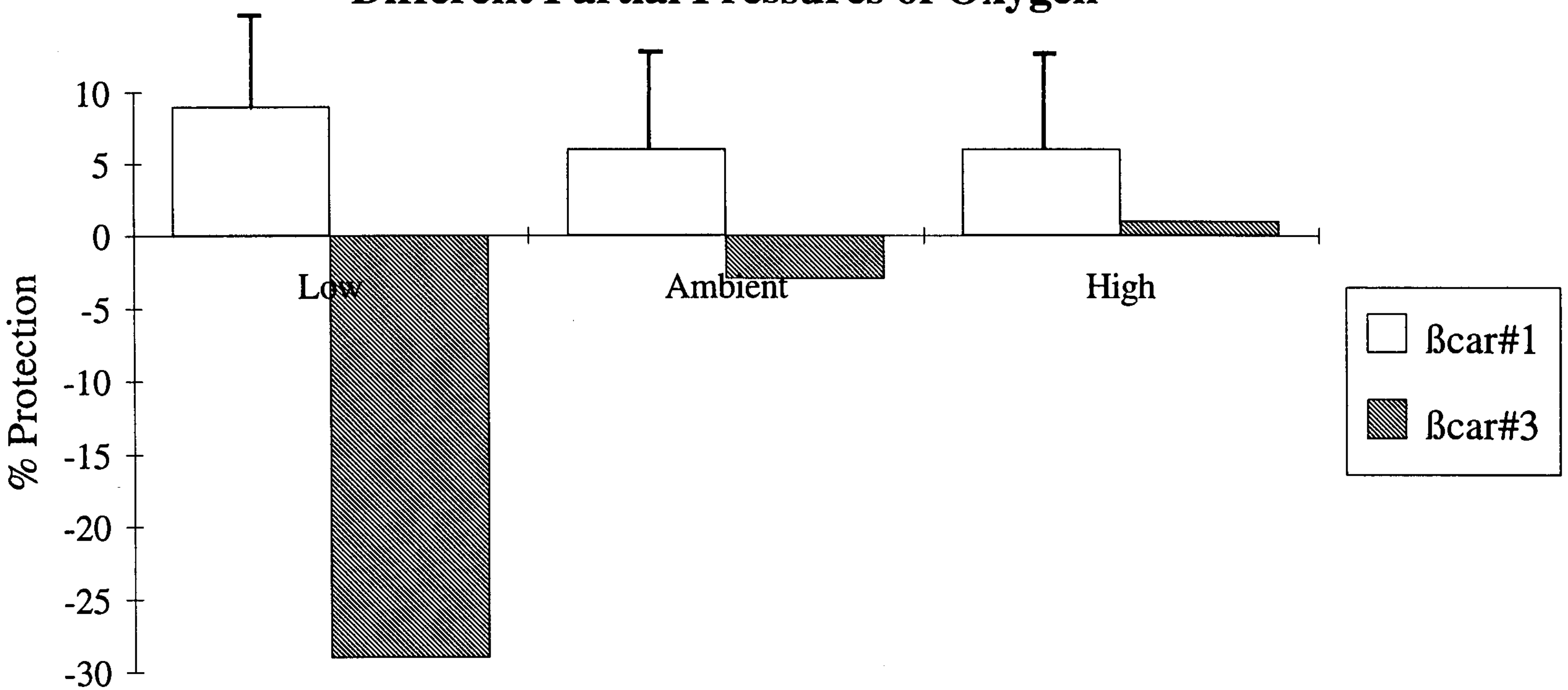


FIGURE 16

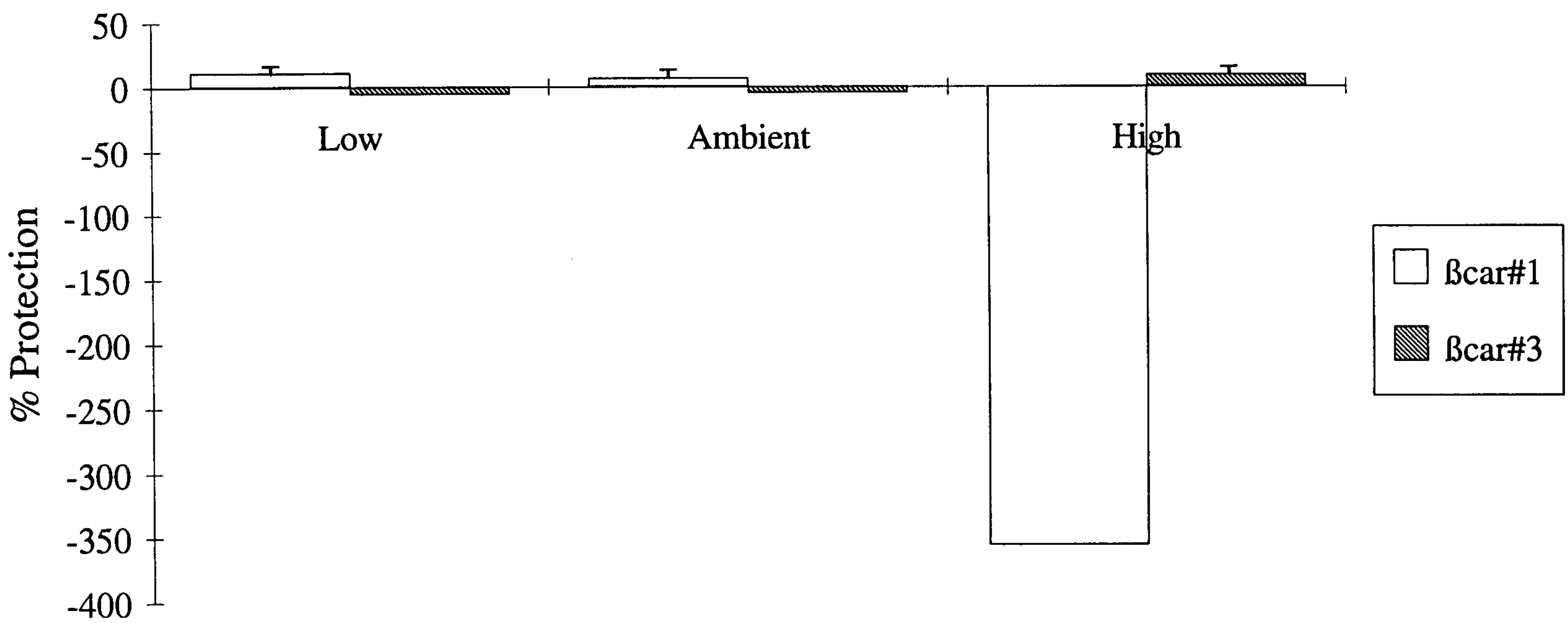


FIGURE 17

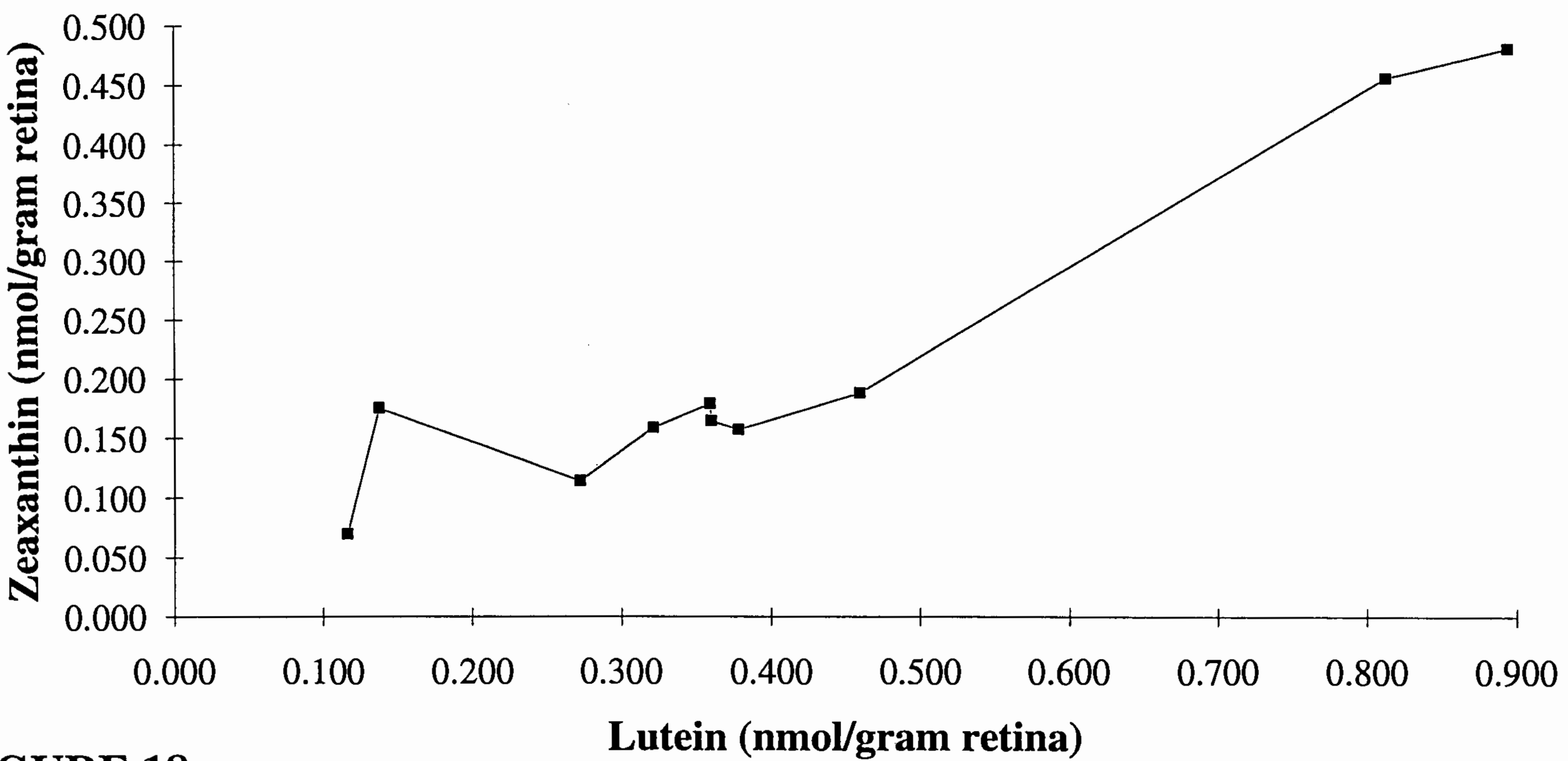


FIGURE 18

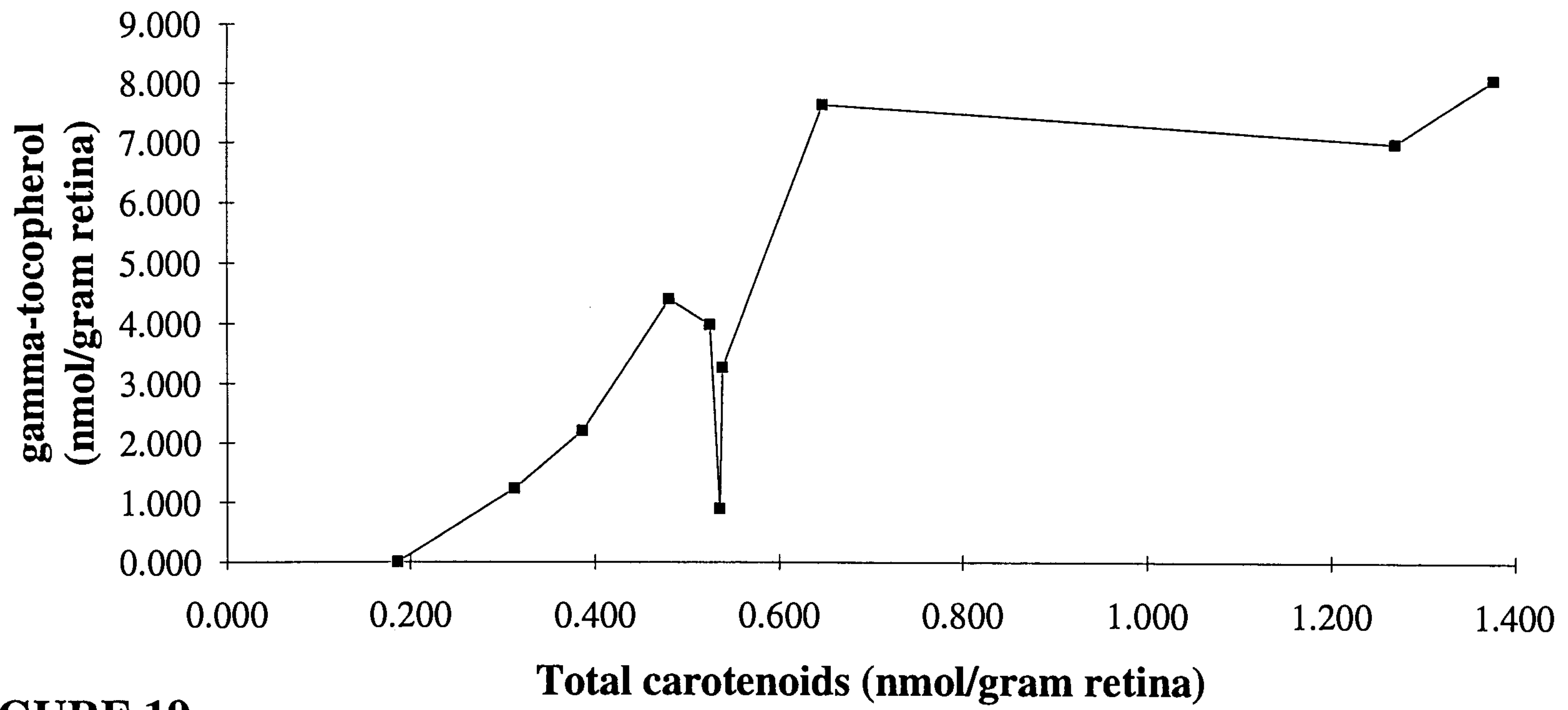


FIGURE 19

General Discussion.

The actions of β-carotene as a singlet oxygen quencher and free radical scavenger against oxygen-mediated stress were reviewed in Chapters 1 and 2. Chapter 2 considered the processes of carcinogenesis and possible mechanisms of action by carotenoids on a biochemical level. Chapter 3 focused on epidemiological findings, rather than *in vitro* experiments and recommendations for supplementation regimen and future studies were discussed. Chapter 4 explored the biochemistry and experimental fundamentals of carotenoid research. The information reviewed in Chapters 1 to 5 formed the groundwork for the experimental studies used in Chapter 6.

In the research described in this thesis, the photosensitized reaction produces mainly singlet oxygen with some propagating species (peroxyl radicals), whereas the metal-catalyzed reaction produces superoxide and hydroxyl radicals, peroxyl radicals, and some singlet oxygen. In the photochemical system, based on the reponse of DABCO (45% protection) a large percentage of damage is caused by species other than peroxyl radicals. Lack of protection by catalase, SOD, and mannitol, and 13% protection by desferrioxamine suggests that a endogenous metals play a role in propagating active species. The known mechanism for this process generates peroxyl radicals. In the metal-catalyzed system, protection by catalase, SOD, and mannitol suggest the presence of hydrogen peroxide, superoxide, and hydroxyl radicals. Total protection by desferrioxamine suggests a Fenton-type reaction is occuring, which would generate peroxyl radicals in the presence of unsaturated lipids. Therefore, the majority of damage in both systems appears, to be propagated by peroxyl radicals in the lipid membranes. Peroxyl radicals diffuse readily throughout living cells and due to their selectivity for

membrane lipids, proteins, and nuclear material, are more dangerous than the more reactive hydroxyl radicals.

Carotenoids are of special interest because of their photochemical and chemical reactivity. α-Carotene, canthaxanthin, astaxanthin, and lycopene are superior antioxidants compared to β-carotene, lutein, and zeaxanthin. Previous studies have concluded that the oxo-groups of canthaxanthin and astaxanthin increases their antioxidant potency. In this study, however, α-carotene and lycopene (carotenoids without oxo-groups) are comparable to canthaxanthin and astaxanthin as antioxidants. The absence of α-carotene, canthaxanthin, astaxanthin, lycopene, and β-carotene in HPLC assays of human retina may indicate that they are unimportant in retina health. Alternatively, these and other carotenoids may cross the blood-retina barrier, only to be quickly destroyed by free radical processes.

Lutein and zeaxanthin do not possess vitamin A activity, however, other vitamin A active carotenoids (such as β-carotene, α-carotene, and cryptoxanthin) may serve as a pool for the never-ending demand for 11-*cis*-retinal. Lutein and zeaxanthin accumulate, providing the yellowness of the "yellow spot" in the *macula lutea,* and their lack of antioxidant activity, singlet oxygen quenching, or converion to retinol would serve to ensure their conservation. Apparently, lutein and zeaxanthin function as filters to attenuate blue light in the macular area, thereby decreasing the production of the initiating species associated with lipid peroxidation. Lower concentrations of lutein and zeaxanthin protected to significance ($p < .05$) against metal-catalyzed damage where higher concentrations did not.

Carotenoids are unstable compounds and special conditions must be used in their storage and handling. Now that more people are supplementing their diets with pure forms of carotene, the question of the effects of carotenoid oxidation products becomes very important. The oxidation products themselves are moderately TBA reactive, however, the greatest damage occurs in the presence of lipid membranes. This suggests that oxidized β-carotene *in vivo* may be deleterious to health. This may be a factor in the recent, highly publicized findings from Finland, where there was an 18 % increase in lung cancer among men supplemented with β-carotene (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). It is possible that β-carotene does not protect against previously formed tumors, or that the presence of smoke-related chemicals increases the autoxidation of β-carotene and/or its oxidation products in oxygenated lung tissue.

Antioxidants may someday be used in the standard therapeutic treatment of patients susceptible to certain retina diseases, including macular degeneration. In cases of trauma or foreign metal particles embedded in the eye, antioxidant treatment may help attenuate the damage incurred. Special attention to diets high in antioxidant-containing fruits and vegetables may be beneficial for people who are exposed to environments with a high risk for inducing oxygen-mediated stress, such as exposure to lasers, high-intensity lighting in medical or industrial settings, laser eye surgery, or in the elderly with lens transplants that are transparent to near UV light.

Ascorbate is superior to the other antioxidants tested in both kinds of oxygen-mediated stress. Our *in vitro* results indicate that, in the human retina, phototoxic reactions and oxygen-mediated damage could be attenuated by the presence of high levels of ascorbate. α-Tocopherol, carotenoids, and retinol may protect to a lesser degree and

their effects in combination with each other or other antioxidants *in vivo* may be more important. *In vitro* trials with retina homogenate in the absence of exogenous antioxidants, indicates that some retinas are naturally more resistant to oxidative stress or possibly contain lower levels of TBA reactive substances.

In the current data, levels of certain endogenous dietary compounds (lutein, zeaxanthin, α-tocopherol, γ-tocopherol, and retinol) were not correlated with protection against photchemical or Fenton-inuced lipid autoxidation of retina homogenate. Interestingly, lutein and zeaxanthin were positively correlated, as were total carotenoids and γ-tocopherol. Endogenous ascorbate in retina tissues may protect agains oxygen-mediated stress, however we were unable to assay this vitamin. Assuming protection by ascorbate, carotenoids, and tocopherol reflects processes *in vivo*, then supplementation with foods high in these antioxidants could be chemically relevant in both treatment and prevention. Epidemiological assessment of high-risk population groups could provide additional basis for intervention trials.

APPENDICES

HPLC Chromatography.

Raw data subject number	Vital statisics subject number
1	X1
2	X11
3	X13
4	X16
5	X18
6	X29
7	X31
8	X35
9	X43
10	X44

Legend to chromatograms.

TA = α-Tocopherol acetate, aT = α-Tocopherol, dT = δ-Tocopherol,
gT = γ-Tocopherol, RA = Retinyl acetate, R = Retinol, L = Lutein, Z = Zeaxanthin.

Tocopherols assayed at 285 nm, carotenoids at 450 nm, retinol at 325 nm.

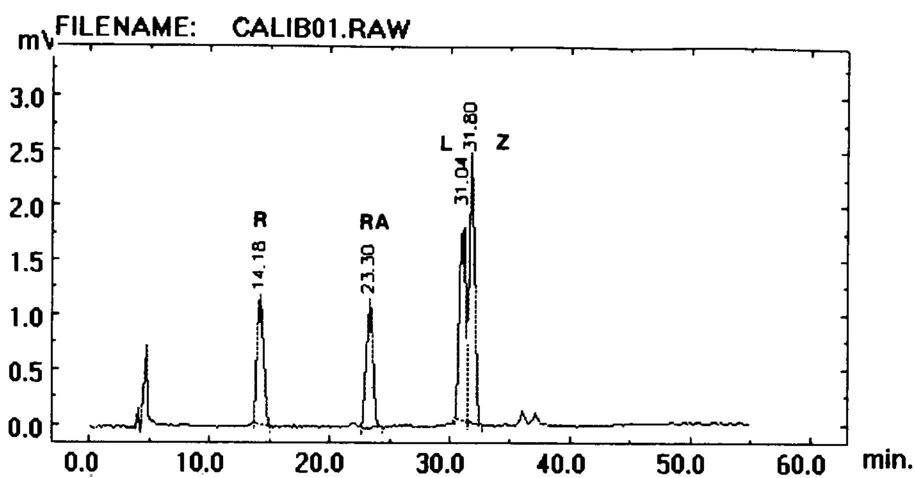
SUBJECT	DATE	AGE (YEARS)	SEX	PM TIME (HRS)	COD	COMMENTS (IRIS COLOUR,OTHER)
X1 *	930521	80	F	19	CHF,DIAB	BLUE
X2	930521	59	F	9	COPD, EMPH	BLUE
X3	930522	36	F	15	GASTRIC CA	BLUE
X4	930523	65	F	18	LUNG DX, RES FAIL	BROWN
X5	930523	57	M	12.5	COLON CA	BLUE - JAUNDICE
X6	930528	58	M	10	COLON CA	BLUE
X7	930607	72	F	4	MLP MYELOMA	BROWN
X8	930609	45	F	17.5	MVA	BLUE-SUBARACH HEMM
X9	930609	66	M	21	CHF	BLUE
X10	930611	58	F	14.5	THYROID CA	BLUE
X11 *	930612	15	F	7	MVA	BLUE
X12	930629	56	M	10	CARD MYOPATHY	BROWN
X13 *	930716	74	F	16	INTRACER HEMM	BROWN
X14	930717	60	M	10	CARDIAC DX	LIGHT GREY
X15	930718	51	M	13	CARDIAC DX	DARK BROWN
X16 *	930804	84	M	10.5	PANC CA	BLUE-BILAT CATARACT EX
X17	930810	69	M	17.5	CEREBRAL HEMM	BROWN
X18 *	930807	23	F	16	RESP ARREST	LIGHT GREEN
X19	930810	48	M	25	CARDIAC DX	BROWN
X20	930815	60	M	24.5	LUNG CA	DARK BROWN
X21	930825	58	M	21.5	LUNG CA	DARK BROWN
X22	930826	69	F	10	BREAST CA	GREY GREEN
X23	930829	53	M	11.5	CARDIAC ARREST	BLUE
X24	930905	71	F	14	BREAST CA	BLUE
X25	930909	60	M	23	LUNG CA	BLUE
X26	930911	62	M	9	RESP ARREST	BLUE
X27	930912	71	F	11	LUNG CA	DARK BROWN
X28	930922	65	F	19	LIVER DX	BLUE

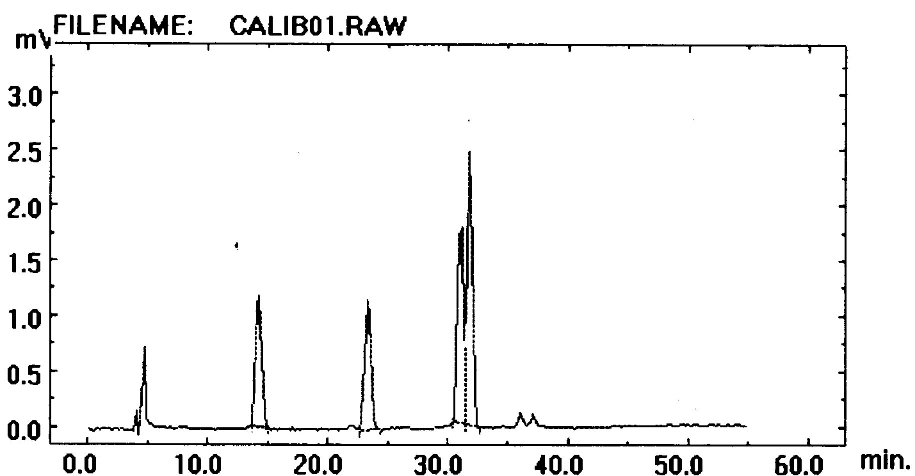
X29 *	930922	53	M	15.5	CARDIAC ARREST	BLUE
X30	930929	59	M	19.5	BOWEL CA	LIGHT GREY
X31 *	931009	88	F	22.5	PNEUMONIA	BLUE
X32	931009	71	F	18.5	CEREB HEMM	GREY
X33	931101	53	M	23.5	RENAL FAILURE	BROWN
X34	931102	58	M	22.5	CHF	
X35 *	931102	41	M	15.5	CARDIAC DX	BLUE
X36	931120	64	M	19	CEREB HEMM	BLUE
X37	931125	68	F	28.5	ESOPHAG CA	BROWN
X38	931128	46	F	9	BREAST CA	BLUE
X39	931203	74	F	22	PULM EMBOLISM	BLUE
X40	931203	66	M	18	PROSTATE CA	BROWN
X41	931210	52	F	25	MULTIPLE TRAUMA	
X42	931210	46	M	22	RENAL CELL CA	LIGHT GREY
X43 *	931213	22	F	25	MELANOMA	BLUE
X44 *	931213	62	F	13	COPD	BROWN
X45	931223	73	M	5	PROSTATE CA	BLUE
X46	940103	70	F	9.5	MYOCARDIAL INF	BLUE
X47	940104	70	M	8.5	CARDIAC ARREST	BLUE
X48	940216	64	M		RET NOT NORMAL	BLUE - PRACTICE ONLY
X49	940216	86	F	7.5	PNEUMONIA	BLUE
X50	940217	66	M	7.5	CARDIAC SHOCK	BLUE
X51	940217	64	M	12.5	PANCREAITIS	BLUE
X52 *	940220	46	F	21	LUNG CA	GREY - MULT SCLEROSIS
X53	940220	70	F	19	LUNG DX	GREY - TYPE II DIABETES
X54	940309	63	M	16.5	CHD	BLUE
X55	940324	71	M	18	CHD	BLUE - VIRUS**DON'T USE
X56	940325	71	M	14	CARDIAC FAILURE	BLUE
X57	940325	64	F	8	LUNG CA	BROWN
X58	940326	50	M	10	RES FAILURE	BLUE

X59	940326		M	22	PROSTATE CA	BLUE
X60	940402	70	M	4	LUNG CA	BROWN
X61	940402	80	M	14	CEREB HEMM	BLUE
X62	940403	49	F	5	LUNG CA	BLUE
X63	940403	71	M	9	LUNG CA	BLUE
X64	940409	74	F	24	PULM EMBOLISM	LIGHT GREY- DIABETES
X65	940409	86	F	8.5	CHD	BLUE
X66	940409	73	F	21.4	CEREB HEMM	LIGHT BLUE
X67	940409	65	M	10	CHD	BLUE

* Right retinas used in stress test, left retinas used in HPLC study.

Abbreviations: PM (post mortem), COD (cause of death), CHF (coronary heart failure), DIAB (diabetes), CA (cancer), DX (disease), RES (respiratory), MLP (multiple), MVA (motor vehicle accident), CARD (cardiac), HEMM (hemmorhage), INTRACER (intracerebral), PANC (pancreatic), ESOPHAG (esophagus), PULM (pulmonary), COPD (chronic obstructive lung disease), INF (infection), RET (retina), CHD (coronary heart disease), CEREB (cerebral), EX (extraction).





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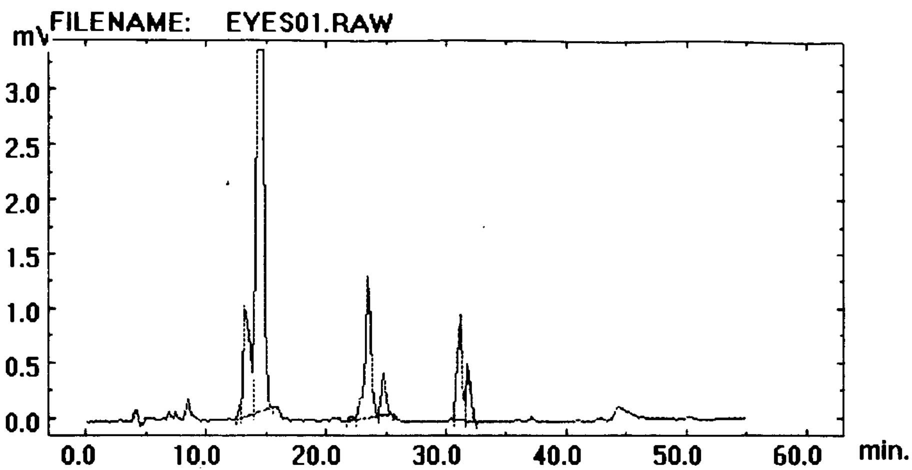
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
retinol	2.022	14.18	88705	0.040	0.609
ret acet.	INTERNAL STD	23.30	87749	1.000	1.000
lutein	1.038	31.04	121474	0.015	1.332
zeaxanthin	1.038	31.80	151837	0.012	1.365
TOTALS	54.098		449765		



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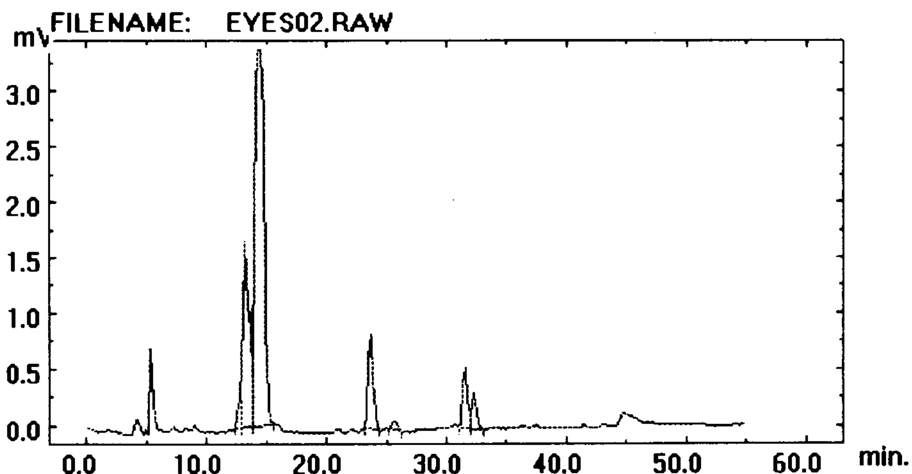
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.75	3546		0.543
2	0.000	13.30	76873		0.566
retinol	20.100	14.42	921392	0.040	0.614
4	0.000	22.06	1848		0.940
ret acet.	INTERNAL STD	23.48	91679	1.000	1.000
6	0.000	24.76	21168		1.054
lutein	0.405	31.08	49527	0.015	1.324
zeaxanthin	0.168	31.83	25623	0.012	1.356
TOTALS	70.673		1191656		



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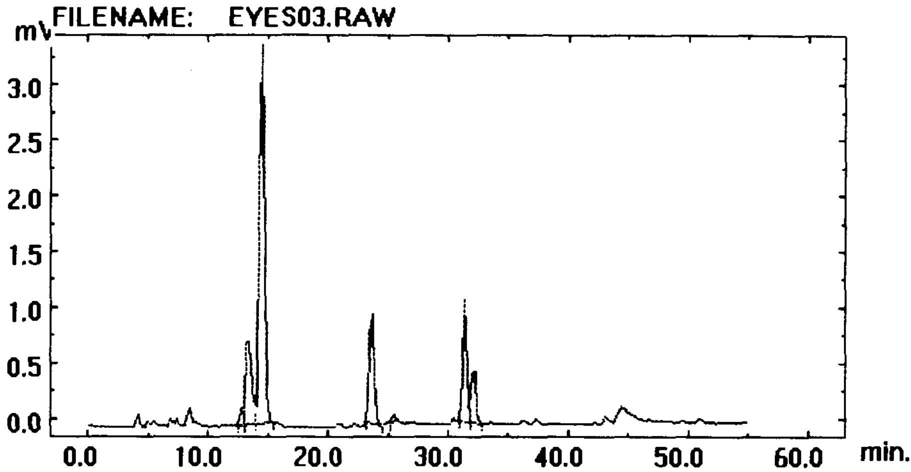
EYES_A CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.64	10095		0.536
2	0.000	13.19	124760		0.559
retinol	22.229	14.33	619218	0.040	0.607
ret acet.	INTERNAL STD	23.59	55713	1.000	1.000
5	0.000	25.63	4635		1.087
lutein	0.423	31.49	31400	0.015	1.335
zeaxanthin	0.193	32.25	17925	0.012	1.367
TOTALS	72.845		863746		



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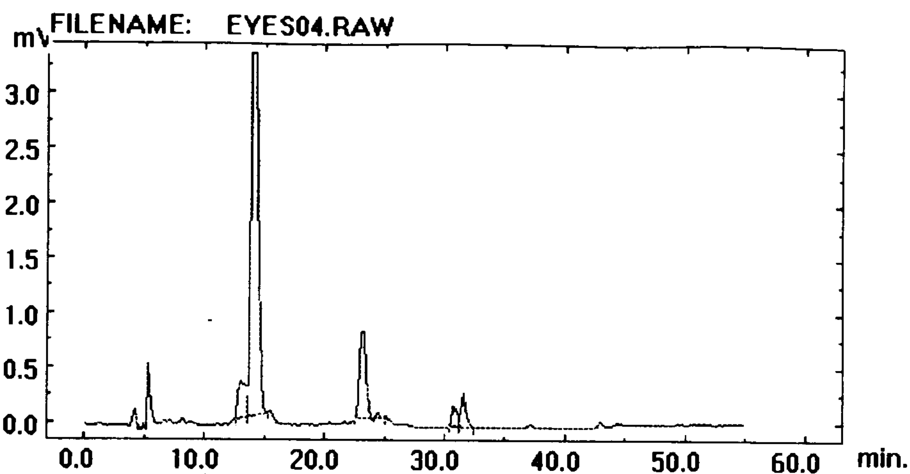
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.84	6002		0.503
2	0.000	13.33	56008		0.523
retinol	6.499	14.47	198780		0.567
ret acet.	INTERNAL STD	23.61	61818		0.925
5	0.000	25.51	366789		1.000
lutein	0.662	31.35	54604		1.229
zeaxanthin	0.270	32.10	27788	0.264	1.259
TOTALS	53.042		771789		



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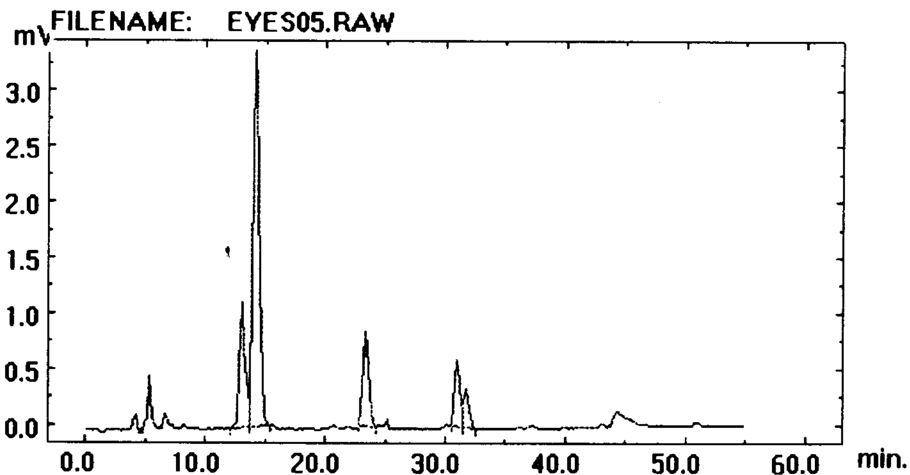
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.91	29990		0.558
retinol	12.668	14.04	382551	0.040	0.607
ret acet.	INTERNAL STD	23.14	60395	1.000	1.000
lutein	0.142	30.77	11432	0.015	1.330
zeaxanthin	0.180	31.54	18118	0.012	1.363
TOTALS	62.990		502486		



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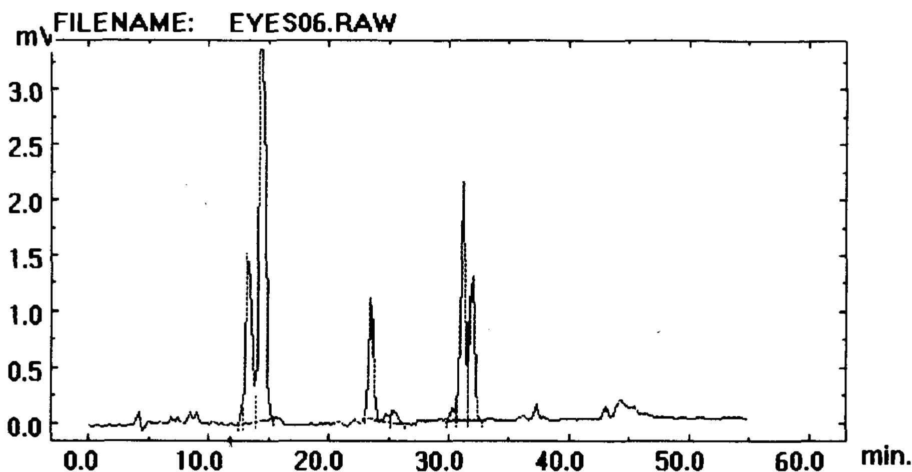
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.98	78277		0.557
retinol	8.936	14.12	268618	0.040	0.606
ret acet.	INTERNAL STD	23.30	60122	1.000	1.000
lutein	0.435	30.94	34856	0.015	1.328
zeaxanthin	0.216	31.68	21599	0.012	1.360
TOTALS	59.586		463472		



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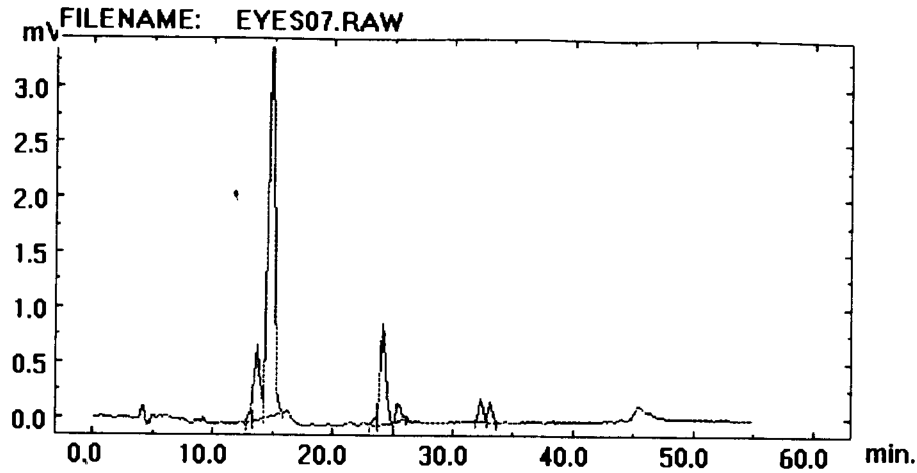
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.79	4479		0.544
2	0.000	13.28	103166		0.565
retinol	10.218	14.43	340263	0.040	0.614
ret acet.	INTERNAL STD	23.52	66602	1.000	1.000
5	0.000	24.84	4516		1.056
6	0.000	25.47	7479		1.083
7	0.000	30.30	5343		1.288
lutein	1.215	31.15	107892	0.015	1.325
zeaxanthin	0.655	31.91	72664	0.012	1.357
TOTALS	62.087		712404		



DISK FILE:EYES07.RAW INJECTED AT: 07/13/94 09:55:28
REPROCESSED: Method: Local

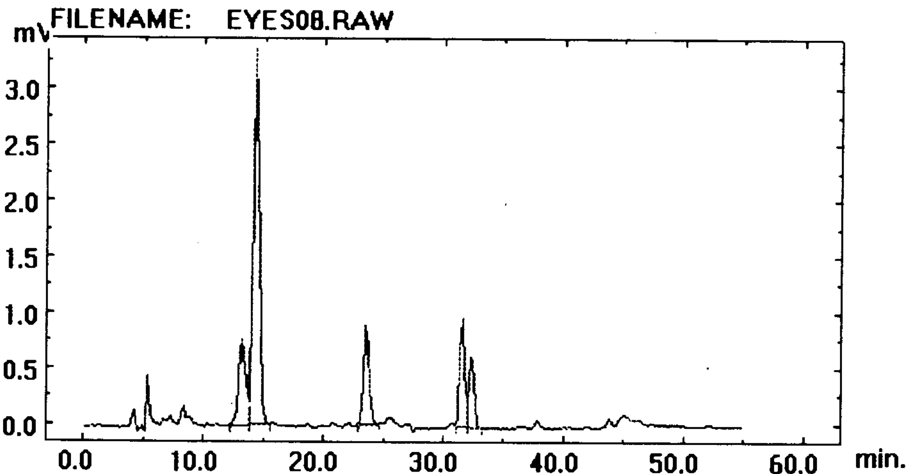
EYES_A CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	13.10	3342		0.542
2	0.000	13.64	46494		0.565
retinol	13.070	14.78	401502	0.040	0.612
4	0.000	23.57	3749		0.975
ret acet.	INTERNAL STD	24.16	61438	1.000	1.000
6	0.000	25.45	8800		1.054
lutein	0.134	32.28	10955	0.015	1.336
zeaxanthin	0.080	33.04	8164	0.012	1.368
TOTALS	63.284		544444		



DISK FILE:EYES08.RAW INJECTED AT: 07/13/94 11:14:06
REPROCESSED: Method: Local

EYES_A CH= PS=1

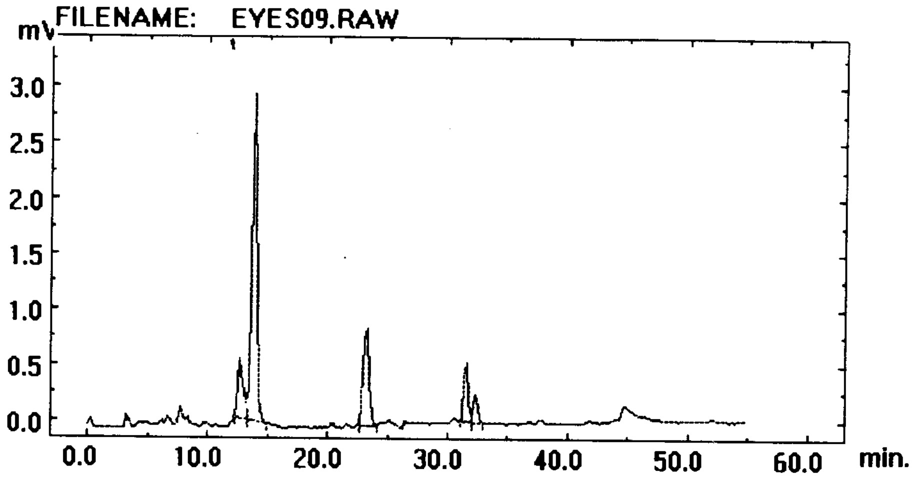
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:

SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	13.12	62894		0.558
retinol	8.054	14.26	242158	0.040	0.607
ret acet.	INTERNAL STD	23.50	60131	1.000	1.000
lutein	0.723	31.51	57975	0.015	1.341
zeaxanthin	0.406	32.29	40666	0.012	1.374
TOTALS	59.183		463824		



DISK FILE:EYES09.RAW INJECTED AT: 07/13/94 12:29:37
REPROCESSED: Method: Local

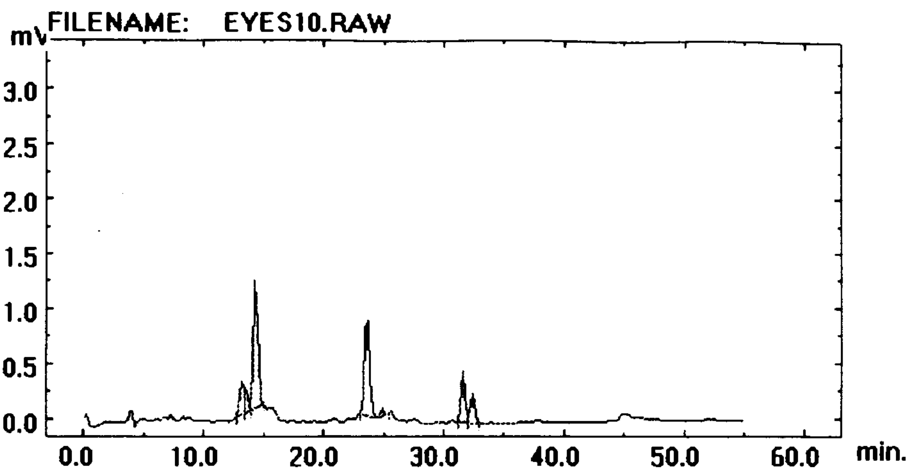
EYES_A CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	12.63	39057		0.545
retinol	5.818	13.80	176969	0.040	0.596
ret acet.	INTERNAL STD	23.16	60836	1.000	1.000
lutein	0.364	31.49	29509	0.015	1.359
zeaxanthin	0.152	32.29	15395	0.012	1.394
TOTALS	56.334		321766		



DISK FILE:EYES10.RAW INJECTED AT: 07/13/94 13:47:42
REPROCESSED: Method: Local

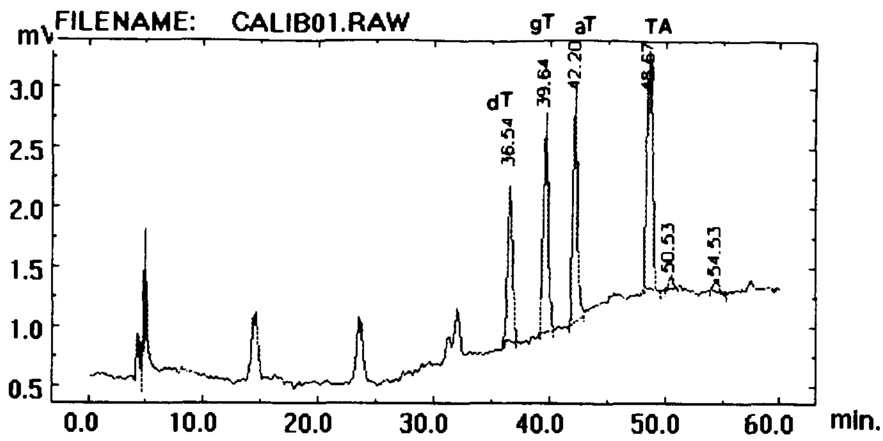
EYES_A CH= PS=1

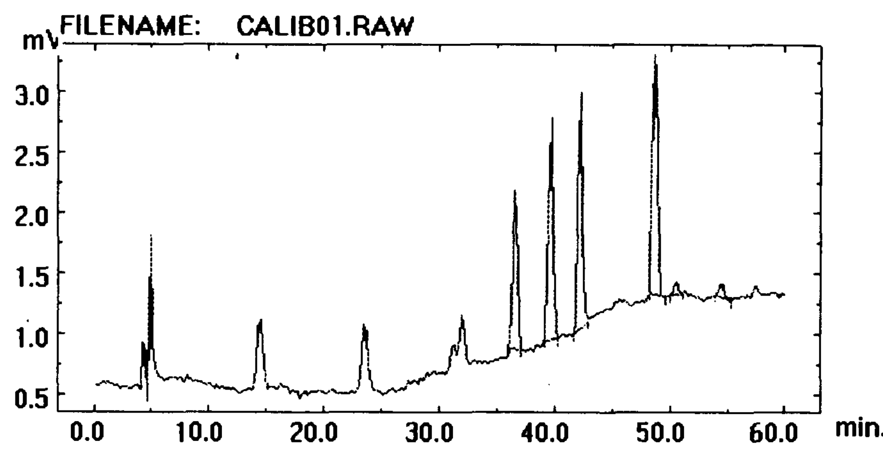
FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

SA	IS	XF
0.000	50.000	0.000

NAME	uM	RT	AREA	RF	RRT
1	0.000	13.16	16394		0.558
2	0.000	13.49	6833		0.572
retinol	2.102	14.32	60436	0.040	0.608
ret acet.	INTERNAL STD	23.57	57511	1.000	1.000
5	0.000	24.85	3119		1.054
lutein	0.296	31.58	22735	0.015	1.340
zeaxanthin	0.146	32.36	14030	0.012	1.373
TOTALS	52.545		181058		



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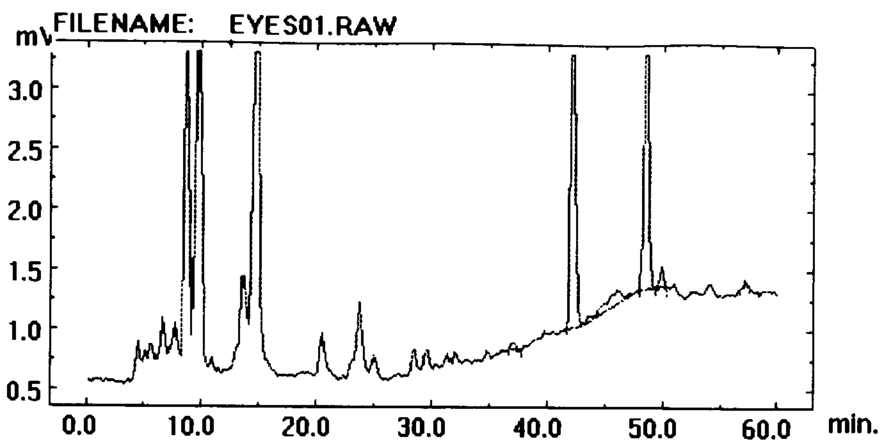
FILE  1        DISK FILE:CALIB01.RAW      INJECTED AT: 07/11/94 11:35:19
REPROCESSED: Method: Local

EYES_B                                                  CH=        PS=1
METHOD 2       RUN   1        INDEX  1

ANALYST:
SAMPLE   1
```

NAME	uM	RT	AREA	RF	RRT
D TOC. ACE	0.462	36.54	73925	0.994	0.750
G TOC. ACE	0.457	39.64	94575	0.769	0.814
A TOCOPH.	0.523	42.20	99362	0.837	0.867
A TOC. ACE	INTERNAL STD	48.67	159033	1.000	1.000
5	0.000	50.53	5163		1.038
6	0.000	54.53	6598		1.121
TOTALS	2.442		438656		



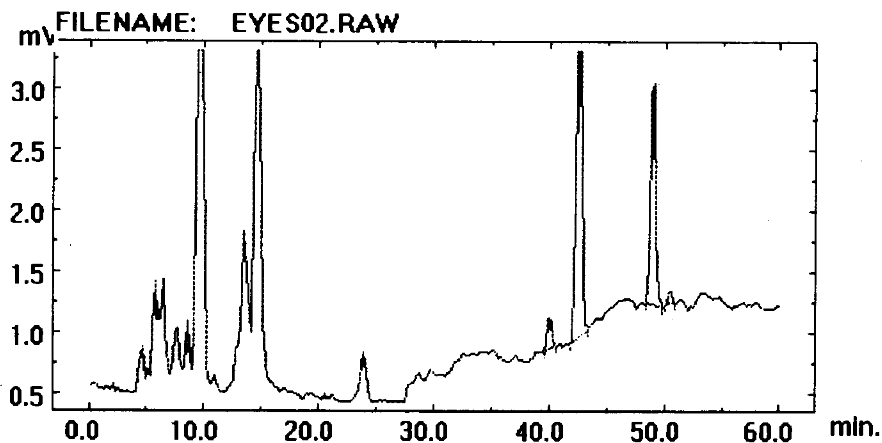
DISK FILE:EYES01.RAW INJECTED AT: 07/11/94 14:31:36
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	0.1959	36.92	2771		0.875
A TOCOPH.	62.880	42.18	215985		1.000
3	0.000	47.91	44924		1.136
A TOC. ACET.	INTERNAL	STANDARD		1	
5	0.000	49.84	6749		1.182
6	0.000	57.07	2418		1.354
TOTALS	1.310		416589		



DISK FILE:EYES02.RAW INJECTED AT: 07/12/94 09:57:10
REPROCESSED: Method: Local

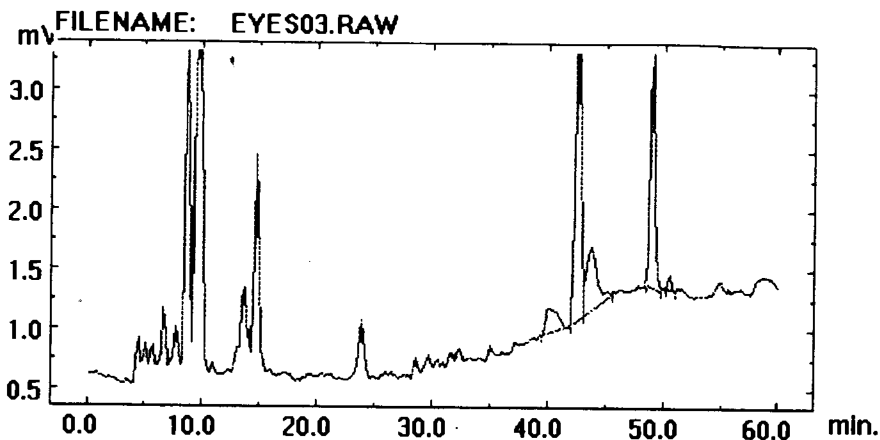
EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT

G TOCOPH.	4.674	39.99	14134		0.941
A TOCOPH.	82.308		228621		
A TOC. ACET.	INTERNAL	STANDARD	116238	1.000	
4	0.000	50.52	5931		1.190
TOTALS	1.000		364923		



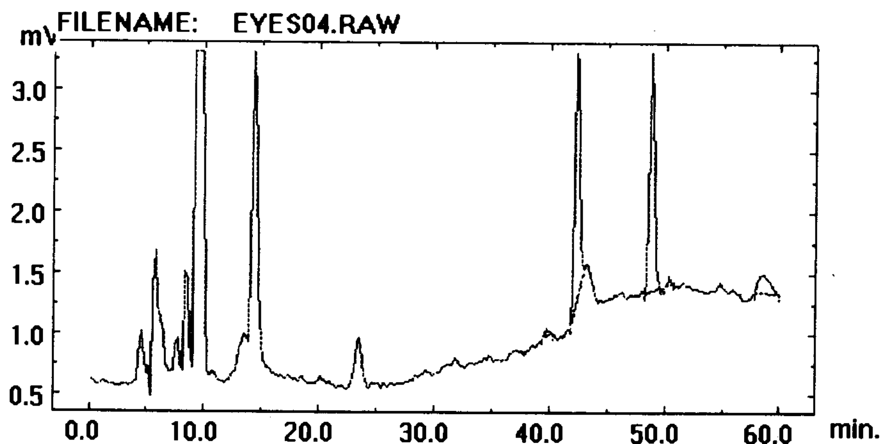
DISK FILE:EYES03.RAW INJECTED AT: 07/12/94 11:13:30
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	10.992	40.03	32666		0.942
A TOCOPH.	86.471	42.49	236010		1.000
3	0.000	43.71	70126		1.029
A TOC. ACET.	INTERNAL	STANDARD	114217		1.153
5	0.000	50.50	5419		1.189
TOTALS	1.000		458438		



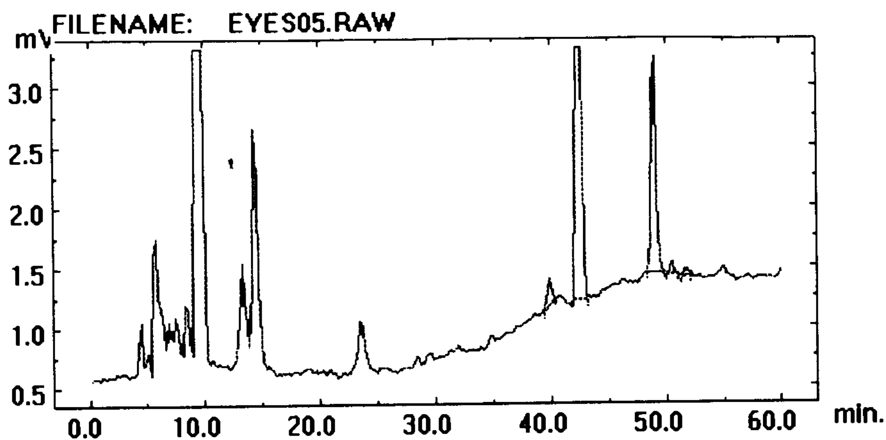
DISK FILE:EYES04.RAW INJECTED AT: 07/12/94 12:29:35
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	1.268	39.78	4008		0.940
A TOCOPH.	43.181	42.32	125409		1.000
A TOC. ACET.INTERNAL STANDARD		48.83	121537		1.154
4	0.000	50.32	2926		1.190
5	0.000	58.62	24072		1.386
TOTALS	1.000		277952		



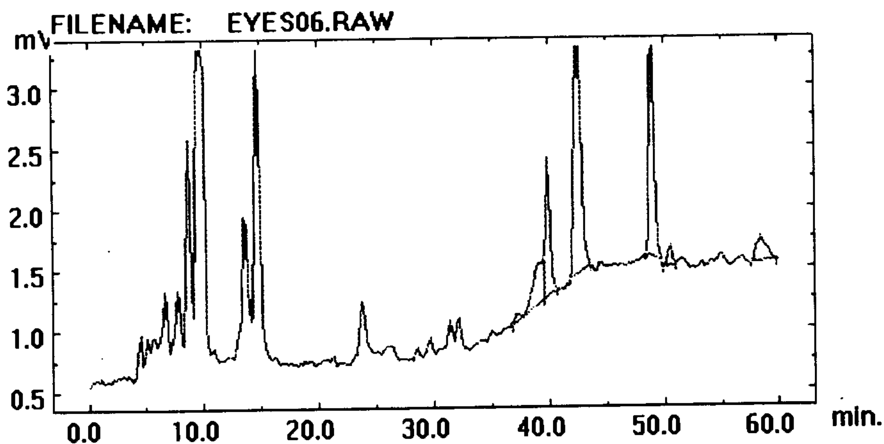
DISK FILE:EYES05.RAW INJECTED AT: 07/12/94 14:12:22
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	3.950	39.88	11498		0.940
A TOCOPH.	37.162	42.44	307256		1.000
A TOC. ACE. INTERNAL STD.		48.98	111890		1.155
4	0.000	50.51	4901		1.191
5	0.000	51.76	2987		1.220
TOTALS	1.000		438532		



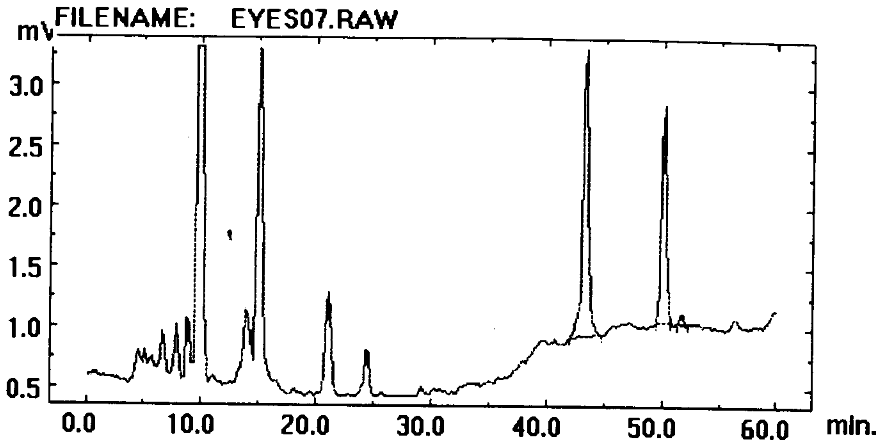
DISK FILE:EYES06.RAW INJECTED AT: 07/12/94 15:35:21
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
1	0.000	39.18	45352		0.922
G TOCOPH.	10.960	39.91	62991		0.939
A TOCOPH.	80.229	42.48	305161		1.000
A TOC. ACE. INTERNAL STD.		48.99	123950		1.154
5	0.000	50.49	8820		1.189
6	0.000	58.45	26679		1.377
TOTALS	4.596		572953		



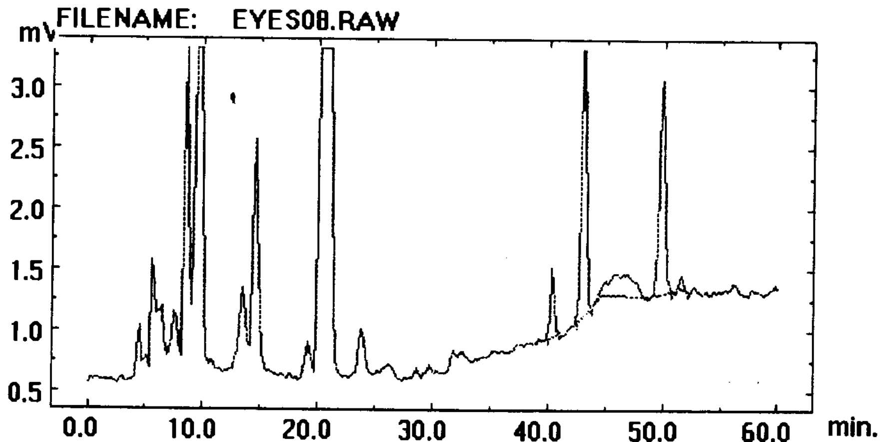
DISK FILE:EYES07.RAW INJECTED AT: 07/13/94 09:55:28
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
A TOCOPH.	26.146	43.26	190116		1.000
A TOC. ACE. INTERNAL STD.		50.07	111635		1.158
3	0.000	51.76	5802		1.197
TOTALS	1.000		307553		



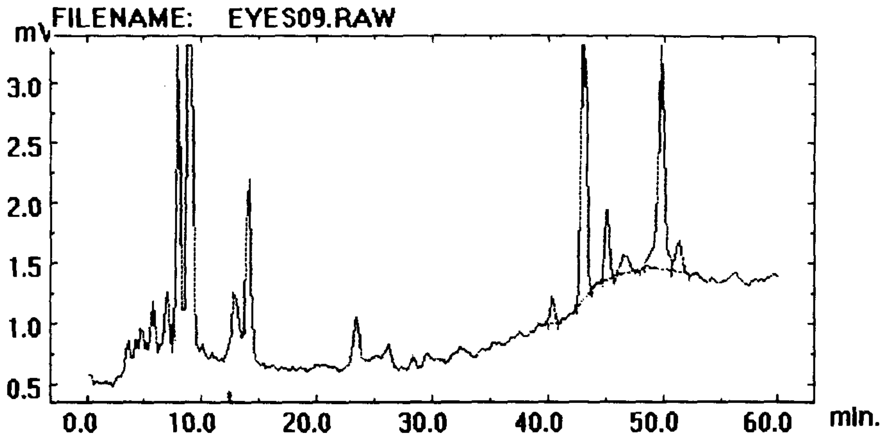
DISK FILE:EYES08.RAW INJECTED AT: 07/13/94 11:14:06
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	6.21	40.31	25697		0.937
A TOCOPH.	47.349	43.01	179940		1.000
3	0.000	46.78	56134		1.088
A TOC. ACE. INTERNAL STD.		49.83	119487		1.159
5	0.000	51.56	5727		1.199
TOTALS	1.000		386985		



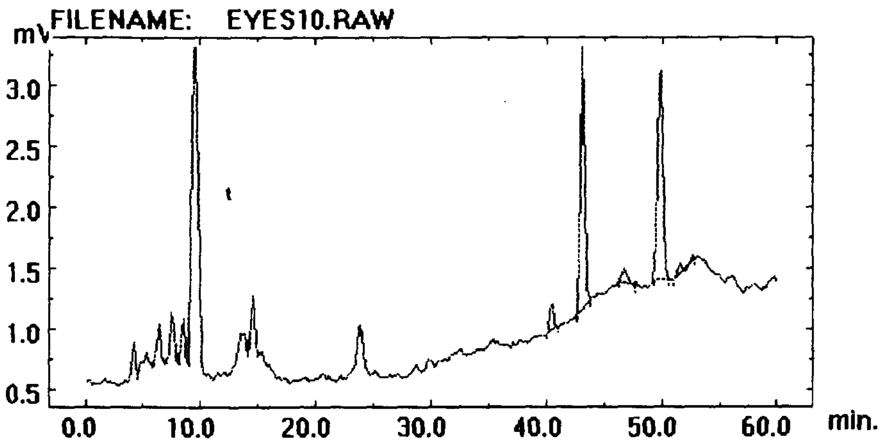
DISK FILE:EYES09.RAW INJECTED AT: 07/13/94 12:29:37
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	2.934	40.37	10714		0.938
A TOCOPH.	75.588	43.04	253529		1.000
3	0.000	45.12	33173		1.049
4	0.000	46.54	14837		1.082
A TOC. ACE. INTERNAL STD.		49.76	140360		1.157
6	0.000	51.43	19825		1.195
TOTALS	1.000		472438		



DISK FILE:EYES10.RAW INJECTED AT: 07/13/94 13:47:42
REPROCESSED: Method: Local

EYES_B CH= PS=1

FILE 1 METHOD 2 RUN 1 INDEX 1

ANALYST:
SAMPLE 1

NAME	uM	RT	AREA	RF	RRT
G TOCOPH.	4.054	40.43	11764		0.939
A TOCOPH.	33.566	43.07	127562		1.000
3	0.000	46.72	6075		1.085
A TOC. ACE. INTERNAL STD.		49.82	111533		1.157
5	0.000	52.69	2873		1.224
TOTALS	1.000		259807		