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Antioxidant functions of beta-carotene

Kennedy, Todd Allen, Ph.D.

The University of Arizona, 1991

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ANTIOXIDANT FUNCTIONS OF BETA-CAROTENE

by

Todd Allen Kennedy

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read
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entitled Antioxidant Functions of Beta-Carotene

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SIGNED: Todd Allen Kennedy

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ABSTRACT

The provitamin A carotenoid β -carotene is an attractive candidate for the prevention of cancer. Indeed, abundant evidence suggests that β -carotene inhibits carcinogenesis. β -Carotene is thought to inhibit carcinogenesis by scavenging free radical involved in tumor formation. However, there is no direct evidence that β -carotene traps radicals under conditions where it inhibits carcinogenesis. The overall objective of this dissertation research was to identify β -carotene oxidation products from β -carotene antioxidant reactions in model systems. Identification of such products will enable the direct measurement of β -carotene antioxidant activity in systems where it inhibits neoplastic transformation. In hexane solution, β -carotene was oxidized by peroxy radicals to 5,6-epoxy- β,β -carotene, 15,15'-epoxy- β,β -carotene, a previously unreported product, and several unidentified polar products. Studies on the kinetics of product formation suggested that polar products are formed by both epoxide-dependent and -independent pathways. Because β -carotene may be localized within lipid bilayers *in vivo*, peroxy radical oxidation of β -carotene in model membranes was examined. In soy phosphatidylcholine liposomes, β -carotene was oxidized by peroxy radicals to the 5,6-epoxide and to unidentified polar products. β -Carotene antioxidant activity in the liposome system was the same at 15 torr and 160 torr O_2 and decreased at 760 torr O_2 . These results suggest that β -carotene provides equal antioxidant protection in all tissues *in vivo*. The relative rates of product formation and β -carotene oxidation at different pO_2 suggested that β -carotene antioxidant activity is governed by the relative

proportions of β -carotene radical trapping and autoxidation reactions, which do not contribute to radical trapping. Therefore, the loss of β -carotene antioxidant action at 760 torr O_2 may result from an increase in β -carotene oxidation by autoxidation pathways. The 5,6-epoxide was formed during both antioxidant reactions and autoxidation reactions and may be marker for the peroxy radical oxidation of β -carotene. Attempts to study β -carotene antioxidant reactions in biological membranes were only partially successful. *In vitro* incorporation of β -carotene into microsomes was attempted by several methods. However, these efforts resulted in only modest β -carotene antioxidant activity in microsomes. These studies provide a basic understanding of β -carotene antioxidant chemistry in model systems. Their results will enable further investigation of β -carotene antioxidant action in biological systems.

CHAPTER 1

INTRODUCTION

The concept of cancer prevention has emerged as a rational approach to effectively reduce cancer mortality in humans (reviewed in Bertram et al., 1987). The inherent difficulties in treating advanced cancer suggest that prevention of tumor formation may offer an alternative strategy to decrease cancer deaths. Chemoprevention, the use of pharmacological agents to inhibit or reverse carcinogenesis, is one means to prevent cancer. Epidemiologic studies suggest that chemopreventive agents exist naturally in the diet. Studies in animals further substantiate this claim. Perhaps most importantly, if effective, chemoprevention will be a much more cost effective and ethical means of reducing cancer mortality than conventional treatment.

One of the most promising chemopreventive agents is β -carotene. β -Carotene is one of almost 600 identified naturally occurring carotenoids (Straub, 1987). Although it is known primarily as an important dietary source of vitamin A (Olson, 1989), β -carotene, has recently attracted greater scientific attention due to its ability to prevent some forms of cancer (reviewed in Temple and Basu, 1988; Krinsky, 1989; Krinsky, 1991). Other carotenoids, some without pro-vitamin A activity, also inhibit carcinogenesis (Mathews-Roth, 1982; Santamaria et al., 1987; Shklar and Schwartz, 1988). Thus, it is thought that β -carotene anticarcinogenic activity is independent of conversion to retinoids. The mechanism of β -carotene anticarcinogenic action is presently unknown. However, several investigators have

suggested that β -carotene suppresses cellular transformation by scavenging free radicals involved in neoplastic conversion (Ames, 1983; Som et al., 1984; Pung et al., 1988; Bertram et al., 1991). Evaluation of this hypothesis is challenging because radical trapping by β -carotene is difficult to measure due to the paucity of knowledge about β -carotene antioxidant chemistry (Krinsky, 1989). The objective of this dissertation research was to investigate the antioxidant chemistry of β -carotene in biological model systems and to identify marker products of β -carotene antioxidant action. β -Carotene was studied as a model carotenoid because it is chemically and biologically the best characterized carotenoid, is a major dietary carotenoid, and is currently being tested in a number of clinical trials (Malone, 1991). An understanding of β -carotene antioxidant chemistry will allow determination of β -carotene oxidation products that result from radical trapping by β -carotene, rather than β -carotene autooxidation, which does not contribute to radical trapping. Identification of these marker products will provide investigators with a specific indicator of β -carotene antioxidant activity. Then β -carotene antioxidant action could be measured under conditions where carcinogenesis is inhibited. The following introductory sections review 1) the evidence for the anticarcinogenic effects of β -carotene, 2) free radical and antioxidant chemistry in biological systems, 3) the role of radicals in carcinogenesis, and 4) the antioxidant properties of β -carotene.

ANTICARCINOGENIC EFFECTS OF β -CAROTENE

The provitamin A carotenoid β -carotene has received much recent attention as a possible chemopreventive agent. Chemopreventive compounds are selected natural and synthetic substances introduced into the diet for the purposes of reducing cancer incidence (Malone, 1991). Indeed, clinical trials are currently underway to test the efficacy of β -carotene in preventing a variety of cancers (Malone, 1991). β -Carotene is well suited for chemoprevention because it is essentially non-toxic (Heywood et al., 1985; Mathews-Roth, 1988; Bendich, 1988) and occurs naturally in many common fruits and vegetables. Used clinically for the treatment of inherited photosensitivities for more than fifteen years, β -carotene at doses of 180 mg/day or more does not cause adverse effects other than skin yellowing (reviewed in Bendich, 1988). Sub-chronic and chronic toxicity studies in animals also have shown no toxicity due to β -carotene (Heywood et al., 1985; Mathews-Roth, 1988). Further, data from pilot clinical chemoprevention studies, where doses are between 15 and 50 mg/day, indicate that β -carotene causes minimal toxicity (Malone, 1991). However, because results from clinical trials will not be available for some time, it will not be known if β -carotene can effectively prevent carcinogenesis in humans.

Epidemiologic Studies

Evidence for the anti-carcinogenic effects of β -carotene was first suggested by epidemiological studies. In 1981, Peto et al. proposed

that dietary β -carotene may prevent cancer independent of its metabolism to vitamin A (Peto et al., 1981). Their proposal was based on their review of epidemiological studies, which found a slight inverse association between β -carotene intake and cancer risk. Because β -carotene intake was estimated from dietary questionnaires, the authors cautioned that this inverse association may be an artifact due to the presence of other chemopreventive substances in β -carotene-rich foods. Nonetheless, their report spawned a surge in epidemiological studies on β -carotene intake, β -carotene blood levels and the risk of cancer.

For convenience, epidemiologic investigations may be classified as either prospective or retrospective studies (Ziegler, 1991). In a retrospective study, patients with a specific cancer are identified and comparable control subjects selected. Then data on the patients' diet and, if available, blood carotenoid levels before onset of disease are collected and compared to the data gathered from the control group. This study design allows the recruitment of a large subject population. However, it may be difficult, if not impossible, to collect blood samples that have not been altered by disease or treatment. In contrast, in a prospective study, data on dietary intake and/or carotenoid blood levels are collected first. Then the study population is followed over time and the incidence of cancer is recorded. In prospective studies, blood analysis is much easier, but only relatively common cancers occur in sufficient numbers to allow proper statistical analysis.

Six prospective studies have investigated the association between β -carotene (carotenoids) intake and cancer (reviewed in Ziegler, 1991).

Dietary intake of carotenoids was estimated by the frequency of consumption of a number of fruits and vegetables. Current food tables from which carotenoid intake is estimated list only total carotenoid content, not individual carotenoid components. This limitation prevents estimation of actual β -carotene intake. In three of the four studies that examined the effect of β -carotene on all cancers combined, cancer risk was inversely related to carotenoid intake (Hirayama, 1985; Colditz et al., 1985; Paganini-Hill et al., 1987). Males and females gave the same results in the two studies in which men and women were evaluated separately (Hirayama, 1985; Paganini-Hill et al., 1987). Of individual cancer sites, the relationship between carotenoids and lung cancer was the most investigated. Four of five studies showed decreased risk of lung cancer with an increased intake of carotenoids (Hirayama, 1985; Shekelle et al., 1981; Kvale et al., 1983; Wang et al., 1985). No protective effect was observed for cancer of the colon or prostate (Shekelle et al., 1981; Paganini-Hill, 1987). Two of three studies also found no association between retinoid intake and lung cancer (Shekelle, 1981; Paganini-Hill, 1987). These results together suggest that high carotenoid intake can reduce the risk of some cancers. Six other prospective studies have evaluated the association between blood carotenoid levels and cancer risk (reviewed in Ziegler, 1991). In five of these studies, blood β -carotene levels were quantitated by HPLC. Risk of lung cancer was reduced in subjects with high β -carotene levels in all five of these studies (Stähelin et al., 1990; Nomura et al., 1985; Menkes et al., 1986; Wald et al., 1988; Connett et al., 1989). In

three studies, high β -carotene levels were associated with a reduced risk of stomach cancer (Stähelin et al., 1990; Nomura et al., 1985; Wald et al., 1988). In none of these six studies, was a decreased risk of cancer associated with retinoid levels. The results of these studies further substantiate the conclusion from dietary investigations that β -carotene can protect against some cancers, particularly lung cancer. Further, these findings suggest that carotenoids act independent of their metabolism to vitamin A because serum retinoid levels did not correlate with decreased cancer risk.

Likewise, retrospective studies have also found an inverse association between β -carotene and cancer (reviewed in Ziegler, 1991). Lung has been the most studied cancer site. A decreased risk of lung cancer with increased intake of carotenoids was observed in all eleven studies of lung cancer and dietary carotenoids. Results of retrospective studies of other cancers and dietary carotenoids were inconclusive.

In all epidemiologic studies, control for smoking has been found to be very important (reviewed in Ziegler, 1991). Plasma β -carotene has been shown to be inversely related to smoking and inversely correlated to smoking intensity (Stryker et al., 1988). Smokers consume less carotenoids, although this may not account entirely for their lower plasma levels. Plasma carotenoid concentrations may be further decreased by the myriad of oxidants present in cigarette smoke. Control for smoking is crucial in epidemiologic studies because smoking is the greatest risk factor for lung cancer. Inadequate control for smoking

therefore would result in false inverse associations between β -carotene and lung cancer.

In summary, both prospective and retrospective studies consistently show an inverse relationship between carotenoid intake and risk of cancer, in particular, lung cancer. Further, these results are supported by prospective studies, which find that lung cancer risk is inversely associated with blood levels of β -carotene. The effects of β -carotene appear to be independent of its metabolism to vitamin A. These results should be considered with caution because they only demonstrate associations and do not prove a causal relationship between β -carotene and cancer prevention. Moreover, results from dietary studies may be confounded by the effects of other carotenoids as well as other compounds that may also affect carcinogenesis.

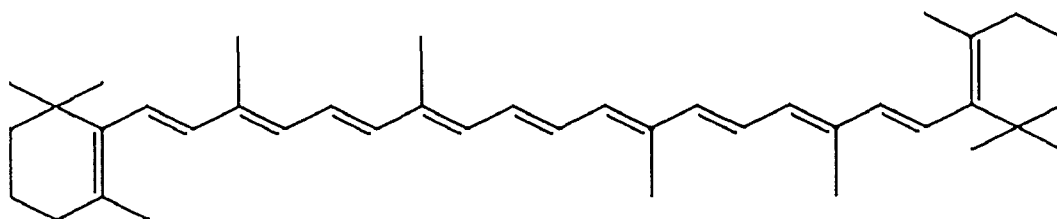
Animal Studies

The limitations of epidemiologic investigations prevent a rigorous approach to determining whether β -carotene can truly prevent cancer. Furthermore, these studies offer little insight into the mechanism(s) of β -carotene antitumor activity. Therefore, several researchers have used animal models of carcinogenesis to further investigate the effect of β -carotene on tumor formation. The use of animals permits far greater control of carcinogen exposure and diet than is possible in epidemiologic studies. Moreover, the administration of defined β -carotene dosages allows a more precise assessment of the role of carotenoids in carcinogenesis.

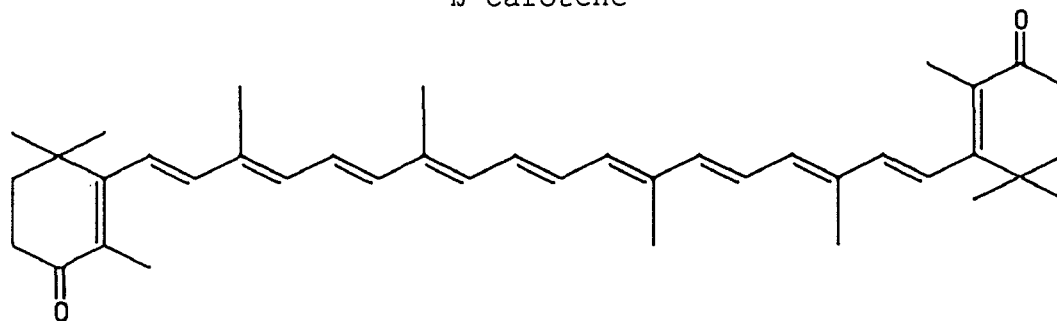
As early as 1977, β -carotene, administered by intraperitoneal injection, was found to reduce tumor growth rate and delay appearance of UV-B induced skin tumors in mice (Epstein, 1977). Mathews-Roth observed similar protection against UV-B induced tumors in mice with orally administered β -carotene and with phytoene and canthaxanthin (4,4'-dioxo- β,β -carotene), two carotenoids that cannot be metabolized to retinoids (Mathews-Roth, 1982). Mathews-Roth also found that β -carotene, but not the other compounds, reduced tumor numbers and delayed appearance of skin tumors in mice that were treated with the initiator 7,12-dimethylbenz(a)anthracene (DMBA) and with the promoters UV-B or croton oil. These results suggested for the first time that β -carotene was effective in protecting against chemical carcinogens alone as well as against combinations of UV light and chemical carcinogens.

Other investigators have shown that the effectiveness of β -carotene as an anticarcinogen is not limited to the mouse skin model of carcinogenesis. Direct application of β -carotene inhibited growth of DMBA-induced tumors in the hamster buccal pouch (Suda et al., 1986) and caused regression of DMBA-induced epidermoid carcinomas (Schwartz et al., 1986). Inhibition by β -carotene was also observed in this model system when tumors were initiated by DMBA and promoted by benzoyl peroxide (Suda et al., 1986). Further investigations by this group have shown that β -carotene, canthaxanthin, α -tocopherol, and a carotenoid-containing algal extract all cause regression of DMBA-induced epidermoid carcinomas in hamster buccal pouches (Shklar and Schwartz, 1988). Temple and Basu showed that dietary β -carotene decreased the incidence

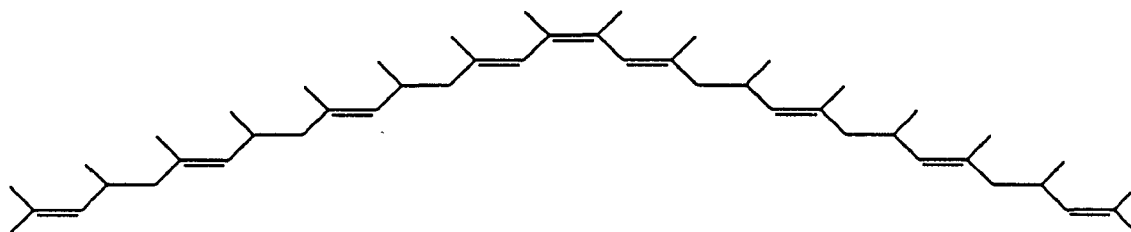
of colon cancer in rats treated with 1,2-dimethylhydrazine (Temple and Basu, 1987). Together, the results of these and other studies strongly suggest that β -carotene, administered orally or applied directly, can protect against tumor formation.



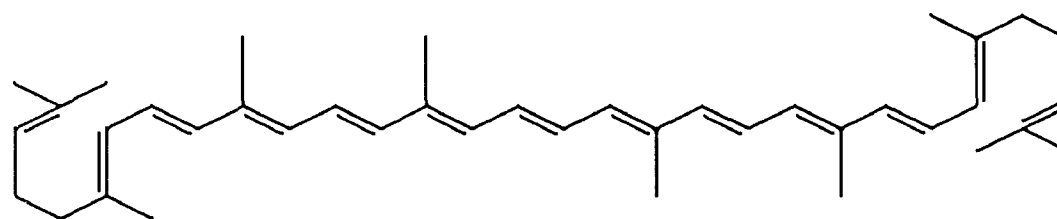
β -Carotene



Canthaxanthin



Phytoene



Lycopene

Figure 1. Structures of some representative carotenoids.

Several animal studies suggest that β -carotene is effective during the promotion or progression stage of carcinogenesis. When administered after an initiating dose of UV-B light, dietary β -carotene or canthaxanthin significantly reduced the number of animals with tumors (Mathews-Roth and Krinsky, 1987). Shklar and Schwartz reported regression of established hamster buccal pouch carcinomas by direct injection of β -carotene, canthaxanthin, α -tocopherol, or algae extract (Shklar and Schwartz, 1988). Because tumor regression was associated with an increased number of TNF- α positive macrophages in the tumor area, these investigators postulated that β -carotene may cause tumor regression by stimulating an immune response. Others have also observed inhibition of progression by β -carotene. Dietary β -carotene or canthaxanthin suppressed progression of preneoplastic foci to infiltrating carcinomas in the stomachs of rats given N-N'-methylnitro-nitrosoguanidine (MNNG) in drinking water (Santamaria et al., 1987). Seifter and colleagues observed that dietary β -carotene improved tumor regression, reduced tumor incidence, and delayed the appearance of Moloney murine sarcoma virus-induced tumors in CBA/J mice (Seifter et al., 1982). This same group found that the combination of X-ray treatment and dietary β -carotene decreased tumor incidence, delayed tumor appearance, and increased survival time of mice inoculated with C3HBA adenocarcinoma cells (Seifter et al., 1983). Further, dietary β -carotene suppressed the growth of preneoplastic lesions in the pancreas of rats treated with azaserine and in the pancreas of hamsters treated with N-nitrosobis(2-oxopropyl)amine (Woutersen and van Garderen-Hoetmer,

1988). These results suggest that β -carotene is active during the promotion or progression stage of carcinogenesis because it inhibits tumor formation by cells that were presumably initiated or caused regression of established tumors. Moreover, results from experiments with canthaxanthin, a non-provitamin A carotenoid, suggest that β -carotene itself suppresses tumor formation. Canthaxanthin is a carotenoid that cannot be metabolized to retinoids. Since canthaxanthin also protects against carcinogenesis, presumably conversion to retinoids is not required for β -carotene to exert its anticarcinogenic effects.

The results of these studies provided the basis of the first clinical studies of β -carotene in the treatment of preneoplastic lesions. Stich and colleagues found that 6 months of oral β -carotene treatment caused regression of oral leukoplakias, a premalignant lesion, and suppressed new leukoplakia formation in tobacco/betel quid chewers (Stich et al., 1988). Most remarkably, the subjects continued throughout the study to chew the tobacco-containing betel quids, which contain many known carcinogens. More recently, Garewal et al. reported a 71 % response rate in patients with oral leukoplakia who received 30 mg/day β -carotene (Garewal et al., 1991). The positive response observed in these clinical studies suggests that oral β -carotene may have therapeutic benefit in the treatment of premalignant oral lesions and the prevention of oral cancer.

Other studies have failed to find a chemoprotective effect of β -carotene. Oral dosing of Syrian hamsters with β -carotene did not protect against respiratory tract tumors induced by intratracheal

instillation of benzo(a)pyrene attached to ferric oxide (Beems, 1987). However, there was a significant increase of plasma retinol levels in animals without tumors vs. animals with tumors, regardless of the dietary treatment. This observation suggests that the tumors induced by this treatment may alter retinoid and therefore β -carotene distribution, which complicates interpretation of these results. Similarly, Jones and colleagues found that oral β -carotene did not protect rats against carcinogenesis induced by orally administered MNNG (Jones et al., 1989). Although the study design was similar to that of Santamaria and colleagues, who found a protective effect at 550 days, this study lasted only 52 weeks. The difference in study duration may account for the conflicting results.

β -Carotene inhibits a wide variety of experimentally induced tumors in animals and appears to cause regression of some premalignant and malignant lesions in animals and humans. These observations are consistent with epidemiologic studies and provide further evidence that β -carotene itself may protect against tumor formation. Studies have shown that canthaxanthin, a carotenoid that can not be converted to vitamin A, also provides protection against carcinogenesis (see above). These results suggest that some intrinsic property of the carotenoids themselves, rather than their metabolism to retinoids, may account for their anticarcinogenic effects. However, most common experimental animals, including rats, mice, hamsters, and rabbits, convert essentially all dietary β -carotene to retinoids. Only after continued feeding of very large amounts of β -carotene can β -carotene levels be

increased in these animals. In contrast, humans metabolize β -carotene much less efficiently and therefore can absorb and accumulate β -carotene and other carotenoids intact. Thus, studies where β -carotene is given orally to animals that are efficient converters may reflect the anticarcinogenic effects of retinoids rather than β -carotene.

In Vitro Studies

To further study the chemopreventive effects of β -carotene, some investigators have used *in vitro* systems of experimental carcinogenesis. By using these systems, the effects of β -carotene on a specific organ or cell population can be tested and other factors that may influence tumor formation *in vivo*, such as hormone levels and immune function, can be eliminated. Moreover, direct administration of β -carotene to target cells may minimize conversion of β -carotene to retinoids.

Som and coworkers examined the effect of β -carotene on DMBA-induced transformation of whole BALB/c mouse mammary glands in culture (Som et al., 1984). Treatment with 10^{-6} M β -carotene during DMBA treatment decreased the incidence of nodule-like alveolar lesions (NLAL) by 68 %. Treatment with β -carotene after DMBA exposure resulted in 49 % inhibition of NLAL. These data suggest that β -carotene may inhibit initiation as well as promotion in this system. Metabolism of β -carotene to retinol by the cultured mammary glands was not detected by fluorescence. For these studies, β -carotene was dissolved in hexane and then added to the culture medium. Although this procedure would

probably cause β -carotene crystallization, mammary gland extracts showed that about 10 % of the added β -carotene was taken up by the glands.

Schwartz and colleagues reported that β -carotene and canthaxanthin selectively altered the proliferation and metabolic state of tumor cell lines (Schwartz et al., 1990). The carotenoids were administered in liposomes to lung carcinoma cells, oral carcinoma cells, or normal human keratinocytes. β -Carotene and canthaxanthin were selectively cytotoxic to both carcinoma cell lines, but did not affect the normal cells. The carotenoids also decreased metabolic viability and [^3H]-thymidine uptake only in the transformed cell lines. Gel electrophoresis of cellular cytosol showed that treatment with β -carotene, but not canthaxanthin, resulted in increased expression of a 70 kD protein in the carcinoma cell lines, but not in normal keratinocytes. These findings are intriguing and represent the first published account of effects of β -carotene on protein expression. However, the induction of the 70 kD protein does not correlate with cytotoxicity or inhibition of proliferation, because canthaxanthin is nearly as effective at growth inhibition as β -carotene, yet canthaxanthin does not appear to cause induction of the 70 kD protein. Further, differences in cellular uptake mechanisms may account for the selective effects on the carcinoma lines. Carotenoid uptake was not examined in this study.

Bertram and colleagues have shown that a variety of carotenoids inhibit chemically- and physically-induced neoplastic transformation of C3H/10T1/2 cells (Pung et al., 1988; Bertram et al., 1991). This murine fibroblast cell line is a well characterized model of carcinogenesis in

vitro and previous work has shown that retinoids reversibly inhibit transformation of these cells. In initial studies, cells were transformed with either X-irradiation or 3-methylcholanthrene (MCA), then treated with a β -carotene or canthaxanthin emulsion in water 7 days after the transforming treatment (Pung et al., 1988). The carotenoid emulsion was prepared from carotenoid-containing beadlets manufactured by Hoffmann-La Roche. Although this delivery method results in a rapid increase in cellular β -carotene, only beadlets containing β -carotene or canthaxanthin are presently available, which limits the study of carotenoid anticarcinogenic activity to these compounds. These beadlets also contained the antioxidants BHT and BHA, so beadlets containing all ingredients except the carotenoids were used for controls. Both carotenoids inhibited MCA-induced transformation in a concentration-dependent manner. Interestingly, canthaxanthin was more effective than β -carotene. Neither compound affected X-ray-induced transformation when added prior to or during irradiation. However, when either carotenoid was given 1 week after X-ray treatment, development of transformed foci was suppressed in a dose-dependent manner. These results suggest that carotenoids slow the progression of cells to the transformed phenotype, since pretreatment of cells had no effect on transformation. Inhibition of MCA-induced transformation by these drugs was also reversible; foci developed if the carotenoids were removed. This result suggests that these compounds do not cause selective toxicity to initiated cells. Conversion of β -carotene to retinoids was not detected in these cells (Rundhaug et al., 1988). More recently, this group has shown that

canthaxanthin, β -carotene, α -carotene, and lycopene inhibit MCA-induced transformation in the above order of potency and in a concentration-dependent manner. Carotenoids were dissolved in tetrahydrofuran (THF) and added to cell cultures. THF is an excellent solvent for carotenoids. Analysis of THF-solubilized β -carotene in aqueous solution by UV-Vis and ^1H NMR spectroscopy suggested that THF formed a cage around β -carotene clusters or microcrystals, which solubilizes the carotenoid in aqueous media. Carotenoids administered by this method displayed the same biological activity as carotenoids administered in beadlets. As in the earlier work, suppression of transformation was not associated with cytotoxicity or inhibition of cell proliferation. These investigators also examined the cell uptake and stability in media of the carotenoids tested. Interestingly, neither cellular uptake nor stability in media correlated with inhibition of transformation. α -Tocopherol also inhibited cellular transformation in this system. However, it was less potent than canthaxanthin, β -carotene, and α -carotene. The authors hypothesized that the anticarcinogenic effects of carotenoids may be due to a combination of carotenoid antioxidant properties and provitamin A activity. Although metabolism to retinoids has not been detected in these cells (Rundhaug et al., 1988), the authors argued that the recent discovery of a family of nuclear retinoic acid receptors with nanomolar affinity for its ligand (Zelent et al., 1989) suggests that only a very small percentage (0.01%) of carotenoid needs to be converted to retinoids to activate these receptors. This low level of conversion to retinoids would not be detected by the

methods used in the previous study (Rundhaug et al., 1988). These investigators further suggested that canthaxanthin may be metabolized to a retinoid analogue, 4-keto-retinoic acid, which was shown to inhibit squamous metaplasia in the hamster trachea (Newton et al., 1980). Oxidation of carotenoids to biologically active products, perhaps structurally similar to retinoids, was also postulated. Because α -tocopherol, a potent lipophilic antioxidant, also inhibited transformation, the authors suggested that carotenoid antioxidant action also may contribute to anticarcinogenic activity.

Other investigators have examined the effect of β -carotene on cellular events associated with neoplastic transformation. Because the late stages of carcinogenesis are associated with alterations of the cellular genomic structure (reviewed in Pitot, 1990), researchers have examined the effect of β -carotene on chromosomal aberrations in cultured cells. β -Carotene inhibited translocations and micro nuclei formation in Chinese hamster ovary (CHO) cells treated with the genotoxins methylmethanesulfonate and 4-nitroquinoline-1-oxide (Stich and Dunn, 1986). Inhibition was concentration-dependent and occurred at concentrations as low as 18 nM. Despite measurable uptake of β -carotene into CHO cells, conversion to retinol was not detected. However, β -carotene was not effective against gallic acid, tannic acid, areca nut extract, or hydrogen peroxide. Weitzman and colleagues have observed similar effects when activated polymorphonuclear leukocytes (PMNL) or xanthine oxidase and hypoxanthine were added to cultures of CHO cells (Weitzman et al.,

1985). β -Carotene suppressed sister chromatid exchange in treated CHO cells. Treatment with hypoxanthine/xanthine oxidase results in superoxide production. Activated PMNL are thought to be cause chromosomal alterations by producing oxidants because a number of antioxidants including superoxide dismutase, catalase, mannitol, sodium benzoate, N-acetylcysteine, and α -tocopherol inhibit SCE formation in PMNL-treated CHO cells (Weitzman and Stossel, 1982). These results suggest that β -carotene can suppress SCE in this system by acting as an antioxidant.

The results of these studies on transformation *in vitro* have important implications regarding the mechanism of β -carotene anticarcinogenic activity. β -Carotene apparently can affect the process of transformation in the absence of an intact immune system. Inhibition of transformation by carotenoids is reversible in 10T1/2 system, which suggests that β -carotene acts within cells to inhibit post-initiation events. Consistent with this observation, β -carotene inhibits chromosomal aberrations associated with neoplastic conversion. Although Bertram and colleagues suggest that conversion to retinoids may be a component of β -carotene anticarcinogenic action, no detectable metabolism of carotenoids to retinoids has been demonstrated in studies where retinoid formation was investigated. Further, canthaxanthin is a more potent inhibitor of transformation, yet cannot be metabolized to retinoids by mammals. Again, the data indicate that the carotenoid molecule itself appears to be responsible for the anticarcinogenic effects of β -carotene.

Hypotheses of Carotenoid Anticarcinogenic Mechanism

The results of these epidemiologic, animal, and cellular studies on the effects of β -carotene on carcinogenesis have led to three hypotheses of the mechanism of β -carotene anticarcinogenic activity: 1) metabolism of β -carotene forms retinoids, known modifiers of tumor formation; 2) β -carotene activation of immune cells, which leads to increased immune-mediated tumor cell killing; and 3) β -carotene antioxidant action scavenges free radicals involved in tumor formation. As mentioned above, the anticarcinogenic actions of β -carotene may be dissociated from its conversion to retinoids by results of epidemiologic studies as well as studies on cell transformation *in vivo* and *in vitro*. Results of epidemiologic studies consistently show a greater inverse association between cancer risk and β -carotene intake or blood levels than between cancer risk and retinoid intake and blood levels (reviewed in Ziegler, 1991). Studies *in vivo* find that direct application of β -carotene to tumors can cause tumor regression (Shklar and Schwartz, 1988), although local metabolism to retinoids has not been entirely ruled out. Investigations with organs or cells in culture also demonstrate the direct anti-tumor effects of β -carotene in the absence of detectable conversion to retinoids (Som et al., 1984; Pung et al., 1988). These results together strongly suggest that β -carotene anticarcinogenic effects are, at least in part, independent of β -carotene pro-vitamin A activity. Watson and colleagues have reported that β -carotene can activate certain immune functions *in vitro* (Abril et al., 1989). Others have found similar results *in vivo* (Shklar and

Schwartz, 1988; Garewal et al., 1991). However, β -carotene also inhibits cellular transformation in cultured cells, in the absence of any apparent immune system (Som et al., 1984; Pung et al., 1988). Although a role for immunostimulation by β -carotene can not be eliminated, these studies suggest that β -carotene can inhibit tumor formation in the absence of an intact immune system. Because β -carotene has known antioxidant properties and prooxidant states are thought to be involved in carcinogenesis, several investigators have hypothesized that the anticarcinogenic effects of β -carotene are due to its antioxidant actions (Ames, 1983; Som et al., 1984; Pung et al., 1988; Bertram et al., 1991). β -carotene protects against oxidative damage in many systems (see below). Further, canthaxanthin, a non-provitamin A carotenoid with antioxidant properties, inhibits carcinogenesis in many of the same experimental systems in which β -carotene is protective. Although these observations are consistent with an antioxidant hypothesis, there is no evidence that β -carotene acts as an antioxidant under conditions where it suppresses transformation. In summary, many lines of investigation suggest that β -carotene inhibits carcinogenesis by suppressing cellular transformation in a manner independent of both an intact immune system and metabolism to vitamin A. Based on these findings, radical scavenging by β -carotene appears to be the most attractive explanation for β -carotene anticarcinogenic activity.

Evaluation of the antioxidant hypothesis of β -carotene chemopreventive effects requires a review of the role of radicals in tumor formation and of the antioxidant properties of β -carotene. Both

of these topics necessitate a discussion of free radicals and radical reactions, particularly with regard to biological systems. The following section reviews basic radical chemistry, its occurrence in biological systems, radical-mediated damage, and cellular defenses against radicals.

Free Radicals in Biology

Free radicals are chemical species that have one or more unpaired electrons. This electron configuration is generally unstable and confers a relatively high reactivity on free radicals, although radical reactivity can vary considerably (see below). Although long studied by chemists as important reaction intermediates, the existence of free radicals in biological systems was considered questionable until the late-1960's. At that time, McCord and Fridovich discovered the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen (McCord and Fridovich, 1969). The existence of an enzyme whose substrate was a radical suggested that radicals were important enough in biological systems for evolution to provide an enzyme to catalyze their destruction. Since the discovery of SOD, radicals have been implicated in a number of pathological processes, most notably, aging, arthritis, atherosclerosis, carcinogenesis, emphysema, ischemia/reperfusion injury, and a number of chemically-induced toxicities. Unfortunately, due to

the transitory existence of most radicals, the evidence for radical involvement in these pathologies is mostly indirect.

Reactive Oxygen Species

In biological systems, free radical chemistry is unavoidably linked to the chemistry of oxygen. Oxygen may be reduced to more reactive forms, both radical and non-radical in nature, that are called active oxygen species or reactive oxygen species. Ground state molecular oxygen is a diradical in an unreactive triplet state (reviewed in Halliwell and Gutteridge, 1990). Since most biological molecules are non-radicals in the singlet state, their reaction with oxygen is spin forbidden and therefore very slow. However, triplet (ground) state oxygen can react rapidly ($10^{-8} \text{ M}^{-1} \text{ sec}^{-1}$) and reversibly with radicals, particularly carbon-centered radicals, to produce peroxy radicals (Pryor, 1986).

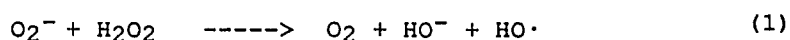
The input of energy, usually in the form of light, will convert triplet oxygen to singlet oxygen, which eliminates spin restrictions on its reaction with biological molecules. Fortunately, formation of singlet oxygen in mammalian systems is usually low, although some disease states cause accumulation of photosensitizing pigments in tissues, which upon light exposure may generate singlet oxygen and cause tissue damage. β -Carotene, an efficient singlet oxygen quencher, has been used effectively to treat patients with these diseases (Mathews-

Roth, 1986). Some small amounts of singlet oxygen may be formed during lipid peroxidation (see below).

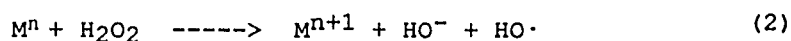
One-electron reduction of oxygen produces superoxide radical. This radical species leaks from the electron transport chains present in mitochondria, chloroplasts, and endoplasmic reticulum. Therefore, superoxide is formed in almost all aerobic cells (reviewed in Halliwell and Gutteridge, 1990). It is produced during the respiratory burst of phagocytic cells by a NADPH oxidase and is thought to contribute to bactericidal effects of these cells. Superoxide is also formed during oxidation of xanthine by xanthine oxidase (McCord and Fridovich, 1968) and by redox cycling of xenobiotic quinoid species such as paraquat and adriamycin (reviewed in Kappus and Sies, 1981). Chemically, superoxide acts as a reducing agent or a weak oxidizing agent in aqueous systems. Although superoxide radical is only moderately reactive compared to most radicals, it is thought to lead to extensive biological damage (reviewed in Fridovich, 1986). However, there is some debate over the amount of injury caused directly by superoxide radical and that caused by other oxygen species that may be formed from other reactions of superoxide radical (reviewed in Halliwell and Gutteridge, 1990). The protonated form of superoxide radical, the hydroperoxyl radical, is more reactive than the unprotonated form and can diffuse across lipid bilayers. However, the pKa of the hydroperoxyl radical is about 4.8, so only about 0.25 % of superoxide radicals are protonated at physiologic pH (Behar et al., 1970).

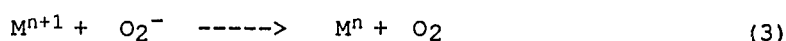
As noted above, enzymatic or non-enzymatic dismutation of superoxide radical forms hydrogen peroxide, another reactive oxygen species (reviewed in Halliwell and Gutteridge, 1990). This oxygen species is not a radical and is moderately reactive by itself. From a biological standpoint, hydrogen peroxide is notable because it can diffuse across biological membranes, whereas the charged superoxide radical cannot. Hydrogen peroxide can be produced by certain bacteria, phagocytic cells, and by mitochondria, microsomes, and chloroplasts (reviewed in Halliwell and Gutteridge, 1990). The toxic effects of hydrogen peroxide on eukaryotic and prokaryotic cells varies and depends on the relative balance between the activity of hydrogen peroxide detoxicating enzymes, such as catalase, and on the rates of production of more toxic oxygen species from hydrogen peroxide.

Both superoxide radical and hydrogen peroxide are thought to cause limited biological injury because of their relatively mild reactivity. These species may react in a Haber-Weiss reaction to give a much more reactive oxygen species, the hydroxyl radical (Haber and Weiss, 1934).



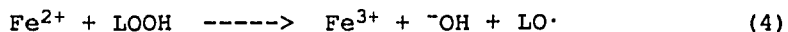
This reaction is very slow in the absence of transition metals (reviewed by Minotti and Aust, 1989) and may be rewritten as equations 2 and 3.



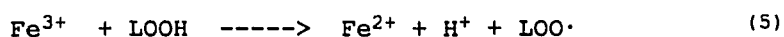


The net reaction (1) is referred to as a metal-catalyzed Haber-Weiss reaction or a superoxide-driven Fenton reaction. Iron, copper, and cobalt are thought to catalyze reactions 2 and 3. Iron is considered to be the most likely metal catalyst of the Haber-Weiss reaction *in vivo*. Because ferric iron is extremely insoluble in aqueous media ($[Fe(III)_{aq}] = 10^{-18}$ M), the iron involved in these reactions is thought to be chelated (Miller et al., 1990). The identity of the physiologic iron chelate is unknown. Hydroxyl radicals produced by these reactions are extremely reactive with an estimated half-life of 10^{-9} sec (Pryor, 1986). In general, it is thought that the hydroxyl radical reacts at a diffusion-limited rate, that is, with the first molecule with which it collides, regardless of the type of molecule. This reactivity makes hydroxyl radicals extremely damaging. However, because hydroxyl radicals react so quickly, the injury they cause is limited to the site of hydroxyl radical formation. Hydroxyl radicals are thought to be the primary toxic species produced by high energy radiation (Schulte-Frohlinde and Von Sonntag, 1985).

Iron ions are involved in the generation of two other reactive oxygen species, specifically alkoxyl and peroxy radicals (reviewed in Minotti and Aust, 1989; Halliwell and Gutteridge, 1990). Ferrous iron can reduce lipid hydroperoxides to form alkoxyl radicals, hydroxide ion, and ferric iron (4).



This reaction proceeds with a rate constant of about $10^3 \text{ M}^{-1} \text{ sec}^{-1}$, an order of magnitude faster than the corresponding reaction between ferrous iron and hydrogen peroxide (Garnier-Suillerot et al., 1984). Formation of lipid alkoxyl radicals by reaction 4 may produce other reactive species, because alkoxyl radicals can react intramolecularly to give mixture of products including aldehydes, carbon-centered radicals, and epoxy allylic radicals (Dix and Marnett, 1985; reviewed in Kappus, 1985). Ferric iron also reacts with lipid hydroperoxides to give lipid peroxy radicals, protons, and ferrous iron (5).



Reaction 5 is slower than reaction 4, although an exact rate constant has yet to be measured (Halliwell and Gutteridge, 1990). Peroxyl radicals are thought to be involved in tumor initiation and promotion (reviewed in Marnett, 1987) and other pathologies because of their relatively long half-life (about 7 sec), which would allow diffusion to distant cellular sites to occur.

At this point, this complex cascade of reactive oxygen species will be summarized. Ground state oxygen reacts only very slowly with biomolecules because of quantum spin restrictions. Reduction of oxygen to superoxide radical removes the spin restriction, yet superoxide radical is only modestly reactive. Hydrogen peroxide has a similarly

moderate reactivity. The hydroxyl radical is the most reactive oxygen species known and is thought to react at the site of its formation. Hydrogen peroxide and superoxide radical can form hydroxyl radicals by the metal-catalyzed Haber-Weiss reaction (reactions 1-3). Iron also catalyzes formation of lipid alkoxyl and peroxy radicals from lipid hydroperoxides (reactions 4 and 5). These radicals are less reactive than hydroxyl radical, yet may cause significant biological injury. Because of their rapid formation from oxygen addition to carbon-centered radicals and their relatively long half-life, peroxy radicals are the most abundant radical species formed during oxygen activation (Table 1).

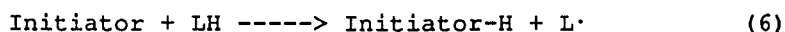
Table 1. Estimates of the half-lives of oxy-radicals and related species (Adapted from Pryor, 1986).

Name	Symbol	Half-life (at 37°C)
Hydroxyl	HO·	10^{-9} sec
Alkoxy	RO·	10^{-6} sec
Peroxy	ROO·	7 sec
Alkyl (Lipid)	R· (L·)	10^{-8} sec
Hydrogen peroxide	H ₂ O ₂	a
Superoxide radical	O ₂ ⁻	a
Singlet oxygen	¹ O ₂	10^{-6} sec

^aThe half-lives of hydrogen peroxide and superoxide radical are very difficult to measure because of their many routes of formation and decomposition. H₂O₂ itself is very stable; it is purchased as a 30 % solution (9 M) in water.

Biological Consequences of Free Radicals

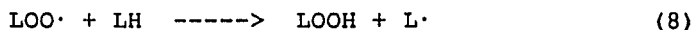
The best studied biological phenomenon involving radicals and active oxygen is lipid peroxidation, sometimes called lipid autoxidation (reviewed in Kappus, 1985; Porter, 1986; Horton and Fairhurst, 1987). The chemistry of lipid peroxidation is well understood for the most part and serves as a tool to understand radical chemistry in biology. Lipid peroxidation is initiated by a species capable of abstracting a bis-allylic hydrogen from a polyunsaturated fatty acid (PUFA). During this step, the double bonds in the PUFA molecule rearrange to give a conjugated diene moiety, which stabilizes L^\cdot . This rearrangement is the basis for a spectrophotometric assay to measure lipid peroxidation.



Since the nature of the initiating species varies among experimental systems and is the subject of much controversy with regard to iron-dependent initiation, initiators will be discussed in a later section. Abstraction of a hydrogen atom produces a lipid radical, which is immediately trapped by oxygen to form a peroxy radical.



The lipid peroxy radical is less reactive than the lipid radical, but is still capable of abstracting a hydrogen atom from another PUFA and thus propagating the radical chain.



Reactions 7 and 8 are the propagation reactions of lipid peroxidation and result in the continued oxidation of PUFA to lipid hydroperoxides. These reactions are examples of the two most common radical reactions in biological systems, hydrogen abstraction and oxygen addition, respectively. It should be noted that reaction 7 is reversible, as are most oxygen addition reactions. Further, reaction 7 is estimated to be 7 orders of magnitude faster than reaction 8, which indicates that peroxy radicals are the most abundant radical species formed during lipid peroxidation (reviewed in Pryor, 1984; see Table 1). Termination of the radical chain is achieved by recombination of any two radicals involved. Using data from autoxidations of linoleic acid in SDS micelles, Pryor has calculated that the concentration of lipid peroxy radicals is about four orders of magnitude greater than the concentration of lipid radicals (reviewed in Pryor, 1984). This estimate suggests that the reaction of two $\text{LOO}\cdot$ to form non-radical products is the most probable termination reaction during uninhibited lipid peroxidation. There is some evidence that singlet oxygen, another active oxygen species, may be formed by this termination reaction (reviewed in Sies, 1987).

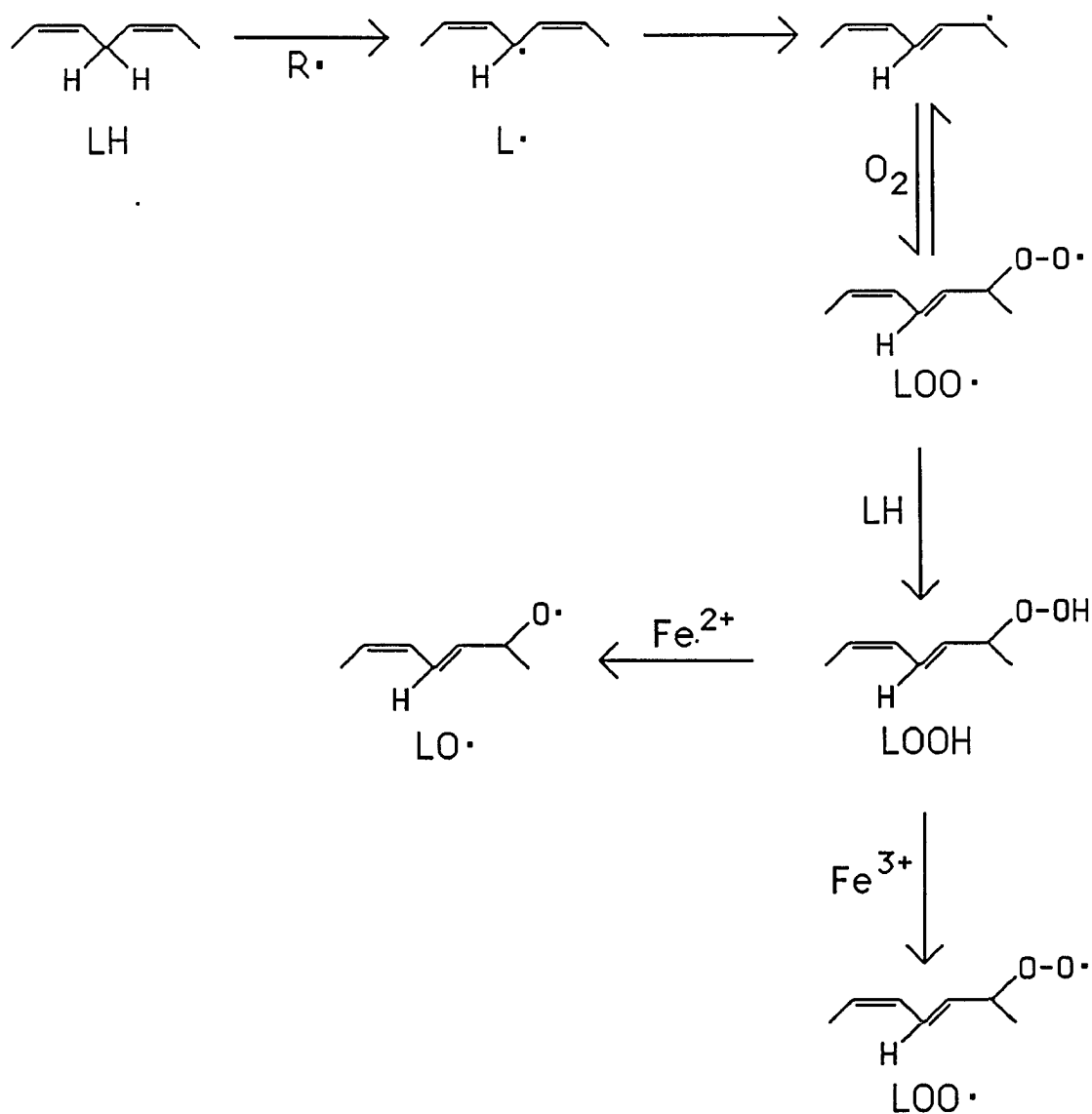


Figure 2. Schematic of the reactions involved in lipid peroxidation initiated by hydrogen abstraction from a PUFA by R^\bullet and including the chain-branching reactions catalyzed by iron. Both LO^\bullet and LOO^\bullet are capable of abstracting hydrogen atoms from other PUFA (see text for discussion).

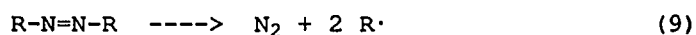
This reaction scheme for lipid peroxidation is simplistic because lipid hydroperoxides are not stable products, especially in the presence of iron. The reaction of both ferrous and ferric ions with lipid hydroperoxides is shown in reactions 4 and 5 above and results in a more complex product mixture than is indicated (reviewed in Kappus, 1985; Horton and Fairhurst, 1987). The most important consequence of iron-catalyzed lipid hydroperoxide decomposition is the production of additional radicals that may initiate radical chains themselves, thus greatly amplifying the rate of lipid peroxidation. This process is referred to as chain branching because new radical chains are started by lipid hydroperoxide-derived free radicals. The radicals formed by iron-catalyzed lipid hydroperoxide decomposition include alkoxyl and peroxy radicals, both of which are capable of abstracting hydrogens to initiate new radical chains. However, the lipid alkoxyl radical has an estimated half-life of 10^{-6} sec vs. 7 sec for the half-life of a lipid peroxy radical (Pryor, 1986). Because of the extreme difference in reactivity between these radicals, alkoxyl radicals have often been suggested to be more damaging. This comparison may be an oversimplification, since PUFA-derived alkoxyl radicals can rapidly rearrange to form less reactive species. Lipid alkoxyl radicals can undergo β -scission to give aldehydes and alkyl radicals, or cyclize to form epoxide radicals (reviewed in Kappus, 1985; Horton and Fairhurst, 1987). Alkyl radicals can abstract hydrogens to form volatile alkanes, which can be measured in expired breath as a non-invasive assay for lipid peroxidation *in vivo*.

(reviewed in Smith and Anderson, 1987). Lipid peroxy radicals can also react intramolecularly to form endoperoxides, which may further decompose to produce malondialdehyde (MDA). MDA formation is often measured as index of lipid peroxidation (reviewed in Smith and Anderson, 1987). Because iron, usually in the form of some chelate, is ubiquitous in most experimental and physiologic systems, the actual end products of lipid peroxidation are a complex array of alkanes, carbonyl compounds, alcohols, and hydroperoxides (reviewed in Horton and Fairhurst, 1987). Lipid peroxidation leads to membrane fluidity and permeability alterations and eventually to destruction of the membrane (reviewed in Kappus, 1985). During lipid peroxidation, a number of proteins are also altered. For example, cytochrome P-450 and glucose-6-phosphatase are destroyed during autoxidation of microsomal lipids. Relatively stable products of lipid peroxidation may diffuse and cause damage elsewhere. MDA can react with both protein and DNA. Marnett and colleagues have shown that lipid peroxidation-derived peroxy radicals can epoxidize the benzo[a]pyrene-7,8-diol to the dihydrodiolepoxide, the ultimate carcinogenic species (reviewed in Marnett, 1987).

The initiation of lipid peroxidation is also quite complex. As discussed above, superoxide radical and hydrogen peroxide are generally considered to be too unreactive to abstract hydrogen atoms and initiate lipid peroxidation. Together, hydrogen peroxide and superoxide radical can initiate lipid peroxidation in the presence of iron by generation of the much more reactive hydroxyl radical (metal-catalyzed Haber-Weiss reaction). Although most investigators agree that hydroxyl radicals are

formed by these reactants, there is much controversy over the identity of the species that initiates lipid peroxidation (reviewed in Minotti and Aust, 1989; Halliwell and Gutteridge, 1990).

Much simpler radical initiators have been used by many investigators to study lipid peroxidation *in vitro*. Azo compounds (reviewed in Niki, 1990) thermolyze unimolecularly to give carbon-centered radicals, which immediately react with oxygen to form peroxy radicals (reactions 9-10).



The structure of R influences both the rate of thermolysis and the relative solubility in polar and nonpolar solvents. 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) is the commonly used water-soluble initiator. 2,2'-Azo-bis(2,4-dimethylvaleronitrile) (AMVN) is often used as a lipid soluble initiator. When incorporated into artificial membranes, AMVN generates radicals within the lipid bilayer. The rate of radical formation by these initiators is governed primarily by temperature, and to a lesser extent by solvent. Radical generation by these compounds is constant at a given temperature, which allows calculation of certain kinetic parameters of lipid peroxidation and antioxidant reactions. In addition, lipid peroxidation is generally more reproducible when initiated by azo compounds than when it is

initiated by iron. Although these compounds are not a great biological hazard, their use has greatly simplified the study of radical reactions in experimental systems.

Biological molecules other than lipids are also targets of radicals. Radical-mediated DNA damage has been extensively studied (reviewed in Schulte-Frohlinde and Von Sonntag, 1985; Floyd, 1990). Damage to DNA by high-energy radiation is caused primarily by radicals, in particular hydroxyl radicals. Attack by hydroxyl radicals can cause strand breaks, release of bases, and modification of bases, including hydroxylation. Hydroxylated bases include 8-hydroxy-2'-deoxyguanine, 5-hydroxymethyluracil, and thymine glycol. Superoxide radical formed from the xanthine/xanthine oxidase combination can also cause DNA strand breaks. Most of these results come from pulse radiolysis studies of bases, nucleosides, and DNA in solution. Assays for oxidative DNA damage *in vivo* have recently been developed (Cathcart et al., 1984; Floyd et al., 1986). Ames and colleagues have used these assays to estimate endogenous oxidative damage to DNA (Cathcart et al., 1984; Adelman et al., 1988). Although the accuracy of these estimates is questionable considering the number of assumptions that must be made concerning the formation and excretion of modified bases, the results of these studies have been offered in support of a role for radicals in aging and chronic disease states (Ames, 1983). The role of oxygen radicals in carcinogenesis will be discussed further in a later section.

Protein is another obvious target of oxygen radicals. Despite the obvious importance of proteins to cell viability, relatively little is

known about radical-mediated protein damage. Proteins exposed to hydroxyl radicals tend to crosslink in the absence of oxygen and fragment in the presence of oxygen (reviewed in Wolff et al., 1986). Exposure to hydrogen peroxide and superoxide is damaging only in the presence of metals. Proteins with metal binding sites tend to suffer relatively minor site-specific damage when exposed to metal-catalyzed oxidation systems (reviewed in Stadtman, 1990). However, even fairly minor changes in a protein may cause severe biological damage. For example, oxidation of a single methionine residue in α -1-protease inhibitor is concomitant with inactivation of this protein and may contribute to increased protease activity and possibly to the development of emphysema (reviewed in Wolff et al., 1986). In general, active oxygen species cause alterations of amino acids, protein cross-linking, and fragmentation, all of which may lead to loss of catalytic activity, and increased susceptibility to enzymatic proteolysis (reviewed in Wolff et al., 1986; Stadtman, 1990). The carbonyl content of proteins also increases upon radical attack and has been used as a measure of protein damage (reviewed in Stadtman, 1990). Radical-mediated protein modification has been implicated in atherosclerosis, aging, cataractogenesis, emphysema, rheumatoid arthritis, stroke, and the toxicity of radical-generating xenobiotics. Evidence for radical involvement in these pathologies is indirect, mainly due to the lack of a specific, sensitive assay for protein oxidation *in vivo*.

Endogenous Sources of Free Radicals

Most endogenous formation of free radicals and active oxygen species involves superoxide radical production as an initial step. Superoxide radical is formed normally from the reduction of oxygen by the various electron transport chains (see above). Several enzymes also produce superoxide radical (reviewed in Jones, 1985). NADPH oxidase of phagocytic cells generates superoxide radical during the respiratory burst. A number of flavoprotein dehydrogenases, including NADPH-cytochrome P-450 reductase, mitochondrial NADH dehydrogenase, and dihydro-orotate dehydrogenase, also reduce oxygen to the superoxide radical. Xanthine oxidase produces superoxide radical during the oxidation of xanthine (McCord and Fridovich, 1968). Autoxidation of hemoglobin and myoglobin also forms superoxide (reviewed in Jones, 1985). Superoxide radicals may lead to the formation of hydrogen peroxide via dismutation and/or hydroxyl radicals via the Fenton reaction (see above).

Other active oxygen species may be generated directly from endogenous sources. Hydrogen peroxide is produced directly from flavin-containing oxidases and copper-containing oxidases (reviewed in Jones, 1985). Many of these hydrogen peroxide-producing enzymes are localized to peroxisomes. Lipid hydroperoxides and peroxy radicals are formed during prostaglandin biosynthesis by prostaglandin H synthase (reviewed in Marnett, 1990). Lipoxygenases also generate lipid hydroperoxides during arachidonic acid metabolism (Hamberg and Samuelsson, 1974). Peroxidases, such as myeloperoxidase, may also add to endogenous free

radical generation. Iron may catalyze production of hydroxyl radicals from other less reactive oxygen species, including hydroperoxides (see above). Normally, the cellular iron pool is carefully controlled by the storage protein ferritin. However, iron can be mobilized from the binding protein by superoxide radical (reviewed in Halliwell and Gutteridge, 1990).

Recently, evidence has accumulated that endothelium-derived relaxing factor (EDRF) is nitric oxide, NO, a relatively unreactive radical (Palmer et al., 1987). EDRF is produced by the endothelium and causes the smooth muscle of the vasculature to relax. Nitric oxide can react rapidly with oxygen to form nitrogen dioxide, which can initiate lipid peroxidation (see below).

Endogenous reactive oxygen species are formed during ischemia/reperfusion injury in which the flow of oxygen to a tissue has been cut for some time, then restored. This type of injury occurs in trauma, myocardial infarction, organ transplantation, and stroke. Damage to tissues occurs during reperfusion, not ischemia, and is inhibited in some animal models by SOD and iron chelates (reviewed in Halliwell and Gutteridge, 1990). Although the source of free radicals and their importance in this injury are yet to be established and may vary among tissues, clearly, oxygen radicals are formed during reperfusion and antioxidants can often attenuate the damage.

Inflammation is another source of endogenous free radical production. Polymorphonuclear leukocytes, macrophages and monocytes use active oxygen species to kill bacteria and, consequently, may cause

damage to surrounding tissue (reviewed in Flohé et al., 1985). These phagocytic cells use enzymes, such as NADPH oxidase and peroxidases, to generate a host of oxidants, including superoxide, hydrogen peroxide, and hypohalous acids (reviewed in Hamers and Roos, 1985).

Exogenous Sources of Free Radicals

Some xenobiotics, in particular air pollutants, are radicals themselves or react with biological molecules to produce radicals (reviewed in Pryor, 1983). The nitrogen oxides, NO and NO₂, are radicals and nitrogen dioxide can react with PUFA to generate radicals *in vitro* and *in vivo*. Ozone, though not a radical, reacts rapidly with biological molecules. Recent work by Pryor and colleagues indicates that ozone reacts with PUFA in aqueous systems to form aldehydes and hydrogen peroxide (Pryor et al., 1991).

Radicals can also be formed by certain xenobiotics, which can intercept electrons from cellular electron transport chains and then reduce oxygen to the superoxide radical. This process is called redox cycling and is common for many quinoid compounds (reviewed in Pryor, 1986), particularly anticancer and antiparasitic drugs. Other foreign compounds may be metabolized to radicals. Carbon tetrachloride is metabolized reductively by cytochrome P-450 to the trichloromethyl radical. Both the trichloromethyl and trichloromethylperoxyl radical formed after the addition of oxygen, can initiate lipid peroxidation (reviewed in Nelson and Pearson, 1990).

Enzyme-Dependent Defenses Against Free Radicals

To defend against the constant barrage of radicals, aerobic life has evolved a vast array of antioxidant defenses (Table 2; reviewed in Cotgreave et al., 1988). These may be conveniently classified as enzymatic and non-enzymatic. These antioxidant categories may be further divided into preventive antioxidants and chain-breaking antioxidants. Preventive antioxidants inhibit radical-mediated damage by inactivating initiators of radical reactions. Chain-breaking antioxidants suppress radical-induced injury by trapping or detoxifying intermediates in deleterious radical reactions.

One of the preventive enzymatic antioxidants is the family of SOD enzymes. These widely distributed, inducible enzymes have been discussed previously. They catalyze the dismutation of superoxide radical with a rate constant of $2 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ (Fridovich, 1983); almost four orders of magnitude faster than spontaneous dismutation (Bielski and Allen, 1977). In eukaryotes, the most prevalent form of the enzyme (CuZnSOD) contains a copper and a zinc atom and is found in the cytosol of almost all eukaryotic cells. A predominantly mitochondrial form of SOD (MnSOD) has a manganese atom at its active site. This isozyme also has been identified in the liver cytosol of higher primates and rats. A third animal isozyme has been found in human plasma and is called extracellular SOD. This copper-containing isozyme has a much greater molecular weight than the CuZnSOD.

Several preventive antioxidant enzymes have evolved to detoxify hydroperoxides (reviewed in Cotgreave et al., 1988). Catalase is

present in most aerobic cells and catalyzes the reduction of hydrogen peroxide to water and oxygen. The enzyme is inducible and is generally localized to peroxisomes within cells. The selenium-dependent glutathione peroxidase (SeGSHPx) also catalyzes the reduction of hydrogen peroxide to water, as well as the reduction of free organic hydroperoxides to the corresponding alcohol. The abundant tripeptide glutathione (GSH) donates the reducing equivalents for the reaction and is consequently oxidized to GSSG. This enzyme links dietary selenium to antioxidant function as four selenocysteine moieties are present in the protein and are involved in catalysis. SeGSHPx is also inducible and is present primarily in cytosol and in mitochondria. To keep the cellular GSH/GSSG ratio high, a NADPH-linked GSH reductase catalyzes the reduction of GSSG to GSH. The NADPH for this enzyme is provided mainly by the pentose phosphate shunt. The enzyme can also catalyze the reduction of other low molecular weight disulfides. Together SeGSHPx and GSSG reductase form the GSH redox cycle, an important cellular mechanism of reducing potentially harmful hydroperoxides.

Other GSH-dependent enzymes may contribute to hydroperoxide detoxication (reviewed in Cotgreave et al., 1988). Non-selenium-dependent GSH transferases also have GSH peroxidase activity, though these enzymes can only reduce organic peroxides and not hydrogen peroxide. Both the selenium-dependent and non-selenium-dependent activities only act on the free fatty acid hydroperoxide. Release from membrane phospholipids by phospholipase A₂ is required before these

Table 2. Cellular enzymatic and non-enzymatic antioxidant defenses
(see text for discussion).

Enzymatic preventive antioxidants

Superoxide dismutase

Catalase

Glutathione peroxidases

Selenium-dependent glutathione peroxidase

Non-selenium-dependent glutathione transferases

Phospholipid hydroperoxide glutathione peroxidase

Non-enzymatic preventive antioxidants

Metal binding proteins

Ferritin

Ceruloplasmin

Glutathione

Ascorbate (vitamin C)

Uric acid

Non-enzymatic chain-breaking antioxidants

α -Tocopherol

Bilirubin and Biliverdin

β -Carotene

peroxidases can be effective. Phospholipid hydroperoxide GSH peroxidase is a selenium-dependent enzyme and can catalyze the reduction of lipid hydroperoxides without prior phospholipase activity. It is thought to be a soluble enzyme that is associated with cellular membranes. Together these enzymes are a major portion of cellular defenses against radicals. Judging from these enzyme activities, enzymatic cellular defense mechanisms have evolved to prevent the production of reactive radicals by eliminating their possible sources, like peroxides and superoxide radical.

Non-enzymatic Defenses Against Free Radicals

Non-enzymatic defenses also play an important role in detoxifying potentially damaging radical species (reviewed in Cotgreave et al., 1988). Metal-binding proteins are particularly important preventive non-enzymatic antioxidants. Ferritin and ceruloplasmin are physiologic storage sites of iron and copper, respectively. Both of these metals are capable of catalyzing the Haber-Weiss reaction, which produces hydroxyl radicals. Other metal-binding proteins may also prevent radical formation by redox-active metals.

Certain small molecules act as preventive antioxidants by reacting directly with reactive oxygen radicals to form relatively stable products. GSH itself can reduce hydrogen peroxide to form water and GSSG (Ross et al., 1985). It can also react with superoxide radical, hydroxyl radical, and alkoxyl radicals to form the thiyl radical, GS \cdot , and, eventually, GSSG (Ketterer, 1986). Another water soluble

antioxidant, ascorbate (vitamin C), can directly reduce oxygen-centered radicals by donating an electron to generate the semidehydroascorbate radical (reviewed in McCay, 1985). Dismutation of the resulting radical gives dehydroascorbate and ascorbate. However, ascorbate can also act as a prooxidant by reducing ferric iron. In this manner, ascorbate, or GSH, can substitute for superoxide radical in the superoxide-driven Fenton reaction and serve as the reductant for iron (Liebler et al., 1986). Uric acid is another water soluble compound that can react directly with radicals (reviewed in Hochstein et al., 1984). It is present at high concentrations in many biological fluids and has been proposed to be a major extracellular antioxidant (Ames et al., 1981).

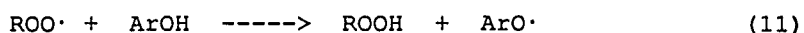
Other small molecules act as chain-breaking antioxidants. The major lipid soluble chain-breaking antioxidant is α -tocopherol (vitamin E) (reviewed in McCay, 1985; Burton and Ingold, 1986). Vitamin E is actually a family of tocopherols of which α -tocopherol is the most effective antioxidant. α -Tocopherol is an effective inhibitor of lipid peroxidation because it reacts with lipid peroxy radicals to form relatively unreactive products (see below). Each tocopherol molecule can scavenge two peroxy radicals. The first radical abstracts the phenolic hydrogen from α -tocopherol to produce the stable tocopheroxyl radical, which may undergo a number of fates (Liebler et al., 1990). A second radical may add to the tocopheroxyl radical to form a stable non-radical adduct called a tocopherone. Alternatively, the tocopheroxyl radical may be reduced by other cellular reductants, such as ascorbate or GSH, to regenerate the parent tocopherol (reviewed in McCay, 1985).

Finally, the tocopheroxyl radical may autoxidize to form epoxy-tocopherones (Liebler et al., 1990). Tocopherones may rearrange to form tocopherylquinone or may complete a two-electron redox cycle and be reduced by ascorbate to α -tocopherol (Liebler et al., 1989). Abundant indirect evidence for tocopheroxyl radical regeneration by ascorbate exists from experiments *in vitro* (reviewed in McCay, 1985). The synergistic action of these antioxidants dramatically increases the ability of α -tocopherol to suppress lipid peroxidation. However, whether regeneration of the tocopheroxyl radical by ascorbate takes place *in vivo* is still debated.

Recently, the bile pigments biliverdin and bilirubin were shown to inhibit lipid peroxidation *in vitro* (reviewed in Stocker et al., 1990). The physiological relevance of the antioxidant activity of these pigments is unknown, since they are considered toxic to most cells. Their antioxidant capabilities may be limited to extracellular fluids like plasma, where they are present as albumin conjugates, and liver, where bilirubin conjugate concentrations are between 20 and 40 μM . The antioxidant β -carotene will be discussed in a later section along with mechanisms of antioxidant activity.

Burton and Ingold have studied many chain-breaking antioxidants, including α -tocopherol, to determine the chemical properties necessary for effective radical trapping (reviewed in Burton and Ingold, 1986). They have found that effective antioxidants react rapidly with peroxy radicals to form well-stabilized products. α -Tocopherol, like most phenolic compounds, is a chain-breaking antioxidant because it

effectively competes with the propagation steps in radical chain reactions such as lipid peroxidation.



Most importantly, the phenoxyl radical formed in the reaction ($\text{ArO}\cdot$) is resonance stabilized and relatively unreactive. Indeed, the antioxidant activity of simple phenolic compounds in model chemical systems correlates well with the degree of phenoxyl radical stabilization and the rate of reaction with peroxy radicals (reviewed in Burton and Ingold, 1986). The phenoxyl radical is eventually consumed by reaction with a second peroxy radical to form non-radical products (see above). Some reductants may be capable of regenerating the parent antioxidant by directly reducing the phenoxyl radical (see above).

Evaluation of Free Radical Processes

Assessing the role of free radicals in pathologic processes is challenging due to the nature of reactive free radicals and their reactions. The most damaging radicals, hydroxyl radicals, alkoxyl radicals, and peroxy radicals, have lifetimes that preclude their direct detection (see Table 1) except by specialized techniques, such as ESR or pulse radiolysis. Measurement of less reactive oxygen species like superoxide radical and hydrogen peroxide is complicated *in vivo* by the many pathways of their formation and destruction (reviewed in Pryor, 1986). These limitations require the use of indirect methods to

determine the effect of free radicals in a biological system. The accurate measurement of even a chemically well-defined free radical process, such as lipid peroxidation, is fraught with complications. Lipid peroxidation is often measured by assaying for products formed during lipid peroxidation, most of which are either labile under assay conditions or non-specific for lipid peroxidation (reviewed in Smith and Anderson, 1987). For example, MDA is often measured as an indicator of lipid peroxidation. However, MDA is also formed during arachidonic acid metabolism and therefore is not a specific indicator for lipid peroxidation *in vivo*. Moreover, MDA forms adducts with protein and DNA *in vivo*, so that much MDA is present in a bound form, which most assays protocols do not measure (reviewed in Draper and Hadley, 1990). Lipid peroxides are among the first non-radical products formed during lipid peroxidation. Unfortunately, these products are metabolized by peroxidases and easily decompose in the presence of metal ions (see above).

Many investigators use indirect methods to determine the extent of radical involvement in a biological process. Usually a series of antioxidants is added to inhibit the process and/or a series of prooxidants is added to mimic or exacerbate the process. Although indirect methodology often provides exciting results, there is always the possibility that the antioxidant or prooxidant used does not function in its anticipated manner. For example, inhibition by specific singlet oxygen scavengers was often used as evidence for singlet oxygen involvement in a biological process. However, Packer and colleagues

used pulse radiolysis to show that many supposedly specific singlet oxygen scavengers also react rapidly with hydroxyl radical and peroxy radicals (Packer et al., 1981). To avoid some of these ambiguities, some investigators have urged identification of specific marker products of both radical-mediated damage and oxidized antioxidants (Halliwell and Gutteridge, 1990; Pryor, 1991). The eventual goal of this strategy is to develop not only unambiguous markers of radical involvement in pathological states, but also to identify the specific radicals involved.

Free Radical Involvement in Carcinogenesis

The role of free radicals in carcinogenesis is suggested by a great deal of indirect evidence. However, the long latency period of most cancers makes it exceedingly difficult to pinpoint an etiologic agent. To cloud the picture even further, numerous factors, including diet, viruses, and environmental contaminants, have been implicated in carcinogenesis. Nevertheless, a framework has emerged to systematically investigate the process of tumor formation. This framework evolved from the study of experimental chemical carcinogenesis (reviewed by Pitot, 1990). Although probably not all human cancers are caused by foreign compounds, these studies provide a useful paradigm for tumor formation.

Studies of mouse skin carcinogenesis in the 1940's demonstrated that tumor formation occurs in stages (Rous and Kidd, 1941; Mottram, 1944; Berenblum and Shubik, 1947). Initially, only two stages were

described. Berenblum and Shubik first expressed these stages as tumor initiation and tumor promotion. Initiation was the exposure of a target tissue to a single carcinogenic polycyclic aromatic hydrocarbon at a subcarcinogenic dose. Promotion was the subsequent repeated exposure of that target to the non-carcinogenic irritant croton oil. Promotion has been further divided into at least two steps, conversion and propagation (Boutwell, 1964; Slaga et al., 1980). More recently, a third stage, called progression, has been described in which a benign lesion becomes a malignant neoplasm (Boutwell, 1983). Subsequent investigations have determined biological characteristics associated with each stage (reviewed in Pitot, 1990).

Initiation is thought to involve a irreversible heritable modification of DNA in the initiated cells because many chemical carcinogens are mutagens (McCann et al., 1975). Many tumor initiators are electrophiles or are metabolized to electrophiles, which then bind to DNA and other cellular macromolecules (Miller and Miller, 1966). Initiation occurs after one dose of initiator and presumably converts only a small percentage of the target cell population to cells that are competent for transformation (Hicks, 1983). The effectiveness of initiation depends on cellular DNA synthesis and cell division (Hennings et al., 1978; Ying et al., 1982). Rapid cell division may not allow DNA repair enzymes time to remove damage caused by initiating agents, which allows DNA damage to become heritable and irreversible (Farber, 1982). Further, different types of damage and different genes are repaired with different efficiencies (reviewed in Bohr and Wassermann, 1988). In

mouse skin carcinogenesis, the cellular Harvey-ras proto-oncogene appears to a major target for initiating mutations, particularly point mutations (reviewed in Balmain and Brown, 1988).

Free Radicals and Initiation

Several lines of evidence suggest that free radicals may be involved in initiation. Free radicals react avidly with DNA and oxidative damage to DNA has been measured *in vivo* (see above). Increased oxygen concentrations cause increased chromosomal breaks and mutations in both prokaryotes (Fenn et al., 1957) and eukaryotes (Kronstad et al., 1959) and may be due to an increased formation of active oxygen species. A tester strain of bacteria detects a number of radical sources, including hydrogen peroxide, other peroxides, X rays, and quinones, as mutagens (Levin et al., 1984). Further, treatment with a variety of known carcinogens, including 4-nitroquinoline oxide, 2-nitropropane, ciprofibrate, chromium (V), and betel quid extract, has been shown to increase 8-hydroxyguanine formation (reviewed in Floyd, 1990). Consistent with this observation, 8-hydroxy-2'-deoxyguanine, a product of oxidative damage to DNA, causes misreading of DNA sequences (Kuchino et al., 1987). Ionizing radiation, a free radical source, is a weak initiator in mouse skin (Jaffe and Bowden, 1987).

Free radicals also may initiate DNA damage indirectly. Malondialdehyde, a product of lipid peroxidation, binds to DNA and is mutagenic (Mukai and Goldstein, 1976). Peroxyl radicals can directly activate carcinogenic compounds. Marnett and coworkers have shown that

peroxyl radicals produced by lipid peroxidation can epoxidize 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to its ultimate carcinogenic form (reviewed in Marnett, 1987). Moreover, in freshly isolated epidermal cells from newborn mouse skin, the peroxyl radical-mediated reaction is the predominant activation pathway in cells from uninduced animals. Prostaglandin H synthase can activate a number of carcinogens by cooxidation (Eling and Krauss, 1985). Cooxidation occurs during the reduction of hydroperoxides by the peroxidase activity of prostaglandin H synthase. Thus, hydroperoxides produced during lipid peroxidation or prostaglandin biosynthesis may activate certain initiators through prostaglandin H synthase.

Many compounds with antioxidant activity have been shown to inhibit initiation (reviewed in Perchellet and Perchellet, 1989). Presumably, these compounds scavenge radicals and electrophiles before DNA damage can occur. α -Tocopherol inhibits DMBA-induced carcinogenesis in the hamster buccal pouch model when applied on alternate days from when DMBA was applied (Trickler and Shklar, 1987). The synthetic antioxidant butylated hydroxyanisole (BHA) inhibits the peroxyl radical mediated-epoxidation of 7,8-dihydroxy-dihydrobenzo[a]pyrene in mouse skin keratinocytes (Marnett, 1987). β -Carotene may protect against initiation by scavenging radicals involved in initiating events. Som and colleagues found that β -carotene, when given along with DMBA, inhibits DMBA-induced transformation in mouse mammary gland cultures (Som et al., 1984). Further investigation with this model found that β -carotene administered during the 24-hour

initiation stage could inhibit SCE caused by DMBA, N-nitrosodiethylamine, and N-methylnitrosurea (Manoharan and Bannerjee, 1985). Similarly, Stich and coworkers found that β -carotene can inhibit the formation of new pre-neoplastic lesions in betel-quid chewers (Stich et al., 1988). However, in many studies it is difficult to determine if initiation is the carcinogenic stage being inhibited because there are no clear phenotypic differences in initiated cells that can differentiate them from normal cells.

Free Radicals and Promotion

Tumor promotion is thought to be a reversible process that does not involve irreversible modification of DNA. Tumor promotion is hypothesized to involve the stimulation of the expression of abnormal genetic information within initiated cells that results in a proliferative advantage over uninitiated neighboring cells (Boutwell, 1978). By definition, tumor promoters are neither carcinogens nor mutagens themselves. Increasing evidence suggests that most tumor promoters act at the membrane to stimulate and alter genetic expression (reviewed in Boutwell et al., 1982). The prototypic and most potent tumor promoter is 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a member of the phorbol ester family of tumor promoters, and a constituent of croton oil. Numerous studies have shown that TPA treatment is associated with an extraordinary range of cellular events including the induction of protein kinase C activity, phospholipid synthesis, prostaglandin release, the synthesis and phosphorylation of histones, an

increase in protease activity, the production of active oxygen species, the induction of polyamine synthesis, and the alteration of gene expression (reviewed in Boutwell, 1982; Perchellet and Perchellet, 1989). A major objective of ongoing tumor promotion research is to identify the critical alterations in cellular functions that result in promotion of initiated cells.

The role of free radicals in tumor promotion has been reviewed numerous times (Demopoulos et al., 1980; Cerutti, 1985; Troll and Wiesner, 1985; Marnett, 1987; Perchellet and Perchellet, 1989; Floyd, 1990). Three distinct, indirect lines of evidence implicate free radical involvement in tumor promotion. First, tumor promoters increase the generation and decrease the detoxication of reactive oxygen species. Second, certain compounds and systems that generate reactive oxygen species have tumor promoting activities and mimic some of the cellular alterations associated with tumor promoters. Third, many antioxidants inhibit the cellular events caused by tumor promoters. Many tumor promoters cause aneuploidy, chromosomal aberrations, SCE, and DNA strand breaks (reviewed in Cerutti, 1985). Because tumor promoters act on the cell membrane and not directly with DNA, it is thought that their effects on DNA are mediated, at least in part, by active oxygen species generated within target cells or by activated phagocytic cells that infiltrate the target area. Perchellet and colleagues have found that TPA treatment of intact epidermal cells rapidly stimulates hydroperoxide formation and a decrease in the GSH/GSSG ratio, indicative of an increased radical challenge (Perchellet and Perchellet, 1989). *In vivo*,

TPA increases hydrogen peroxide formation in mouse skin (Goldstein et al., 1983). Hydrogen peroxide may be formed directly or from dismutation of superoxide radical. Consequently, any system that generates superoxide also generates hydrogen peroxide. It is well known that TPA can activate the respiratory burst of phagocytic cells. Inflammation also supports promotion, perhaps, because of the active oxygen species generated by phagocytic cells (see previous section). Many anti-tumor promoting agents can suppress the production of reactive oxygen species by TPA-stimulated leukocytes (reviewed in Perchellet and Perchellet, 1989). Benzoyl peroxide is known to generate free radicals and is an effective tumor promoter (Slaga et al., 1981). Other experiments indicate that tumor promoters can inhibit the activity of both catalase and SOD (Solanki et al., 1981), and that catalase and SOD-mimics can inhibit some of the cellular changes induced by tumor promoters (Friedman and Cerutti, 1983; Kensler et al., 1983). The superoxide radical-generating system xanthine/xanthine oxidase mimics the effects of TPA on ornithine decarboxylase induction (Fisher et al., 1988) and tumor promotion (Zimmerman and Cerutti, 1984). A variety of antioxidants, including BHT, mannitol, α -tocopherol, disulfiram, and a lipid soluble ascorbate analogue, ascorbyl palmitate, can inhibit some of the effects of TPA on cellular processes (reviewed by Perchellet and Perchellet, 1989). Arachidonic acid metabolism, an endogenous source of peroxides, also appears to be involved in TPA-stimulated inflammation, DNA synthesis, and cell differentiation and is thought to contribute to the tumor promoting effects of TPA (Fischer, 1984). Shklar and

colleagues found that β -carotene inhibited growth of DMBA-initiated tumors that were promoted with benzoyl peroxide (Suda et al., 1986). Retinoids modulate tumor promotion and react readily with peroxy radicals, although they may also contribute to peroxy and alkoxy radical formation. Marnett has speculated that the dichotomous antioxidant and prooxidant properties of retinoids may explain the anti-promoting and promoting activities that have been reported (Marnett, 1987).

Unfortunately, for all the indirect evidence, there is still little direct proof that the oxygen radical-generating properties of tumor promoters are related to their ability to promote initiated cells. Some basic questions remained to be firmly answered. For instance, do tumor promoters cause free radical formation under conditions where they promote tumor formation? Do antioxidants actually trap free radicals under conditions where they inhibit tumor formation? And finally, how do increases in free radical formation cause the changes in gene expression associated with tumor promotion? Some investigators are beginning to address these questions. Kensler and Taffe detected methyl radicals by spin trapping methods and ESR after addition of cumene hydroperoxide to isolated mouse epidermal cells (Kensler and Taffe, 1986). Samokyszyn and Marnett have identified a peroxy radical oxidation product of 13-cis-retinoic acid (Samokyszyn and Marnett, 1987). Because of the relative ease of retinoid oxidation, Marnett has suggested that 13-cis-retinoic acid could be used as a probe to

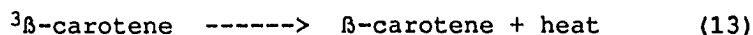
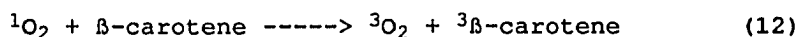
determine the effects of promoters and antipromoters on peroxy radical formation in promotion-sensitive cells (Marnett, 1987).

Recent work suggests that cellular redox status may regulate directly the expression of some genes. Both prokaryotes and eukaryotes have inducible oxidative defenses, however, the mechanism of induction has yet to be elucidated. Ames and coworkers have recently identified a gene, *oxyR*, and the protein it produces, OxyR, that positively regulate genes induced by oxidative stress in two species of bacteria (Storz et al., 1990). Their findings suggest that direct oxidation of the OxyR protein results in induction of oxidative stress genes. In human cells, another regulator of gene expression, the iron-responsive element (IRE), is sensitive to oxidation *in vitro* (Hentze, et al., 1989). This regulator controls the translation of ferritin mRNA. The IRE binding protein requires free sulfhydryl groups for binding to IRE's. Recent findings suggest that oxidants may also play a role in transcriptional activation by the proto-oncogenes *c-fos* and *c-jun*. Their protein products, Fos and Jun, form heterodimers that interact with DNA regulatory elements that regulate several genes. Abate and colleagues have found that oxidation of a single cysteine residue prevents DNA binding by the Fos-Jun complex *in vitro* (Abate et al., 1990). These results suggest that cellular redox status may directly regulate gene transcription and translation. Such regulatory mechanisms suggest a pathway whereby radical-mediated alterations of cellular redox status could lead to altered gene expression and subsequent transformation.

Antioxidant Properties of β -Carotene

The ability of β -carotene to protect against damage from singlet oxygen, an active form of oxygen, has been appreciated for many years. In fact, the protective effect of β -carotene in a particular system was often used as evidence of the involvement of singlet oxygen. In the last 10 years, solid evidence has appeared that β -carotene can protect against other reactive oxygen species as well as singlet oxygen (reviewed in Krinsky, 1990).

In 1968, Foote and Denny found that β -carotene could quench singlet oxygen, which was produced by the interaction of ground state oxygen and a photosensitizing dye (Foote and Denny, 1968). Singlet oxygen quenching by β -carotene appears to have both a physical component and a chemical component. The physical component is catalytic in that β -carotene is not consumed during the reaction. Singlet oxygen transfers excitation energy to β -carotene ($k_Q = 1.3 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$), which releases the excess energy through rotational and vibrational interactions with solvent molecules. Isomerization may occur during this process.



This type of quenching is common for carotenoids and is maximal for those compounds with 9 or more conjugated double bonds (Foote et al., 1970a). β -Carotene is also oxidized by singlet oxygen ($k_R \approx 10^4 \text{ M}^{-1} \text{ sec}^{-1}$), which ultimately limits the amount of singlet oxygen β -carotene can quench (Foote et al., 1970b). Some products of photosensitized oxidation of β -carotene in homogenous organic solutions have been identified (Tsukida et al., 1969; Seely and Meyer, 1971). However, singlet oxygen oxidation products of β -carotene have not been identified in any biological system. Quenching of singlet oxygen by β -carotene and other carotenoids protects bacteria, algae, plants, and animal, including humans, from the harmful combination of oxygen, light, and photosensitizing pigments, which produces singlet oxygen (reviewed in Krinsky, 1982).

In 1981, Packer and colleagues demonstrated that β -carotene, as well as other reportedly "selective" singlet oxygen scavengers, could also react rapidly with hydroxyl radical and the trichloromethyl peroxy radical (Packer et al., 1981). In these pulse radiolysis studies, β -carotene reacted with the peroxy radical with a rate constant of $1.5 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$, which is near the diffusion-controlled rate. At about the same time, Krinsky and Deneke observed that β -carotene could inhibit free radical-initiated lipid peroxidation in liposomes as well as singlet oxygen-initiated lipid peroxidation (Krinsky and Deneke, 1982). Previous work had demonstrated that β -carotene could suppress lipid peroxidation initiated by photosensitizing dyes, presumably by scavenging singlet oxygen (Anderson and Krinsky, 1973; Anderson et al.,

1974). Krinsky and Deneke showed that β -carotene could inhibit liposome lysis induced by K_2CrO_8 , a generator of singlet oxygen, hydroxyl radical, and superoxide (Wörner et al., 1979), and could suppress ferrous iron-initiated lipid peroxidation. Together, these two separate studies indicated that β -carotene could protect against active oxygen species other than singlet oxygen. The findings of Packer and coworkers indicate that β -carotene has the intrinsic reactivity necessary for effective radical trapping. The investigations of Krinsky and Deneke suggest that β -carotene can offer significant antioxidant protection against active oxygen species in a biomimetic system.

Many investigations have confirmed that β -carotene can suppress radical-mediated reactions in model systems. Kellogg and Fridovich found that β -carotene inhibits linoleic acid peroxidation stimulated by xanthine oxidase and acetaldehyde (Kellogg and Fridovich, 1975). At the time, they concluded that the antioxidant effect of β -carotene was due to singlet oxygen scavenging, although now it appears that β -carotene may have scavenged other reactive oxygen species. Others have found that β -carotene suppresses autoxidation of liposomes, although no protection was observed in the presence of ferrous chloride (Cabrini et al., 1986). *In vivo*, pretreatment with β -carotene and a number of other antioxidants suppressed expiration of ethane and pentane, products of lipid peroxidation, from carbon tetrachloride-treated guinea pigs (Kunert and Tappel, 1983). Presumably, β -carotene trapped radicals involved in carbon tetrachloride-initiated lipid peroxidation. Weitzman and colleagues have shown that β -carotene and other antioxidants inhibit

SCE in CHO cells treated with either TPA-activated leukocytes or xanthine/xanthine oxidase, both excellent sources of active oxygen species (Weitberg et al., 1985). In yeast, the addition of β -carotene blocks growth inhibition induced by either hyperoxic conditions (80 % O_2) or duroquinone, a redox cycling quinone (Moore et al., 1989). β -Carotene inhibits the loss of retinol, alpha-tocopherol, and PUFA from isolated rat liver mitochondria treated with NADPH, ADP, and ferric iron (Parker, 1985). To increase mitochondrial β -carotene levels rats were fed supplemental β -carotene. Stocker and coworkers evaluated the antioxidant activity of β -carotene and other antioxidants in homogenous solutions of linoleic acid or in phosphatidylcholine liposomes under air and 2 % O_2 (about 15 torr) (Stocker et al., 1987). Oxidations were initiated with the azo compound AMVN. α -Tocopherol was the most effective antioxidant in both systems. β -Carotene was modestly effective in these systems. Decreasing the partial pressure of oxygen (pO_2) increased inhibition by β -carotene in liposomes to about 35 % from about 20 % observed under air. Similar results have been achieved in microsomes (Vile and Winterbourne, 1988). β -Carotene suppressed adriamycin-stimulated, iron-dependent lipid peroxidation by 50 % under air and by 70 % at 4 torr O_2 in rat liver microsomes. However, significant inhibition was achieved only at very high concentrations (approximately 4 mol % or greater, based on phospholipid). These results suggest that *in vitro* incorporation of β -carotene into biological membranes is difficult and may require special manipulations.

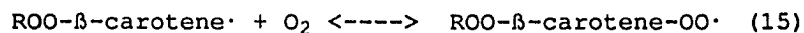
Experiments on the effect of pO_2 on β -carotene activity were spurred by chemical studies on the mechanism of β -carotene radical scavenging (Burton and Ingold, 1984). In chlorobenzene solution, β -carotene inhibited the azo-bis(isobutyronitrile)-catalyzed oxidation of methyl linoleate or tetralin at 30°C. Oxidation was measured by oxygen uptake. Inhibition by β -carotene was greater at 15 torr O_2 than at 150 torr O_2 . Further, at 760 torr O_2 (100 % O_2), β -carotene apparently increased rates of oxidation after a lag period. These data have been interpreted to suggest that β -carotene is a better antioxidant at low pO_2 than under air, and that β -carotene may be a prooxidant at high pO_2 . This conclusion warrants close inspection since it implies that β -carotene may be an effective antioxidant in tissues at low pO_2 , such as the liver, but may be a prooxidant in tissues at high pO_2 , such as the lung (Kessler et al., 1982).

Several separate aspects of this work suggest that a more cautious interpretation may be advised. These experiments were performed in a homogenous organic solution and results may be somewhat different in biological membranes, although others have observed a modest pO_2 effect in model membrane systems (see above). The data were presented in absolute terms and were not corrected for the effects of reduced pO_2 on the uninhibited oxidation. For example, at 150 torr O_2 , the initial rate of oxygen uptake in the presence of 50 μM β -carotene was $33 \times 10^8 M \text{ sec}^{-1}$. At the same β -carotene concentration, the corresponding rate at 15 torr O_2 was $20 \times 10^8 M \text{ sec}^{-1}$. However, when corrected for the effect of pO_2 alone, inhibition at 150 torr O_2 was 25 % vs. 20 % inhibition at

15 torr O₂. At 760 torr O₂, β-carotene does cause increases in oxidation rates after a lag period, but only after an initial period of inhibition. Even when oxidation rates have increased after the lag period, the rates of oxidation are still less than those without β-carotene. These considerations suggest that the effect of pO₂ on β-carotene antioxidant action in this system is quite modest and that extrapolation of these results to *in vivo* requires further investigations. These contentions aside, Burton and Ingold have proposed an interesting antioxidant mechanism for β-carotene. Based on the studies mentioned and the pO₂ dependence of β-carotene autoxidation, they suggest that β-carotene reacts with peroxy radicals to form a relatively stable β-carotene-radical adduct.



Although the adduct is a carbon-centered radical, it is relatively unreactive because of resonance stabilization by the extensive polyene system. This radical adduct can also react reversibly with oxygen, like lipid radicals, to form a reactive peroxy radical.



The pO₂ of the system would then effect the equilibrium of reaction 15. At high pO₂, the equilibrium would lie to the right and favor formation of a β-carotene-derived peroxy radical. At low pO₂, the reaction would

lie to the left, which favors the stability of the trapped radical adduct. These studies, combined with those of Packer and colleagues, suggest that β -carotene possesses two important antioxidant properties, rapid reactivity with peroxy radicals and formation of a resonance-stabilized radical product.

Burton and Ingold used the kinetics of oxygen uptake to investigate the mechanism of β -carotene antioxidant action. Another useful approach is to identify products formed during the reaction of β -carotene and radicals. Identification of oxidation products should allow deduction of the reactions that led to their formation. Moreover, oxidation products of β -carotene may also be marker products of β -carotene antioxidant action. Investigators then could determine if β -carotene acts as an antioxidant under conditions where it inhibits carcinogenesis. Little is known about the reaction between β -carotene and specific radicals because previous investigations have used systems that generate poorly defined oxidizing species.

β -Carotene autoxidation results in the formation of a number of products (Hunter and Krakenberger, 1947; El-Tinay and Chichester, 1970). Autoxidation occurs when a substrate oxidizes in air or oxygen in the absence of any apparent catalyst. This process consumes antioxidant, yet does not contribute an antioxidant effect. Hunter and Krakenberger let β -carotene autoxidize in benzene or arachis oil under oxygen at 50°C and reported the formation of 5,6-epoxy- β , β -carotene, 5,8-epoxy- β , β -carotene, 5,6,5',6'-diepoxy- β , β -carotene, 5,6,5',8'-diepoxy- β , β -carotene, 5,8,5',8'-diepoxy- β , β -carotene, semi- β -carotenone and

β -carotenone (Hunter and Krakenberger, 1947). El-Tinay and Chichester observed similar products when β -carotene was autoxidized in toluene at 60°C (El-Tinay and Chichester, 1970). β -Carotene autoxidation is thought to have a free radical nature because it is accelerated by radical initiators and inhibited by antioxidants. The radical initiator azobis(isobutyronitrile) was found to accelerate β -carotene autoxidation, but no products were reported for the reaction with the initiator (El-Tinay and Chichester, 1970). Similarly, the antioxidant diphenylamine inhibited β -carotene autoxidation (El-Tinay and Chichester, 1970). However, the exact nature of the initiating radical in β -carotene autoxidation is not known. The 5,6-epoxide, the 5,6,5',6'-diepoxide, and a series of conjugated polyene carbonyl compounds were produced during the ferrous phthalocyanine-catalyzed oxidation of linoleate (Friend, 1958). β -Carotene is cooxidized by lipoxygenases to low molecular weight carbonyl compounds, in addition to β -carotene epoxides (reviewed in Krinsky, 1989). Again, it is difficult to determine if these products were formed by antioxidant reactions of β -carotene or by β -carotene autoxidation because the identity of the oxidizing species is undetermined.

The overall objective of this dissertation research was to describe the antioxidant chemistry of β -carotene in model systems. The first specific aim was to identify peroxy radical oxidation products of β -carotene in homogenous solution. A solution system provides chemically defined conditions for investigating radical reactions. These studies are described in Chapter 2. The second specific aim was

to determine the effect of pO_2 on β -carotene antioxidant activity and oxidation product formation in a biomimetic system. The liposome model membrane system provides a physiologically relevant reaction environment and permits the accurate control of antioxidant concentration, prooxidant concentration, and pO_2 . This work is presented in Chapter 3. The final specific aim was to develop a method to load microsomes *in vitro* with β -carotene that would allow study of β -carotene antioxidant reactions in biological membranes. *In vitro* supplementation provides a more exact and less time consuming method of loading biomembranes than dietary supplementation. The final chapter will summarize the results of this dissertation research and discuss their contribution to the literature that has been reviewed here.

CHAPTER 2

PEROXYL RADICAL OXIDATION OF β -CAROTENE: FORMATION OF
 β -CAROTENE EPOXIDES

INTRODUCTION

Several lines of evidence suggest that the provitamin A carotenoid β -carotene (Fig. 3) and other carotenoids inhibit carcinogenesis (reviewed in Temple and Basu, 1988). Epidemiological studies suggest that human cancer risk, particularly lung cancer risk, is inversely correlated with dietary intake (Peto et al., 1981; Ziegler, 1989) and blood levels of β -carotene (Ziegler, 1989). Consistent with these findings, treatment with β -carotene caused remission of oral pre-cancerous lesions in tobacco/betel quid chewers (Stich et al., 1988). β -Carotene also inhibited chemically- and physically-induced neoplastic transformation of cells in culture (Pung et al., 1988) and caused regression of chemically-induced buccal pouch carcinoma in hamsters (Shklar and Schwartz, 1988).

β -Carotene is thought to protect against tumor formation by scavenging oxidants involved in carcinogenesis (Ames, 1983). Although β -carotene is a well known $^1\text{O}_2$ quencher, it, like other $^1\text{O}_2$ quenchers, also reacts rapidly with peroxyl radicals (Packer et al., 1981). β -Carotene is thought to trap peroxyl radicals because it inhibits lipid peroxidation *in vitro* and *in vivo* (Krinsky and Deneke, 1982; Kunert and Tappel, 1983; Stocker et al., 1987). Although β -carotene suppresses the

deleterious effects of peroxy radicals, the radical trapping mechanism of β -carotene has not been identified nor have the products of radical trapping by β -carotene been characterized (Krinsky, 1989).

Although the products of the reaction between specific peroxy radicals and β -carotene have not been identified, the autoxidation of β -carotene has been studied in a variety of chemical systems. Products of β -carotene autoxidation in arachis oil and in benzene (Hunter and Krakenberger, 1947), and in toluene (El-Tinay and Chichester, 1970) included 5,6-epoxy- β,β -carotene, 5,6,5',6'-diepoxy- β,β -carotene and their corresponding furanoid rearrangement products, 5,8-epoxy- β,β -carotene and 5,8,5',8'-diepoxy- β,β -carotene. The radical initiator azobis(isobutyronitrile) stimulated the rate of autoxidation (El-Tinay and Chichester, 1970), although the products of the reaction with the radical initiator were not reported. The 5,6-epoxide and the 5,6,5',6'-diepoxy and a mixture of aldehydes and ketones also were formed during the ferrous phthalocyanine-catalyzed autoxidation of β -carotene in benzene and from the enzymatic oxidation of β -carotene by lipoxygenase (Friend, 1958).

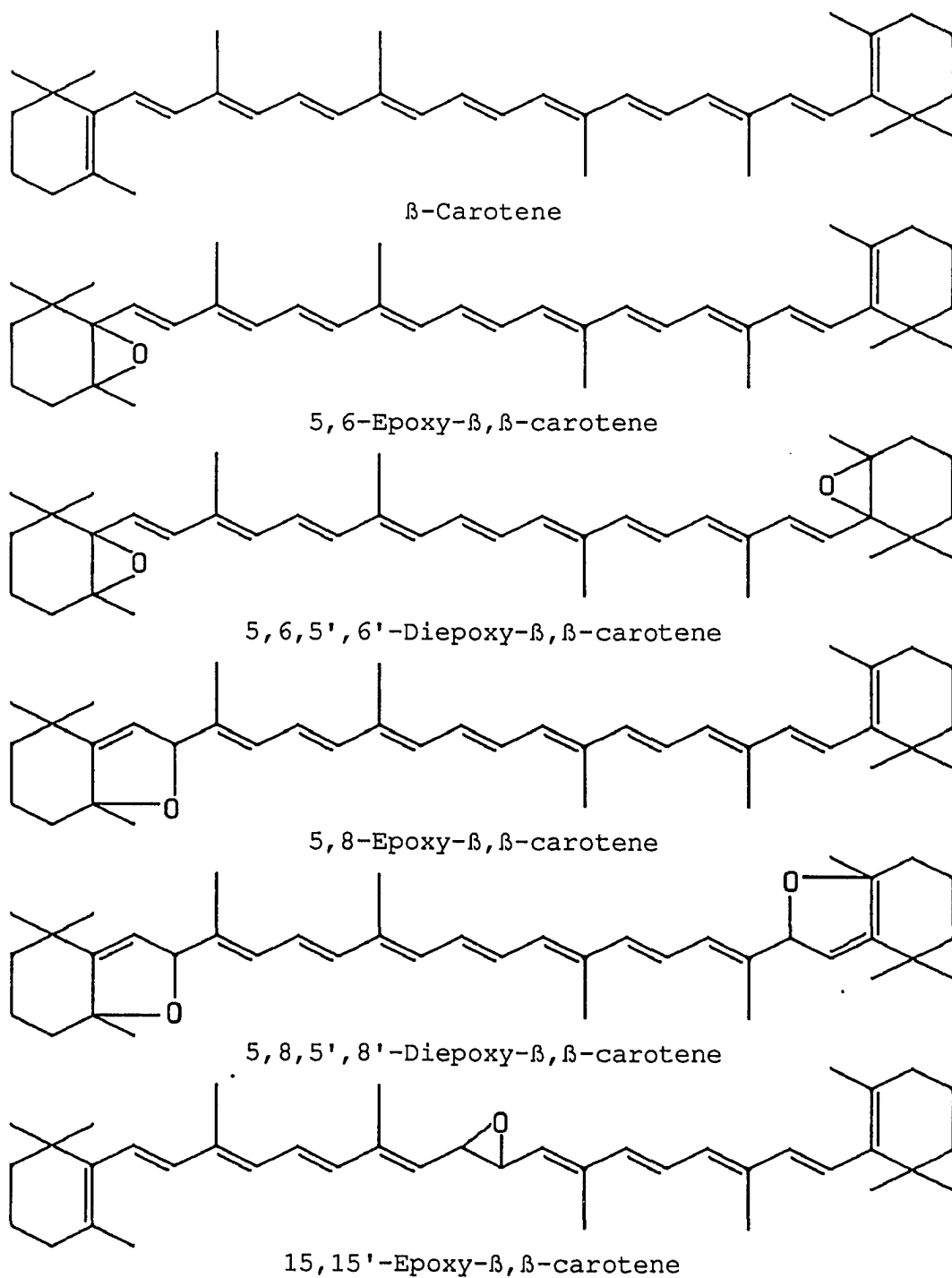


Figure 3. Structures of β -carotene and its epoxides.

The purpose of this study was to identify the major products formed by peroxy radical oxidation of β -carotene in a well defined model system. We chose hexane as our solvent to ensure the solubility of β -carotene and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) and to minimize the generation of solvent-derived radicals. We found that AMVN-derived peroxy radicals react with β -carotene to form the 5,6-epoxide, 15,15'-epoxy- β , β -carotene, a previously unreported product, and polar appear to result from peroxy radical addition to β -carotene and are stable enough to accumulate. Epoxide formation may indicate a mechanism of peroxy radical trapping by β -carotene. Moreover, epoxides may be marker products for the antioxidant action of β -carotene.

EXPERIMENTAL PROCEDURES

Chemicals. β -Carotene (Fluka Chemical Corp., Ronkonkoma, NY) was stored at -20°C under Ar. Only β -carotene from newly opened vials was used for incubations. There were no detectable differences in DAD-HPLC chromatogram profiles at 450, 330, and 280 nm between HPLC-purified β -carotene and β -carotene from newly opened vials. $[10,10',11,11'\text{-}^{14}\text{C}]\text{-}\beta$, β -Carotene (53.7 mCi/mmol) was a generous gift from Drs. Lawrence J. Machlin and Peter F. Sorter, Hoffman-LaRoche Co. and was purified by reverse phase HPLC on a Whatman Partisil ODS-2, 10 micron, 4.6 x 250 mm column eluted with methanol/hexane (85:15, v/v) at 1.5 ml min^{-1} immediately prior to use. AMVN was purchased from Polysciences, Inc., Warrington, PA, and was used as received. Hexane for oxidations was

HPLC grade (Baxter/Burdick and Jackson, Muskegon, MI). All other solvents were of highest quality available. Oxidation products of β -carotene were stored with solvent removed at -20°C under Ar to prevent degradation. All procedures involving β -carotene and its oxidation products were performed under reduced light.

Apparatus. HPLC was performed with a Spectra Physics model 8810 pump and a Hewlett-Packard model 1040A diode array detector system (DAD-HPLC) operated by a HP 79994A ChemStation equipped with HP ChemStation software. HPLC columns containing Spherisorb and Whatman packings were purchased from Alltech (Deerfield, IL). UV-Vis spectroscopy was performed with a Hewlett-Packard model 8452A diode array spectrophotometer run with HP operating software. Mass spectrometry was done with a Finegan MAT-90 instrument in the College of Pharmacy Mass Spectrometry Center. Samples were introduced by direct probe insertion and ionized with a 70 eV electron beam. ^1H -NMR spectra were obtained with a Bruker AM-500 instrument equipped with an Aspect 3000 computer and operating at 500.13 MHz for ^1H . Spectra were recorded in CDCl_3 (Cambridge Isotope Laboratories, Woburn, MA) with tetramethylsilane as a reference standard.

Oxidation of β -carotene. β -Carotene (50 mg; 93 μmol) and AMVN (23 mg; 93 μmol) were dissolved in 100 ml air-saturated hexane. The mixture was shielded from light and incubated under air at 37°C in an orbital shaking incubator. Small aliquots of the reaction mixture were

taken periodically for analysis of β -carotene by reverse phase DAD-HPLC. When less than 10 % of the initial β -carotene remained, the reaction mixture was evaporated to dryness *in vacuo* and dissolved in HPLC mobile phase for product purification.

HPLC Analysis and Purification of β -carotene and its Oxidation Products. β -Carotene and its oxidation products were separated and analyzed by reverse phase HPLC on a Whatman Partisil ODS-2, 10 micron, 4.6 x 250 mm analytical column eluted with methanol/hexane (85:15, v/v) at 1.5 ml min⁻¹. Products in fractions B and C from reverse phase analyses were further purified with a Spherisorb CN, 5 micron, 4.6 x 250 mm analytical column. Fraction B was eluted with hexane/ethyl acetate (99.5:0.5, v/v) at 1.5 ml min⁻¹. Fraction C was eluted with hexane/ethyl acetate (99.99:0.01, v/v) at 1 ml min⁻¹.

Quantification of Product Distribution. Unlabeled β -carotene and AMVN were incubated as described above with the addition of [¹⁴C]- β -carotene (2 μ Ci). Periodically, aliquots were taken and hexane was evaporated under a stream of N₂. The residue was dissolved in mobile phase for analysis by reverse phase HPLC as described above. Radiolabeled products were detected by collecting 0.4 min fractions, which were assayed for [¹⁴C] by liquid scintillation counting.

Kinetics of Loss of Fractions B and C. AMVN (460 μ M) and fraction B products (approximately 460 μ M 5,6-epoxide) or fraction C

products (approximately 460 μM 15,15'-epoxide) were dissolved in hexane and incubated in a screw-cap test tube at 37°C. The concentration of fraction B was estimated spectrophotometrically using $\epsilon = 125,000 \text{ M}^{-1}\text{cm}^{-1}$ (Acemoglu and Eugster, 1984). The concentration of fraction C was estimated similarly with $\epsilon = 25,000 \text{ M}^{-1}\text{cm}^{-1}$. The loss of each fraction was quantitated by reverse phase HPLC, as described above, with detection at 450 nm for fraction B and detection at 330 nm for fraction C.

RESULTS

Peroxyl radical oxidation of β -carotene

Peroxyl radicals generated by thermolysis of AMVN oxidized over 99% of β -carotene in hexane during a 24 h incubation under air. The color of the incubation mixture changed from dark amber to light orange over the course of the incubation. Preliminary experiments indicated that complete bleaching of the reaction mixture resulted in the formation of only polar, volatile products. Oxidation was radical dependent; without AMVN a similar loss of β -carotene required approximately 200 h.

Analysis of fractions A and B

Three fractions of products were separated by reverse-phase HPLC (Fig. 4). Fraction A was poorly retained on the reverse phase column and preliminary studies of this fraction indicated that it contained a mixture of volatile, low molecular weight aldehydes and ketones. This product fraction was not further characterized. Fraction B absorbed strongly at 450 nm and eluted as a broad peak between 4 and 7 min on the reverse phase column. Consecutive DAD-HPLC spectra taken during elution of this fraction indicated that it contained several components. Fraction B was resolved into 1 major peak and at least 7 much smaller peaks by cyanopropyl column HPLC (Fig. 5). The major peak was identified by UV-Vis and electron ionization mass spectrometry as 5,6-epoxy- β,β -carotene. The UV and mass spectra obtained matched those previously published (Tsukida and Zechmeister, 1958; Eschenmoser and Eugster, 1978). The 5,6-epoxide displayed UV maxima at 424, 446, and 474 nm (in hexane) and mass spectral signals at m/z 552 (M^+ , 100 %), 550 (71), 472 (38), 406 (11), 336 (24), 205 (68), and 165 (23). Further, treatment of the 5,6-epoxide in 1 ml hexane with 5 drops of concentrated formic acid led to a 20 nm hypsochromatic shift in its UV spectrum consistent with rearrangement to 5,8-epoxy- β,β -carotene (Liaanen-Jensen and Jensen, 1971). One of the minor peaks resolved by cyanopropyl column DAD-HPLC displayed UV maxima at 384, 404, and 426 nm, which match the published UV spectrum of 5,8,5',8'-diepoxy- β,β -carotene (Tsukida and Zechmeister, 1958).

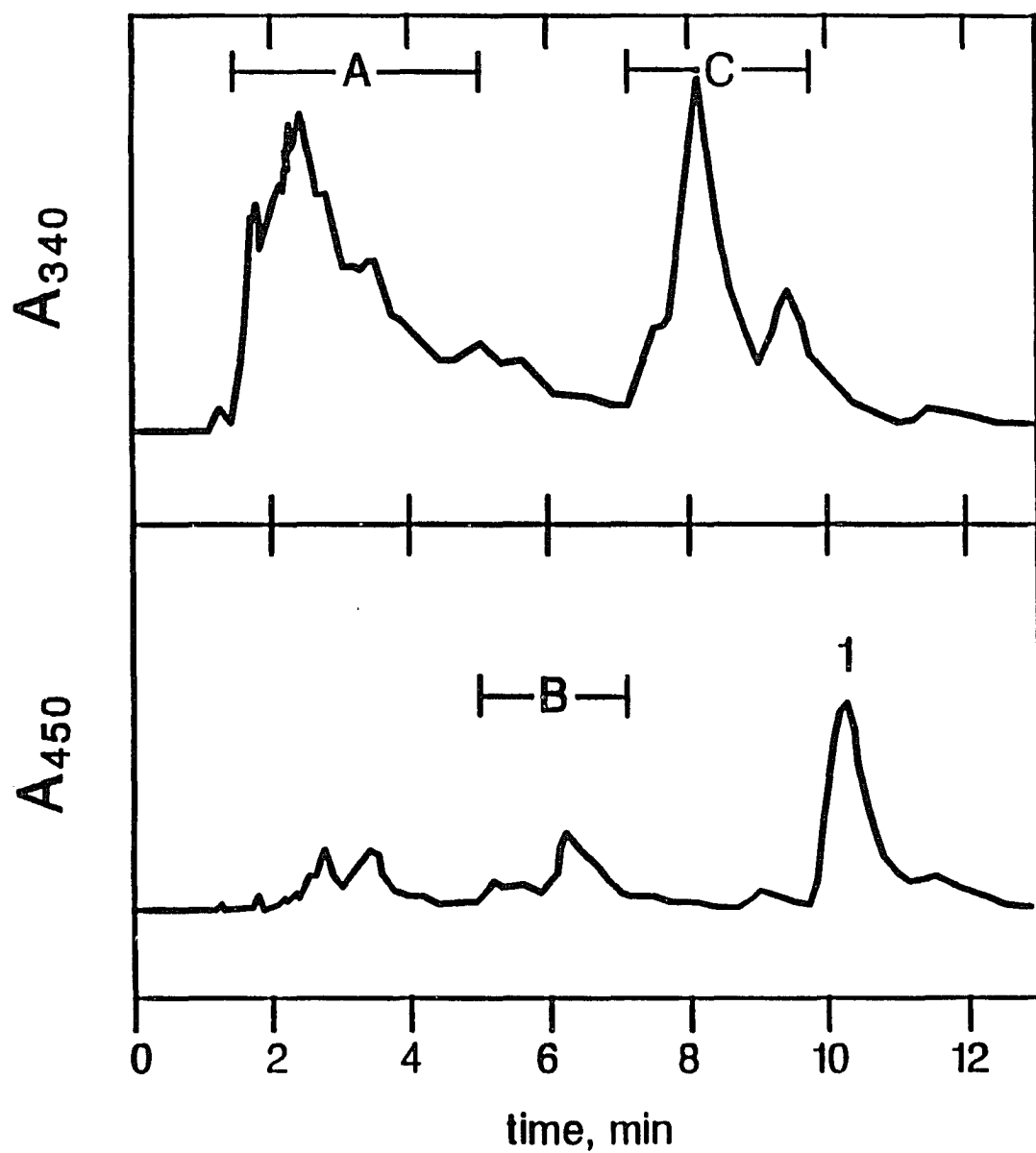


Figure 4. Reverse-phase DAD-HPLC analysis of incubation mixture of β -carotene and AMVN at 24 h. Fractions A, B, and C, and β -carotene (1) are indicated.

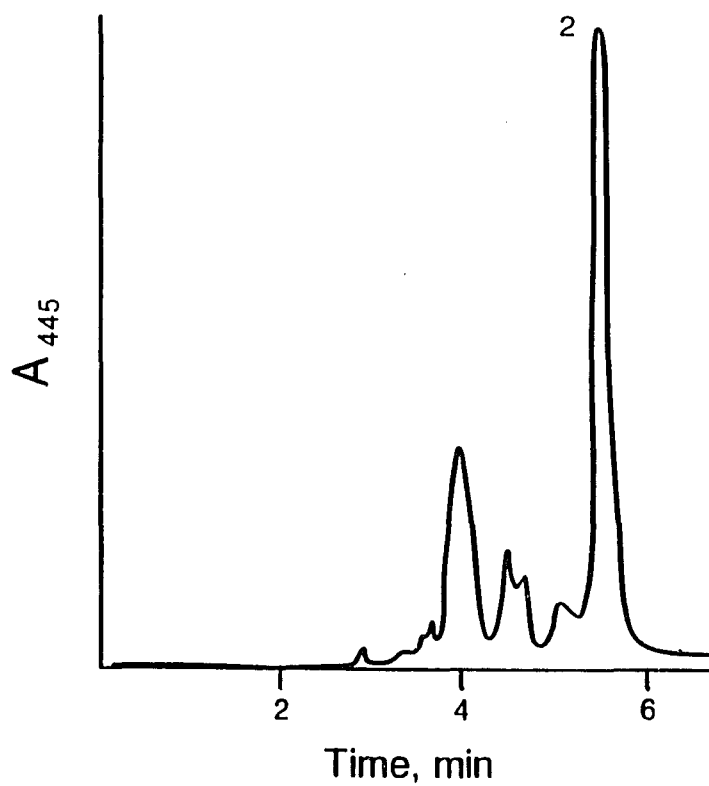


Figure 5. Cyanopropyl HPLC of fraction B at 445 nm. The 5,6-epoxide is designated as 2.

Analysis of fraction C

Fraction C eluted between 7 and 10 min on the reverse phase column, just prior to β -carotene. DAD-HPLC analysis indicated that this fraction absorbed strongly at 340 nm but not at 450 nm (Fig. 4). Cyanopropyl column HPLC of this fraction yielded 2 major peaks (Fig. 6). These peaks were identified by UV-Vis, mass spectrometry and $^1\text{H-NMR}$ spectroscopy as the *cis* and *trans* isomers of 15,15'-epoxy- β,β -carotene. The peak (6a) eluting at 3.5 min (on the cyanopropyl column) displayed a UV maximum at 330 nm ($\epsilon_{330} = 6,200 \text{ M}^{-1}\text{cm}^{-1}$) with an indistinct band at 258 nm (in hexane). The peak (6b) eluting at 6 min displayed a UV maximum at 330 nm ($\epsilon_{330} = 25,000 \text{ M}^{-1}\text{cm}^{-1}$) without the additional band. Extinction coefficients were calculated from the UV spectra of solutions of known concentration. Electron ionization mass spectrometry yielded nearly identical spectra for both peaks with signals for peak 6a at m/z 552 (M^+ , 100 %), 534 (7), 444 (3), 378 (25), 349 (10), and 255 (14), and for peak 6b at m/z 552 (M^+ , 100 %), 534 (13), 444 (8), 378 (58), 349 (32), and 255 (13) (Fig. 7). High resolution electron ionization mass spectrometry yielded M^+ 552.4363 for peak 6a and M^+ 552.4308 for peak 6b (calc'd. $\text{C}_{40}\text{H}_{56}\text{O}$ 552.4331). $^1\text{H-NMR}$ (CDCl_3) of 6a gave resonances at 1.03 (s, 1,1'- CH_3), 1.46 (m, 2,2'- CH_2), 1.60 (m, 3,3'- CH_2), 1.71 (s, 5,5'- CH_3), 1.94 (s, 9,9',13,13'- CH_3), 2.02 (m, 4,4'- CH_2), 2.11 (m, 15,15'-CH), 5.95 (m, 14,14'-CH), 6.01 (m, 10,10'-CH), 6.12 (m, 7,7',8,8'-CH), 6.25 (m, 12,12'-CH), 6.50 (m, 11,11'-CH). $^1\text{H-NMR}$ (CDCl_3) of 6b yielded resonances at 1.04 (s, 1,1'- CH_3), 1.48 (m, 2,2'- CH_2), 1.60 (m, 3,3'- CH_2), 1.71 (s, 5,5'- CH_3), 1.95 (d, 9,9',13,13'- CH_3), 2.02

(s, 4,4'-CH₂), 2.10 (m, 15,15'-CH), 5.80 (m, 14,14'-CH), 6.07 (m, 7,7'- or 8,8'-CH), 6.18 (m, 7,7'- or 8,8'-CH), 6.18 (m, 10,10'-CH), 6.42 (m, 12,12'-CH), 6.62 (m, 11,11'-CH). Most of the resonances were identical to those of the corresponding nuclei in β -carotene (Vetter et al., 1971), which facilitated resonance assignment. Significant differences were noted in the resonances of 7,7'-H or 8,8'-H, 12,12'-H, 14,14'-H and 15,15'-H. Peak assignments in the olefinic region were confirmed by phase-sensitive, double quantum filtered ¹H-COSY analysis (not shown). The spectrum contained a crosspeak between resonances at 2.10, 15,15'-H, and 5.80, 14,14'-H, consistent with the 15,15'-epoxide structure. We were unable to measure $J_{15-15'}$ and, therefore cannot assign *cis* and *trans* configurations on that basis. A tentative assignment of configuration may be made on the basis of the UV/Vis spectra of the isomers. The molar absorptivity at 330 nm of epoxide **6b** is approximately 5-fold that for the epoxide **6a** (see above). Zechmeister et al. (Zechmeister et al., 1943) found that *cis*-carotenoids displayed weaker absorptivities than the corresponding all-*trans*-carotenoids. We therefore tentatively assign epoxide **6a** as the *cis* isomer and **6b** as the *trans* isomer.

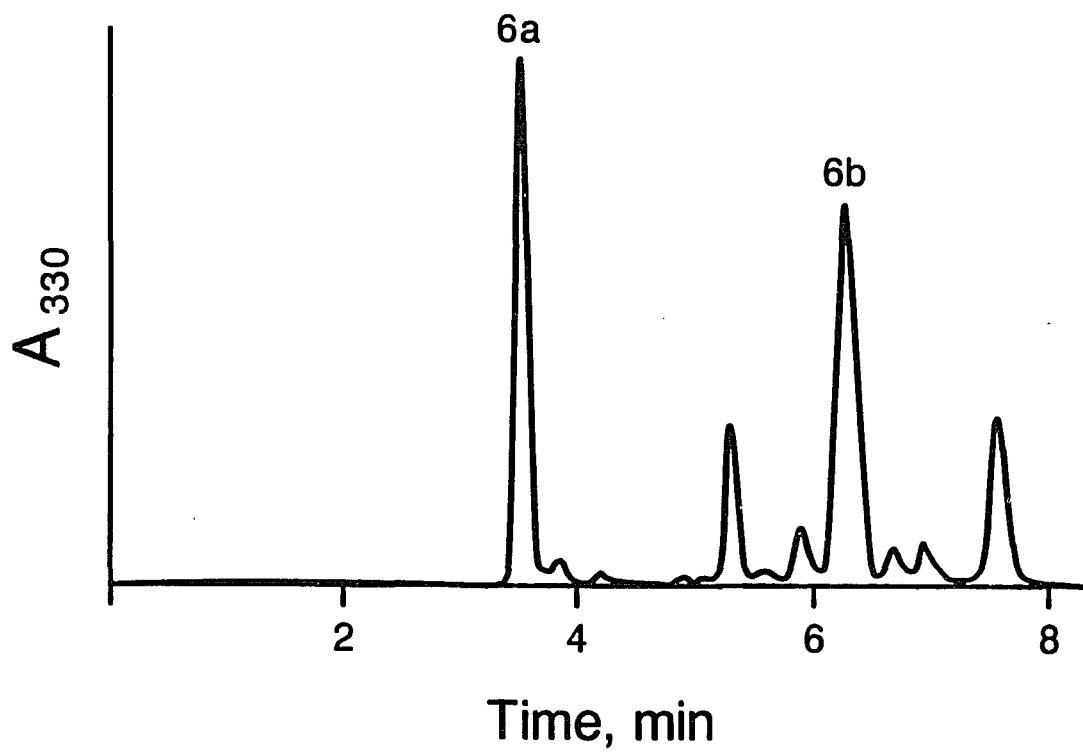


Figure 6. Cyanopropyl HPLC of fraction C at 330 nm. The *cis* and *trans* isomers of the 15,15'-epoxide are indicated as **6a** and **6b**.

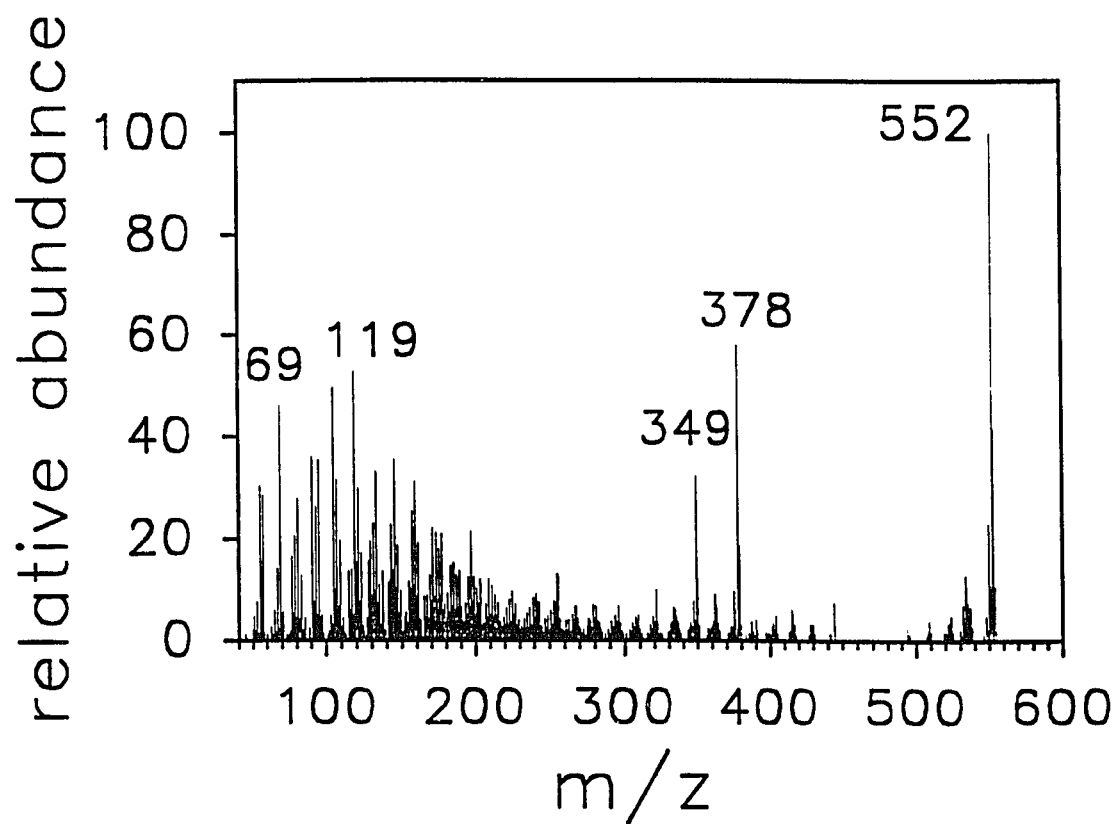


Figure 7. Electron ionization MS of 15,15'-epoxy- β,β -carotene purified from fraction C.

Kinetics of product formation and disappearance

To determine the distribution of products and the relative rates of formation of each product fraction, incubations were carried out with [^{14}C]- β -carotene. Aliquots of the reaction mixture were taken during the reaction and analyzed by reverse phase HPLC. The amount of radiolabel associated with fraction A increased rapidly with time with no apparent lag phase. At 13 h, 58 % of the total radiolabel eluted with fraction A (Fig. 8). The amount of radiolabel associated with fractions B and C increased with time at a much slower rate. By 13 h, fraction B and fraction C each contained 8 % of the total radiolabel. The increase in radiolabel associated with fractions A, B, and C paralleled the decrease in radiolabel eluted as unchanged β -carotene.

DAD-HPLC analysis of incubations with unlabeled β -carotene suggested that the 5,6-epoxide and the 15,15'-epoxide gradually disappeared from the reaction mixture (data not shown). These epoxides therefore may be intermediates in the formation of fraction A from β -carotene. To further explore this possibility, we studied the stability and fate of the 5,6-epoxide and 15,15'-epoxide under the reaction conditions. Both epoxides were prepared by oxidation of β -carotene (see above) and each was incubated separately with AMVN, without β -carotene, under the original reaction conditions. The initial concentration of epoxide in each experiment was approximately 5 times that formed by oxidation of β -carotene (Fig. 8) to provide sufficient quantities of unlabeled epoxide to permit accurate DAD-HPLC quantitation. The initial AMVN concentration was then set equal to the epoxide concentration to

approximate conditions at about 6 h in Figure 8. At various times, the reaction mixture was analyzed by DAD-HPLC. Both epoxides were oxidized by AMVN to products that eluted with fraction A (data not shown). The initial rates of epoxide consumption appeared to be approximately 3.5 mol h^{-1} for both epoxides, although no attempt was made to determine the reaction order (Fig. 9). Thus, to the extent that this experiment approximates the early stages of β -carotene oxidation, approximately $7 \text{ mol epoxides h}^{-1}$ may be oxidized to fraction A products.

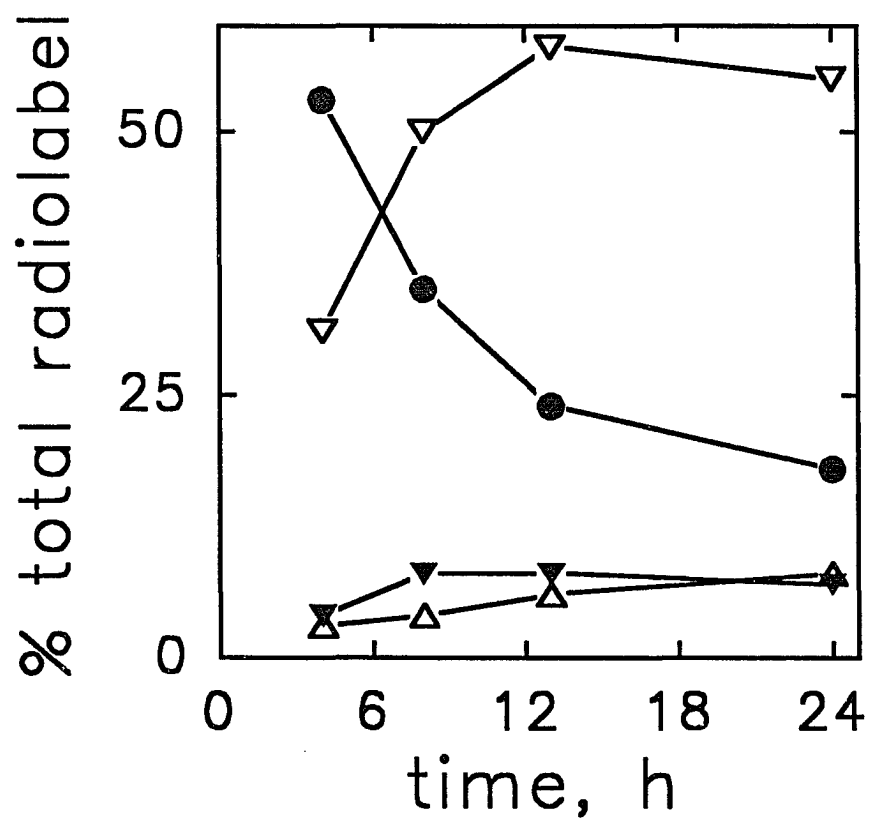


Figure 8. Kinetics of AMVN dependent oxidation of β -carotene (•) and formation of fractions A (▽), B (▼), and C (Δ).

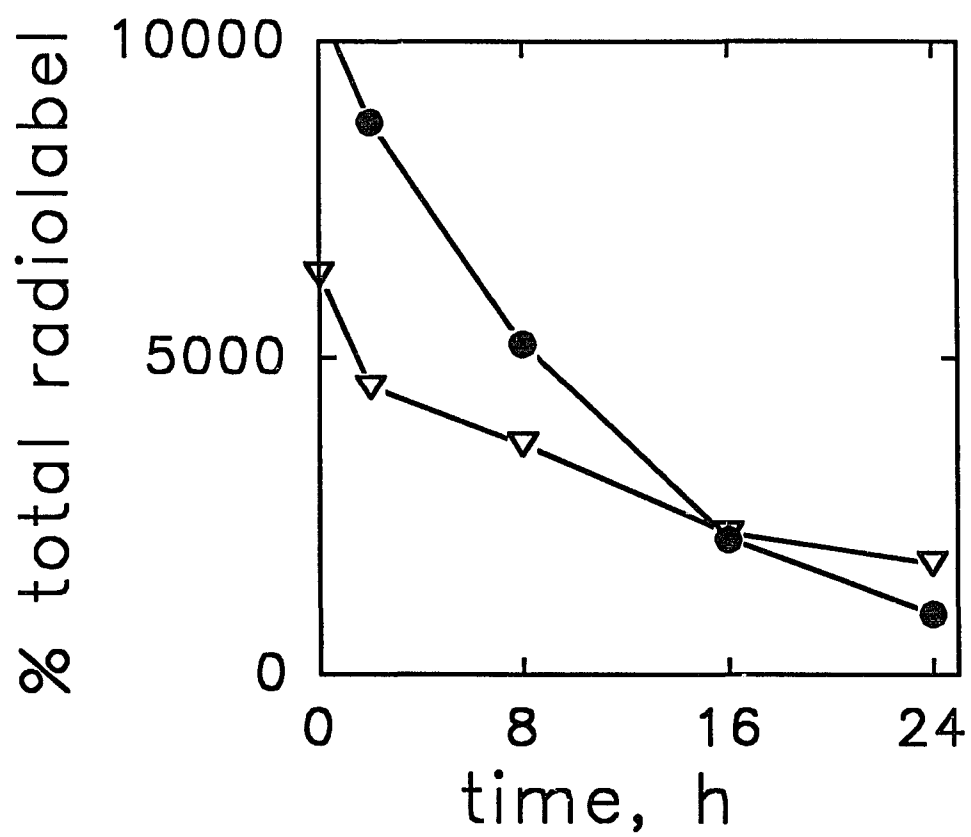


Figure 9. Kinetics of AMVN dependent loss of 5,6-epoxy-β,β-carotene (●) and 15,15'-epoxy-β,β-carotene (▽).

DISCUSSION

β -Carotene is widely considered an antioxidant because it protects against oxidative damage in a variety of systems (Krinsky and Deneke, 1982; Kunert and Tappel, 1983; Stocker et al., 1987, Krinsky, 1989). The antioxidant action of β -carotene is often assumed to be due to $^1\text{O}_2$ quenching, because this property is well documented by previous work (Foote and Denny, 1968). However, like other $^1\text{O}_2$ quenchers, β -carotene also reacts readily with oxy-radicals (Packer et al., 1981), which may cause more oxidative damage than $^1\text{O}_2$, even when $^1\text{O}_2$ is present. Thus, while $^1\text{O}_2$ scavenging by β -carotene may contribute to its antioxidant activity, oxy-radical trapping by β -carotene may be an important component of the antioxidant action of β -carotene. The objective of this investigation was to identify the products formed by reaction of β -carotene with peroxy radicals in a chemically defined system. Our results indicate that 5,6-epoxy- β,β -carotene and 15,15'-epoxy- β,β -carotene are nonradical products formed by peroxy radical oxidation of β -carotene. The 5,6-epoxide was identified previously as an autoxidation product of β -carotene (Hunter and Krakenberger, 1947; El-Tinay and Chichester, 1970). The 15,15'-epoxide is reported here for the first time. The 5,6-epoxide and 15,15'-epoxide comprise about 20% of the radiolabeled products formed. The major product group (fraction A, Fig. 4) contained a mixture of polar products. Preliminary studies suggest that several volatile, low molecular weight carbonyl compounds comprise this fraction. Some of these contain ^{14}C radiolabel, which was

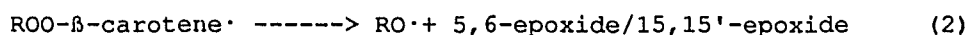
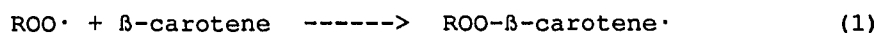
present at C₁₀, C_{10'}, C₁₁, and C_{11'} of [¹⁴C]-β-carotene, but others may not. Additional efforts to characterize this product fraction are underway in our laboratory.

Radical addition to conjugated polyene systems usually occurs at the terminal carbons, where electron density is greatest (reviewed in March, 1985). The resulting polyene radical adduct possesses maximum resonance stabilization. Epoxide formation consequently follows at the site of addition; formation of the 5,6-epoxide is consistent with this mechanism. In contrast, the 15,15'-epoxide is formed by epoxidation of the central double bond, the least electron dense double bond in a polyene system (Zechmeister et al., 1943). The formation of the 15,15'-epoxide may be explained by the greater accessibility of the 15,15'-double bond compared to that of the 5,6-double bond. The di-substituted 15,15'-double bond provides a less sterically hindered approach to an attacking radical than the 5,6-double bond, which has four alkyl substituents, the 5-CH₃, 4-CH₂, 1-C, and 7-CH. The site of radical attack thus may be governed by the accessibility of the 15,15'-double bond, the high electron density of the 5,6-double bond, and the structure of the attacking radical.

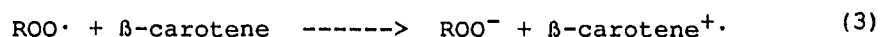
Epoxide formation in this system apparently is initiated by peroxy radical addition to the polyene rather than by a process initiated by hydrogen atom abstraction. This conclusion is based on the work of Samokyszyn and Marnett, who found that hydroperoxide-dependent cooxidation of 13-*cis*-retinoic acid by prostaglandin synthetase involved both hydrogen abstraction and peroxy radical addition mechanisms

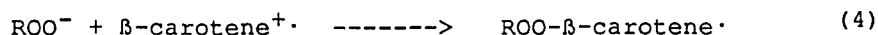
(Samokyszyn and Marnett, 1987). Hydrogen abstraction was mediated exclusively by the enzyme and ultimately yielded 4-hydroxy-13-*cis*-retinoic acid, whereas peroxy radical addition resulted in epoxidation of the 5,6-double bond, as we observed here. We did not observe any reaction product with characteristics expected of a 4-hydroxy- β,β -carotene product, even though such a product would most likely elute in our HPLC systems near the 5,6-epoxide and the 15,15'-epoxide. Consequently, it appears that hydrogen abstraction did not initiate product formation in our system.

Radical addition is followed by homolytic cleavage of the peroxide bond to yield an epoxide and release an alkoxyl radical (eq. 1 & 2).



A peroxy radical may add to the polyene chain to form the radical addition product directly. Alternatively, the peroxy radical may abstract an electron from the polyene to form an intermediate ion pair consisting of a peroxide ion and the radical cation of β -carotene, which then combine to form an radical addition product (eq. 3 & 4).





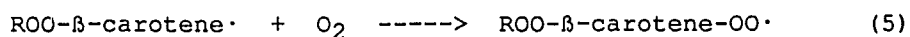
This alternative mechanism is supported by the observation that pulse radiolysis-generated $\text{CCl}_3\text{O}_2 \cdot$ radicals oxidized β -carotene to a transient product with a near IR absorption band attributed to the radical cation of β -carotene (Packer et al., 1981). The radical cation of β -carotene was also formed by electrochemical oxidation of β -carotene (Grant et al., 1988). Although the scheme presented in equations 1 and 2 results in no net radical trapping, peroxy radical oxidations could be inhibited if the rate of radical adduct formation (eq. 1) is significantly greater than the rate of alkoxy radical release (eq. 2).

Analysis of product formation indicated that fraction A products accumulated rapidly whereas the 5,6-epoxide and the 15,15'-epoxide accumulated somewhat more slowly during the oxidation of $[\text{}^{14}\text{C}]\text{-}\beta\text{-carotene}$ (Fig. 8). The very rapid formation of fraction A products suggests that these may be formed directly from β -carotene. On the other hand, the experiment depicted in Figure 9 indicated that the epoxides are converted under the reaction conditions to products that elute with fraction A. Continued oxidation of the epoxides may account for the failure of the epoxides to accumulate to higher levels. The polar products of epoxide oxidation have not been well characterized and may not be identical to the fraction A products formed when β -carotene is the starting substrate. However, it seems plausible that β -carotene,

the 5,6-epoxide and the 15,15'-epoxide may be oxidized to some of the same fraction A products. The experiment depicted in Figure 9 approximates the conditions under which epoxide oxidation might occur during incubations with β -carotene. Comparison of the data in Figures 8 and 9 suggests that epoxide oxidation may contribute significantly to fraction A accumulation. However, epoxide disappearance may be faster in Figure 9 than in experiments where β -carotene was the substrate (e.g., Fig. 8) because 5-fold higher concentrations of epoxides were employed and because β -carotene was not available to suppress epoxide oxidation. Certainly, more work will be necessary to estimate the relative contributions of these two putative pathways to forming fraction A products.

Recently, Samokyszyn and Marnett (Samokyszyn and Marnett, 1990) suggested that the carbon-centered radical product formed during peroxy radical scavenging by 13-*cis*-retinoic acid, a product analogous to the radical product of equation 1 above, can react with a second peroxy radical to form a *bis*-peroxy adduct. A similar reaction scheme may describe our system, where polar products (fraction A) appear to arise from epoxide-dependent and -independent pathways (Fig. 10). In this scheme, β -carotene reacts sequentially with two peroxy radicals to yield a *bis*-peroxy adduct that breaks down to yield polar products without the intermediacy of an epoxide. Alternatively, β -carotene may react with a single peroxy radical to yield an epoxide (see above), which may be oxidized further to polar products. The peroxy radical

adduct $\text{ROO-}\beta\text{-carotene}\cdot$ could also add oxygen to form a reactive peroxy radical (eq. 5).



This peroxy radical could form polar products either by intramolecular or intermolecular reactions. Reaction 5 would be accelerated at high oxygen concentrations, which diminish the antioxidant effects of β -carotene (Burton and Ingold, 1984). However, since β -carotene exerts significant antioxidant actions at the oxygen pressures used in this study, reaction 5 probably is not the principal fate of $\text{ROO-}\beta\text{-carotene}\cdot$.

Epoxides may be useful marker products of β -carotene oxidation by peroxy radicals. The epoxides are nonradical products formed by peroxy radical oxidation and are structurally different from the retinoid metabolites of β -carotene. We are currently investigating the significance of epoxide formation in membrane systems.

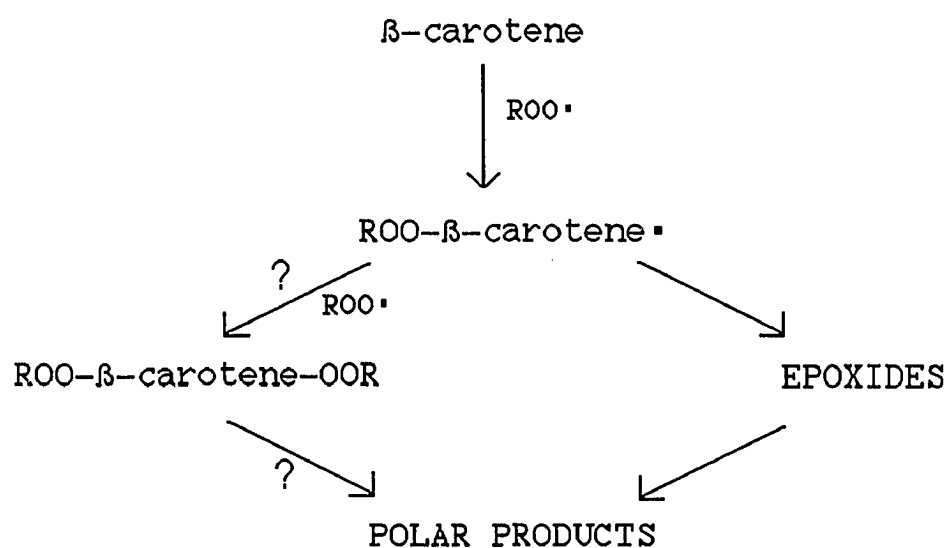


Figure 10. Proposed mechanism for peroxy radical oxidation of β -carotene to polar products (see text for discussion).

CHAPTER 3
PEROXYL RADICAL SCAVENGING BY β -CAROTENE IN LIPID
BILAYERS: EFFECT OF OXYGEN PARTIAL PRESSURE

INTRODUCTION

β -Carotene and other carotenoids are attractive agents for cancer chemoprevention. These pigments are essentially non-toxic (Heywood et al., 1985; Bendich, 1988; Mathews-Roth, 1988) and occur naturally in most fruits and vegetables (Ziegler, 1991). Most importantly, several lines of investigation provide evidence for the anticarcinogenic activity of β -carotene. Epidemiologic studies suggest that human cancer risk, particularly lung cancer risk, is inversely correlated with dietary intake (Peto et al., 1981; Ziegler, 1991) and blood levels (Ziegler et al., 1991) of β -carotene. *In vivo*, β -carotene protects against chemical carcinogenesis in rodent models (Mathews-Roth, 1982; Temple and Basu, 1987; Alam and Alam, 1987; Shklar and Schwartz, 1988) and causes regression of oral pre-cancerous lesions in humans who chew betel quid (Stich et al., 1988). *In vitro*, β -carotene inhibits physically- (Pung et al., 1988) or chemically-induced (Pung et al., 1988; Som et al., 1984) neoplastic transformation of cultured cells.

β -Carotene is thought to protect against carcinogenesis by scavenging radicals involved in tumor formation (Ames, 1983). Indeed, in several systems β -carotene inhibits radical-mediated damage (reviewed in Krinsky, 1989). β -Carotene suppresses CCl_4 -induced lipid

peroxidation *in vivo* (Kunert and Tappel, 1983). The pigment improves viability in yeast under oxidative stress induced by either hyperoxia or duroquinone, a redox cycling quinone (Moore et al., 1989). In liposomes, β -carotene inhibits lipid peroxidation initiated by singlet oxygen and by iron salts (Krinsky and Deneke, 1982). β -Carotene inhibits peroxy radical-initiated autoxidation of both tetralin and methyl linoleate in solution (Burton and Ingold, 1984). These investigators (Burton and Ingold, 1984) also reported that in solution β -carotene was a more effective antioxidant when pO_2 was 15 torr than when pO_2 was 150 torr. This effect could have important implications *in vivo*, because pO_2 variations within tissues could affect the extent to which β -carotene affords protection against radical injury. Others have observed a modest increase in β -carotene antioxidant action in liposomes at 15 torr O_2 (Stocker et al., 1987) and at about 4 torr O_2 in microsomes (Vile and Winterbourne, 1988).

Recently, we showed that in solution β -carotene reacts with peroxy radicals to form 5,6-epoxy- β,β -carotene and 15,15'-epoxy- β,β -carotene, as well as several unidentified polar products (Kennedy and Liebler, 1991). The purpose of this study was to compare the effect of pO_2 on β -carotene antioxidant activity and on β -carotene oxidation in a biomimetic system, and to identify peroxy radical oxidation products of β -carotene in lipid bilayers. Because β -carotene is associated with membrane fractions *in vivo*, we chose phospholipid liposomes to model the lipid bilayer environment. β -Carotene more effectively inhibited AMVN-initiated lipid peroxidation at 160 torr or 15 torr O_2 than at 760 torr

O₂. The rates of β -carotene depletion and β -carotene oxidation product formation in liposomes also were affected by pO₂. Although increased pO₂ may attenuate β -carotene antioxidant efficiency, β -carotene exerted significant antioxidant effects throughout the physiologic range of pO₂.

EXPERIMENTAL PROCEDURES

Chemicals. Soybean phosphatidylcholine (SPC) (type III-S) was purchased from Sigma; 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) from Polysciences (Warrington, PA); β -carotene from Fluka (Ronkonkoma, NY). [10,10',11,11'-¹⁴C]- β -carotene (53.7 mCi/mmol) was a generous gift from Drs. Lawrence J. Machlin and Peter F. Sorter, Hoffman-LaRoche Co. and was purified by HPLC immediately prior to use (Kennedy and Liebler, 1991). All other chemicals were of the highest grade available. All procedures with β -carotene and its oxidation products were performed under reduced light.

Liposome Preparation and Incubation Conditions. Multilamellar liposomes were prepared by modification of published methods (Deamer and Ulster, 1983). A SPC stock solution in CHCl₃, an AMVN stock solution in hexane and a freshly prepared CHCl₃ solution of β -carotene were added to a silanized glass chamber and evaporated under N₂. In some experiments, about 45 nCi [¹⁴C]- β -carotene was added prior to solvent evaporation. The resulting thin, dry lipid film was resuspended in 10 ml air-saturated, Chelex-treated 50 mM Tris HCl, 100

mM NaCl, pH 7.0, by brief bath sonication to a final SPC concentration of 0.5 mM. The AMVN concentration was 20 mol % based on phospholipid. It should be noted that liposomes containing more β -carotene than 0.5 mol % are difficult to prepare because β -carotene tends to crystallize out in the aqueous buffer during sonication. For some experiments, the buffer was sparged at 37°C with either 100% O₂ or a N₂/O₂ mixture where pO₂ = 15 torr prior to liposome preparation. Liposome suspensions were incubated in the dark at 37°C with stirring in a YSI Standard Bath Assembly (Yellow Springs Instruments, Yellow Springs, OH). Incubations were performed under air (pO₂ = 160 torr), or a constant humidified flow of either 100% O₂, or a N₂/O₂ mixture where pO₂ = 15 torr. The pO₂ of the incubations was measured with a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, OH) equipped with Clark type electrodes prepared with high sensitivity membranes.

Identification of β -carotene Oxidation Products. To identify β -carotene oxidation products, a 100 ml-liposome suspension was incubated under air at 37°C for 4 h, then extracted three times with 150 ml hexane:isopropanol (3:2, v/v). The combined hexane extracts were evaporated *in vacuo*, then dissolved in HPLC mobile phase and analyzed by reverse phase DAD-HPLC on a 4.6 x 250 mm, 10 micron, Whatman Partisil ODS-2 analytical column eluted with methanol:hexane (85:15, v/v) at 1.5 ml min⁻¹ (Kennedy and Liebler, 1991). The 5,6-epoxide was further purified with a Spherisorb CN, 5 micron, 4.6 x 250 mm analytical column eluted with hexane:ethyl acetate (99.96:0.04, v/v) at 1.5 ml min⁻¹. The

purified sample was collected and analyzed by mass spectrometry in the College of Pharmacy Mass Spectrometry Center. The sample was introduced by direct probe insertion and ionized with a 70 eV electron beam.

Assays. β -Carotene and its oxidation products were assayed radiochemically as described (Kennedy and Liebler, 1991). In all product formation experiments, the total β -carotene concentration was 0.5 mol %. Liposomes were incubated as described above and aliquots (1 ml) of liposomes were extracted twice with 1.5 ml hexane:isopropanol (3:2, v/v) and the extracts were evaporated under N_2 . The residue was dissolved in HPLC mobile phase and analyzed by reverse phase HPLC as described above. Radiolabelled β -carotene and its products were detected by collecting 0.4 min fractions, which were assayed for [^{14}C] by liquid scintillation counting. All-*trans*- β -carotene and *cis* isomers of β -carotene were quantified together as total β -carotene since *cis*- β -carotene is not an oxidation product, although isomerization may be radical-catalyzed. Conjugated dienes were measured by modification of published methods (Pryor and Castle, 1984). Aliquots (0.35 ml) of liposome incubations were extracted twice with 1.0 ml chloroform:methanol (2:1, v/v) and 1 ml of the chloroform (lower) layer was evaporated under N_2 . The residue was dissolved in 1 ml hexane and the absorbance at 230-236 nm was measured.

RESULTS

Effect of pO_2 on AMVN-initiated Lipid Peroxidation

At 37°C, the lipid soluble azo compound AMVN thermolyzes to yield two carbon-centered radicals that react immediately with oxygen to form the corresponding peroxy radicals. The prooxidant effects of AMVN-derived peroxy radicals on SPC liposomes were assessed by measuring conjugated diene formation. At a concentration of 20 mol %, AMVN initiated a nearly linear increase in conjugated diene formation from liposomes incubated under air (Fig. 11). The time course of conjugated diene formation was linear for 6 h under all three pO_2 conditions (data not shown), however, the net increase in conjugated diene absorbance increased with increasing pO_2 (Table 3). Increasing the pO_2 from 160 torr (air) to 760 torr (100 % O_2) increased conjugated diene formation by 33%. Decreasing the pO_2 from 160 torr (air) to 15 torr (low pO_2) decreased diene production by 35 %.

Effect of pO_2 on Inhibition of Lipid Peroxidation by β -carotene

Incorporation of 0.38 mol % β -carotene into SPC liposomes partially suppressed AMVN-dependent lipid peroxidation under air (Fig. 11). Net conjugated diene formation at 6 h was inhibited 69 % vs. a parallel incubation without β -carotene. Using net conjugated diene formation at 6 hr as an index of lipid peroxidation, we examined the effect of β -carotene concentration on AMVN-initiated lipid peroxidation

in SPC liposomes. Liposomes with and without β -carotene were incubated for 6 hr at 37°C under 160 torr, 760 torr, or 15 torr O_2 . Incubations without β -carotene served as controls to correct for the dependence of lipid peroxidation rate on pO_2 . Under air or low pO_2 , β -carotene inhibited conjugated diene formation in a concentration-dependent manner at concentrations less than 0.3 mol % (Fig. 12). At β -carotene concentrations greater than 0.3 mol %, inhibition remained constant at approximately 70 %. In contrast, at 760 torr O_2 , β -carotene inhibition of lipid peroxidation apparently was not concentration-dependent (Fig. 12). Further, under these conditions, the maximum inhibition achieved was approximately 40 % vs. control and the degree of inhibition was far less consistent than under 160 torr or 15 torr O_2 .

Table 3. Effect of pO_2 on conjugated diene formation from AMVN-challenged SPC liposomes. SPC liposomes with 20 mol % AMVN were incubated at 37°C for 6 h under the indicated atmosphere. Conjugated dienes were measured as described in **Experimental Procedures**.

	760 torr O_2	160 torr O_2	15 torr O_2
Increase in A230-236	0.445 ± 0.103^a	0.335 ± 0.068^b	0.220 ± 0.054^c

^amean \pm SD, n=22

^bmean \pm SD, n=28

^cmean \pm SD, n=18

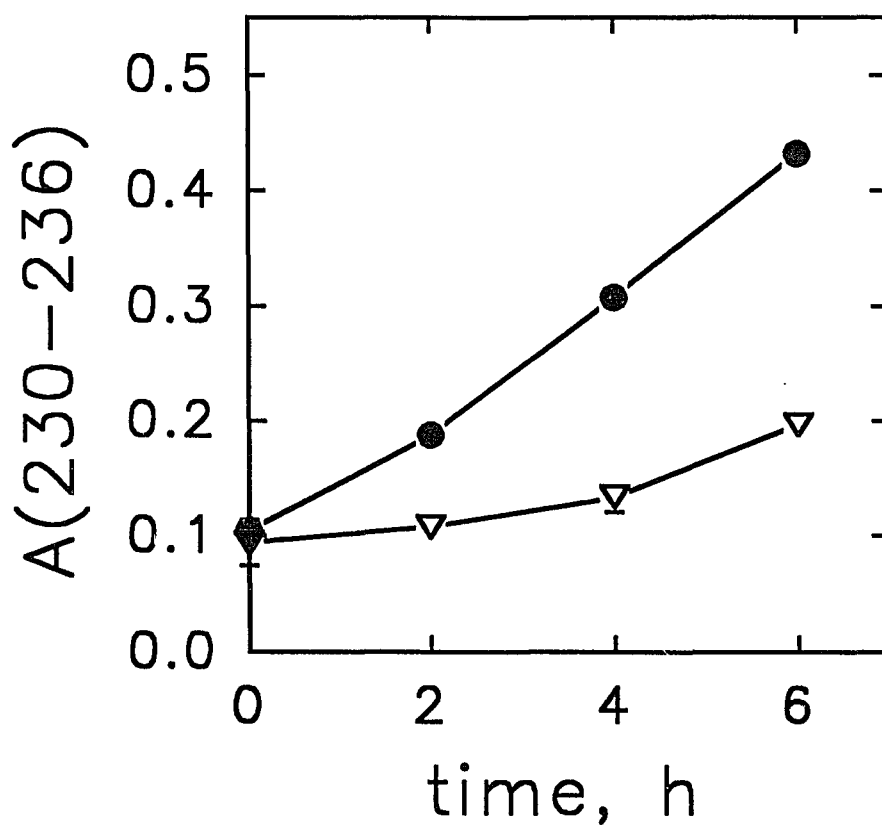


Figure 11. Effect of β -carotene on conjugated diene formation in SPC liposomes challenged with AMVN-derived peroxy radicals. Liposomes contained 20 mol % AMVN and (•) no β -carotene, or (▽) 0.38 mol % β -carotene. Results are expressed as the mean \pm SD ($n=3$).

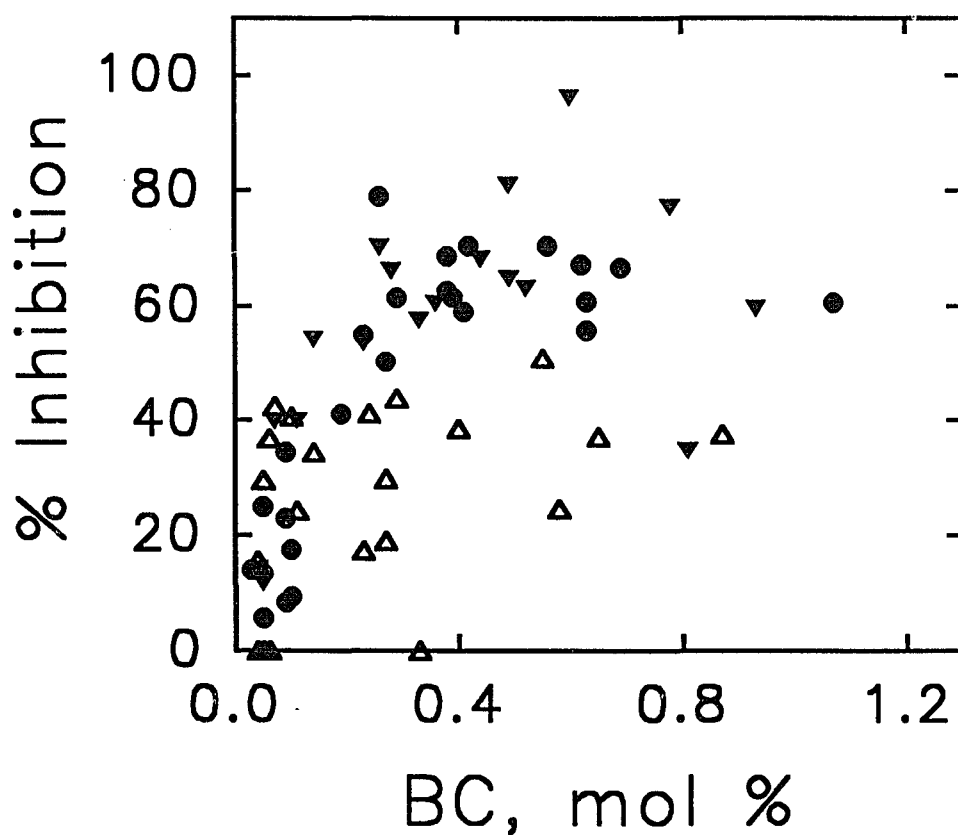


Figure 12. Effect of pO_2 on inhibition of conjugated diene formation by B-carotene in SPC liposomes challenged with 20 mol % AMVN. Liposomes were incubated at (●) 160 torr, (△) 760 torr, or (▼) 15 torr O_2 as described in Experimental Procedures. Each point represents one 6-h experiment.

Effect of pO_2 on AMVN-independent β -Carotene Depletion

To determine the background rate of β -carotene depletion in phospholipid bilayers, β -carotene-loaded SPC liposomes were prepared without AMVN and incubated at 37°C. At 6 and 12 h, aliquots were taken and analyzed as described above. The rate of β -carotene depletion from liposomes without AMVN increased as pO_2 increased (Table 4). After 6 h, at 160 torr O_2 the extent of β -carotene depletion was only 64 % of that at 760 torr O_2 . Likewise, at 15 torr O_2 the extent of β -carotene depletion was 78 % of that at 160 torr O_2 . At 12 h, β -carotene was further depleted and the extent of depletion was similarly pO_2 -dependent (data not shown). HPLC chromatograms of the products of from these incubations appeared identical with those observed for AMVN-dependent β -carotene depletion (see below), except that smaller amounts of products were present (data not shown). However, the apparent product yield did increase with increasing pO_2 .

Table 4. Effect of pO_2 on AMVN-independent β -carotene depletion in SPC liposomes. SPC liposomes without AMVN were incubated at 37°C for 6 h under the indicated atmosphere and β -carotene was quantitated as described in **Experimental Procedures**. Results are expressed as the mean \pm SD (n=3).

	760 torr O_2	160 torr O_2	15 torr O_2
β -carotene, % total radiolabel	42 \pm 7	63 \pm 6	71 \pm 4

β -Carotene Oxidation Products Formed in SPC Liposomes

To determine the β -carotene oxidation products formed during β -carotene antioxidant protection of lipid bilayers, SPC liposomes containing 0.5 mol % β -carotene and 20 mol % AMVN were incubated under air at 37°C for 4 h. β -Carotene and products were then extracted and analyzed by reverse phase DAD-HPLC. Two fractions of products were separated. Fraction A was poorly retained on the reverse phase column and contained several peaks that absorbed strongly between 200 and 350 nm. Previous studies of the corresponding polar product fraction from AMVN-initiated β -carotene oxidation in hexane suggest that this fraction contains a mixture of volatile, low molecular weight aldehydes and ketones (Kennedy and Liebler, 1991). This product fraction was not further characterized. Fraction B eluted as a single peak at 6 min on the reverse phase column and absorbed strongly at 450 nm. DAD-HPLC analysis of this peak indicated a UV spectrum with a maximum at 444 nm (in mobile phase) consistent with published spectra of 5,6-epoxy- β,β -carotene (Kennedy and Liebler, 1991). This product was purified further for mass spectral analysis by cyanopropyl column chromatography (Kennedy and Liebler, 1991). This fraction was actually subjected to cyanopropyl column HPLC several times as chromatography conditions were optimized. This repeated chromatography resulted in the rearrangement of the 5,6-epoxide to the corresponding furanoid derivative, 5,8-epoxy- β,β -carotene, which was identified by UV spectroscopy and mass spectrometry. The UV and mass spectra obtained virtually matched those previously published (Tsukida and Zechmeister, 1958; Budzikiewicz et al., 1970).

The 5,8-epoxide displayed UV maxima (in hexane) at 404, 426, and 452 nm and mass spectral signals at m/z 552 (M^+ , 100 %), 550 (76), 472 (43), 406 (14), 336 (27), 205 (49).

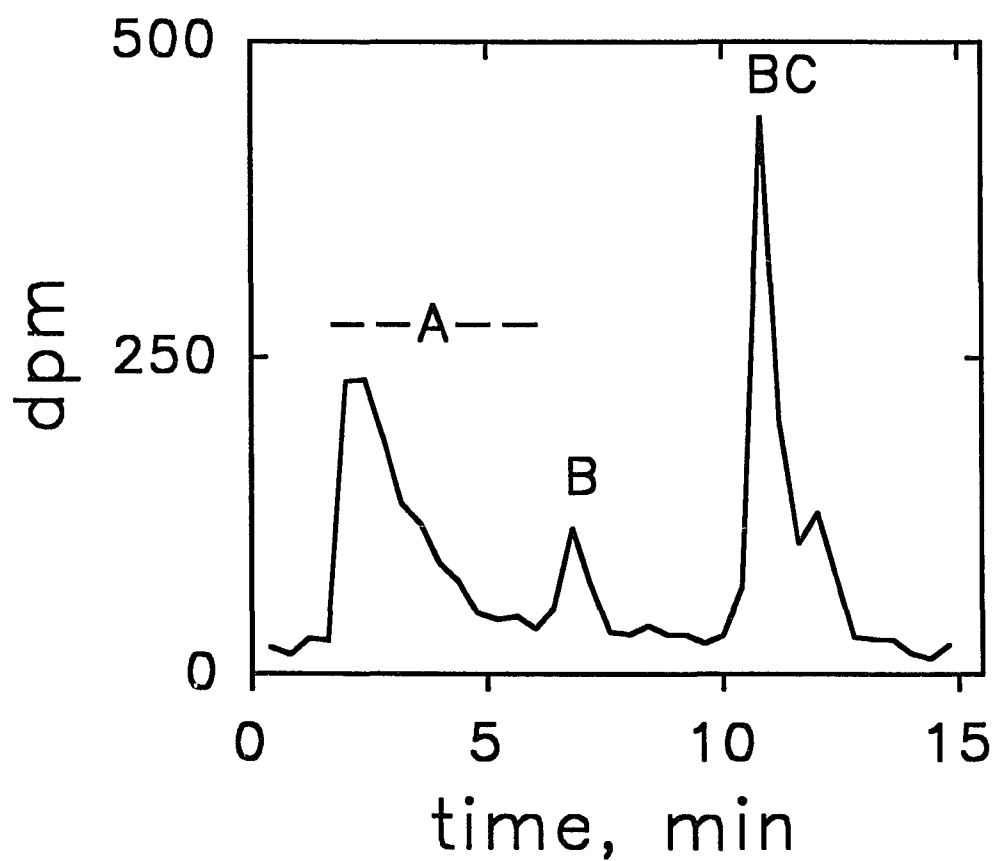


Figure 13. HPLC analysis of products formed from [^{14}C]- β -carotene and AMVN-derived peroxy radicals in SPC liposomes. Liposomes were incubated as described in **Experimental Procedures**. HPLC of the liposome extracts separated the radiolabelled products into (A) polar products, (B) 5,6-epoxy- β , β -carotene, and (BC) β -carotene, the all-*trans* isomer followed by a mixture of *cis* isomers.

Effect of pO_2 on β -Carotene Oxidation Product Formation in SPC Liposomes

To further examine how pO_2 affects β -carotene antioxidant reactions, we investigated the effect of pO_2 on the formation of β -carotene oxidation products. Liposomes with 0.5 mol % [^{14}C]- β -carotene were subjected to AMVN-initiated peroxidation at 160 torr, 760 torr, or 15 torr O_2 . Aliquots of the liposome suspensions were then extracted and analyzed by reverse phase HPLC. Peroxyl radical oxidation of β -carotene in liposomes yielded both 5,6-epoxide and fraction A products (Fig. 13) under all pO_2 conditions. Although [^{14}C]- β -carotene was purified immediately prior to use, a small percentage of β -carotene (about 15 %) was oxidized during liposome preparation. The rates of β -carotene consumption and product formation all increased with pO_2 (Figs. 14-16). The rates of β -carotene depletion and β -carotene oxidation product formation were nearly identical at 160 torr and 760 torr O_2 . The rate of β -carotene consumption was pO_2 -dependent (Fig. 14). β -carotene levels were depleted to less than 20 % of total radiolabel in 6 h under 160 torr and 760 torr O_2 . Under low pO_2 conditions β -carotene was oxidized less rapidly, to just 47 % of total radiolabel in 6 h. Under all pO_2 conditions, the amount of radiolabel associated with fraction A increased rapidly throughout the incubation. However, at 160 torr and 760 torr O_2 , the rate of fraction A formation was approximately twice that 15 torr O_2 (Fig. 15). Similarly, the rate of epoxide formation and disappearance was similar at 160 torr and 760 torr O_2 (Fig. 16). Under these conditions the amount of epoxide reached a

maximum at 4 h and then declined. In contrast, at 15 torr O₂ epoxide levels increased more slowly throughout the incubation. Thus, at 160 torr and 760 torr O₂ rates of β -carotene depletion and product formation were virtually identical.

Preliminary experiments demonstrated that our extraction method yielded greater than 95 % recovery of [¹⁴C]- β -carotene from SPC liposomes. However, in all experiments we observed a consistent decrease over time in the amount of radiolabel recovered during β -carotene oxidation. Further, the recovery of radiolabel also decreased with increasing pO₂. The minimum radiolabel recovery was 53 % at 6 h under 760 torr O₂. Additional experiments indicated that the unrecovered radiolabel remained in the aqueous phase of the extractions (data not shown). These findings suggest that the unrecovered radiolabel was present as oxidation products that were too polar to be efficiently extracted into hexane:isopropanol (3:2, v/v). If the unrecovered radiolabel would elute with HPLC fraction A, we may have underestimated the size of the polar product fraction at 6 h by about 15 % at 760 torr O₂ and by 15 % and 40 % at 160 torr and 15 torr O₂, respectively. However, the data in Figs. 14-16 represent the distribution of extractable radiolabel only. Measurements taken at later time points are most affected by this finding.

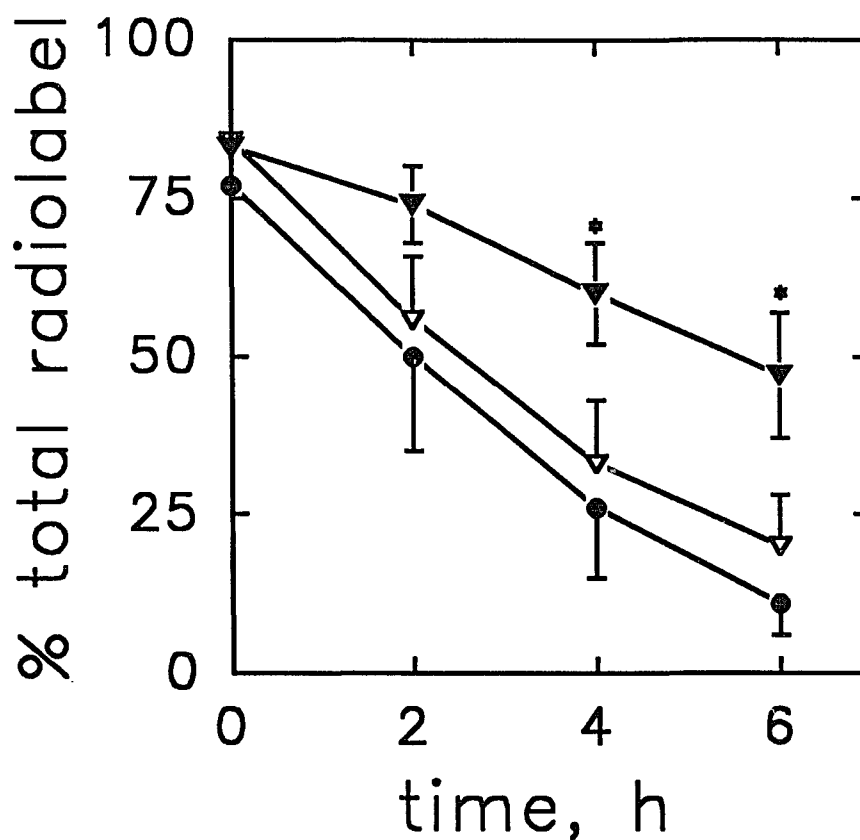


Figure 14. Effect of pO_2 on the rate of $[^{14}C]$ - β -carotene depletion from AMVN-challenged SPC liposomes. Liposomes were incubated at (●) 760 torr, 160 torr (▽), or 15 torr O_2 (▼) as described in **Experimental Procedures**. Results are expressed as the mean \pm SD ($n=3$), except at time zero, where the results are the mean of two determinations. Asterisks indicate significant difference ($p<0.05$) between points at 15 torr O_2 vs. 160 torr or 760 torr O_2 .

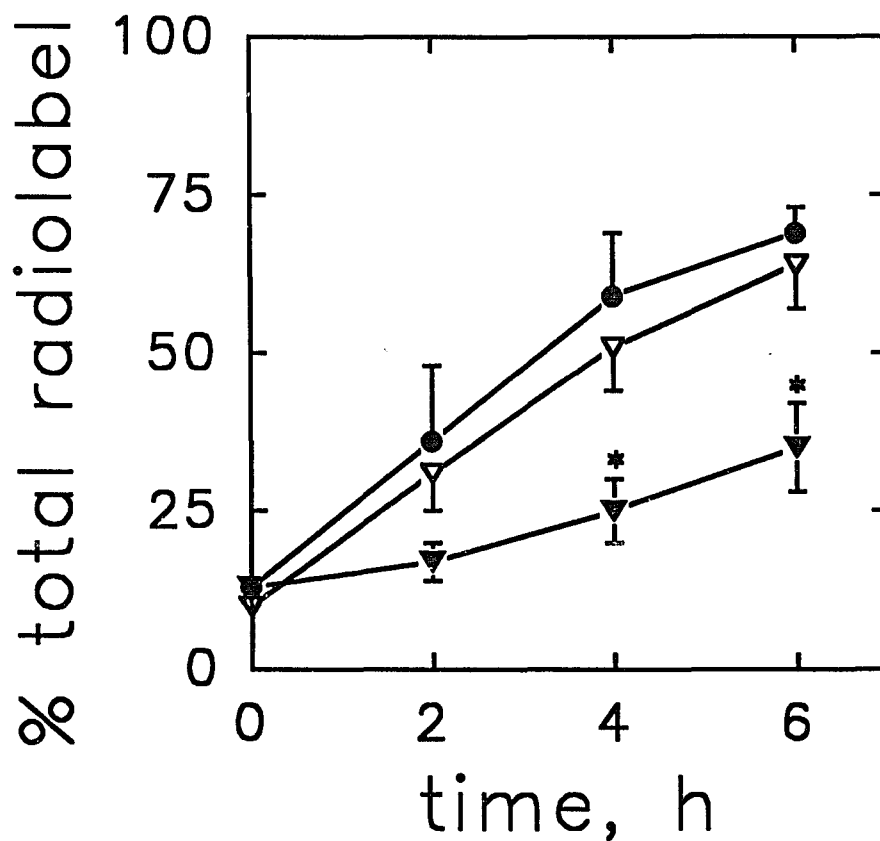


Figure 15. Effect of pO_2 on the rate of polar product formation from $[^{14}C]$ - β -carotene in AMVN-challenged SPC liposomes. Liposomes were incubated at (\bullet) 760 torr, 160 torr (∇), or 15 torr O_2 (\blacktriangledown) as described in **Experimental Procedures**. Results are expressed as the mean \pm SD ($n=3$), except at time zero, where the results are the mean of two determinations. Asterisks indicate significant difference ($p<0.05$) between points at 15 torr O_2 vs. 160 torr or 760 torr O_2 .

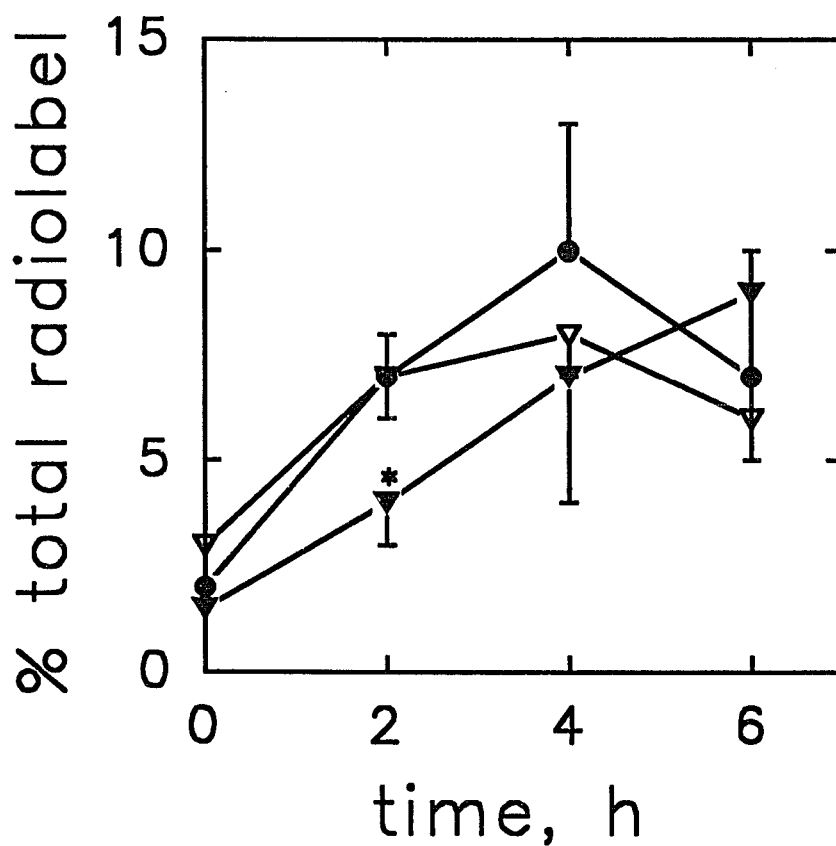


Figure 16. Effect of pO_2 on the rate of 5,6-epoxy- β,β -carotene formation from $[^{14}C]$ - β -carotene in AMVN-challenged SPC liposomes. Liposomes were incubated at (●) 760 torr, 160 torr (∇), or 15 torr O_2 (▼) as described in **Experimental Procedures**. Results are expressed as the mean \pm SD ($n=3$), except at time zero, where the results are the mean of two determinations. Asterisk indicates significant difference ($p<0.05$) between points at 15 torr O_2 vs. 160 torr or 760 torr O_2 .

DISCUSSION

An ideal antioxidant is consumed only by radical trapping reactions that produce an antioxidant effect. Autoxidation reactions, which consume antioxidant without trapping radicals, produce no antioxidant effect. Antioxidant efficiency thus depends on the balance between radical trapping and autoxidation reactions. Factors that enhance autoxidation of an antioxidant diminish its efficiency. The work of Burton and Ingold suggested that pO_2 may be an important determinant of β -carotene antioxidant efficiency (Burton and Ingold, 1984). We found that pO_2 affects β -carotene oxidation product formation as well as β -carotene antioxidant efficiency. At pO_2 above the physiologic range, β -carotene antioxidant efficiency was diminished and the rate of β -carotene oxidation and oxidation product formation was accelerated (Figs. 14-16). However, the loss of β -carotene antioxidant efficiency at high pO_2 appeared to be due to increased β -carotene oxidation by reaction pathways that do not contribute antioxidant activity. Therefore, pO_2 apparently affects β -carotene antioxidant efficiency by increasing β -carotene autoxidation.

Radical scavenging by β -carotene in this system is most effective in the physiological range of pO_2 . When corrected for the effect of pO_2 alone, β -carotene antioxidant activity was the same at either 160 torr or 15 torr O_2 ; at either pO_2 , β -carotene inhibited lipid peroxidation by up to 70 % (Fig. 12). Vile and Winterbourne (Vile and Winterbourne, 1988) have also shown β -carotene antioxidant activity to be the same at

similar pO_2 . They and others have investigated the effect of pO_2 on β -carotene antioxidant action (Burton and Ingold, 1984, Stocker et al., 1987, Vile and Winterbourne, 1988). These studies, done with different experimental systems and with different lipid peroxidation assays, indicate that improvement in β -carotene antioxidant activity is modest when the data is corrected for the effect of low pO_2 alone. At 760 torr O_2 , β -carotene was a much less effective antioxidant (Fig. 12). Such poor antioxidant performance at high pO_2 has been observed in other experimental systems (Burton and Ingold, 1984, Pryor et al., 1988). This loss in antioxidant efficiency may be explained in part by the effect of increased pO_2 on the rate of uninhibited lipid peroxidation. At 760 torr O_2 , the rate of lipid peroxidation in liposomes without β -carotene was increased by 33 % vs. that at 160 torr O_2 (Table 3). The increased radical flux at 760 torr O_2 could conceivably accelerate β -carotene consumption and overwhelm its antioxidant effect. Were this true, the rate of β -carotene depletion at 760 torr O_2 would be much greater than at 160 torr O_2 . However, β -carotene was consumed at nearly identical rates at 160 torr O_2 and 760 torr O_2 (Fig. 14).

A comparison of β -carotene antioxidant action with β -carotene depletion kinetics at each pO_2 suggests an alternative explanation for the loss of β -carotene antioxidant effectiveness at high pO_2 (Figs. 12 and 14). Our results suggest that β -carotene may be consumed by two radical trapping reactions and by an autooxidative pathway (Fig. 17). Previous work (Kennedy and Liebler, 1991) suggested that β -carotene exerts antioxidant effects by at least two pathways, A and B. The first

step in both pathways is peroxy radical addition to the polyene chain. In pathway A, the peroxy adduct collapses to form an epoxide and an alkoxy radical. This pathway may produce a kinetically significant antioxidant effect if the formation of the peroxy radical adduct is much faster than release of the alkoxy radical. Pathway A may not provide a dominant contribution to β -carotene antioxidant action, however, since epoxides account for less than 20 % of the products formed. In pathway B, a second peroxy radical adds to the β -carotene peroxy adduct to form a bis-peroxy adduct. Both of these pathways trap peroxy radicals at the expense of β -carotene. In pathway C, on the other hand, β -carotene is depleted by autoxidation. In this pathway, oxygen adds to the β -carotene peroxy adduct to form a reactive peroxy radical. Therefore, a new peroxy radical is formed for every radical initially trapped. Consequently, at high pO_2 , when the rate of oxygen addition is greatest, pathway C greatly diminishes β -carotene antioxidant efficiency. The decrease in β -carotene antioxidant efficiency observed at 760 torr O_2 may reflect a shift in β -carotene consumption to pathway C from pathways A and B, even though the overall rate of β -carotene consumption may not be changed.

Although AMVN greatly increased overall oxidation rates, β -carotene also was depleted in liposomes without AMVN (Table 4). β -carotene was significantly depleted in the absence of AMVN, even though conjugated dienes accumulated very slowly. The rate of AMVN-independent β -carotene depletion also increased with pO_2 . The great sensitivity of β -carotene to oxidation suggests that β -carotene oxidation in the

absence of AMVN proceeds largely through an autoxidative pathway, i.e., pathway C. In the absence of AMVN, β -carotene oxidation is probably initiated by traces of lipid hydroperoxide present in the SPC. The rate of AMVN-independent β -carotene oxidation was greater in liposomes prepared with stored SPC than in liposomes prepared with freshly HPLC-purified SPC. Catalysis by trace metal ions, particularly iron, would slowly initiate peroxy radical formation (reviewed in Miller et al., 1990). Our data suggest that a background level of autoxidation contributes to β -carotene depletion in any system and that the autoxidation rate depends on pO_2 , the generation of initiators, and possibly on other factors.

Peroxy radical oxidation of β -carotene in liposomes results in a product profile similar to that in solution (Kennedy and Liebler, 1991). One notable difference is that the 15,15'-epoxy- β , β -carotene was not detected in any liposome incubation, although it was a major epoxide product formed by peroxy radical oxidation of β -carotene in solution (Kennedy and Liebler, 1991). The absence of this product suggests that the relative orientations of β -carotene and the attacking peroxy radical are important in determining the oxidation products formed. The orientation of β -carotene in lipid bilayers appears to vary with the phospholipid composition of the lipid bilayer (van de Ven et al., 1984). Nevertheless, attack at the terminal double bonds of β -carotene seems to be preferred in this model system. Other studies suggest that β -carotene may also aggregate within the lipid bilayer (Yamamoto and Bangham, 1978). Aggregation of β -carotene may explain the observed

plateau in β -carotene antioxidant activity at high β -carotene concentration.

Polar products comprised the major product fraction in liposomes (Fig. 13). Although the polar products formed from β -carotene oxidation in liposomes have not been characterized, a similar product fraction formed by β -carotene oxidation in solution was found to contain volatile, low molecular weight carbonyl compounds (Kennedy and Liebler, 1991). In liposomes, as in solution, polar products were formed from the initial stages of β -carotene oxidation. The rapid rate of polar product formation suggests that polar products may arise directly from β -carotene (pathway B) as well as from epoxide oxidation (pathway A). Polar products were also formed during AMVN-independent β -carotene depletion in liposomes (see Results). Although some of these products may be derived from epoxide oxidation (see above), most are probably products of pathways B and C. It is reasonable to postulate that at least some of these polar products are unique to a given pathway. Therefore, further characterization of the polar products may support this hypothesis.

The antioxidant effectiveness of β -carotene in living tissues was postulated by Burton and Ingold to depend on tissue pO_2 (Burton and Ingold, 1984). Their studies in homogenous solutions indicated that β -carotene antioxidant efficiency diminished with increasing pO_2 and that β -carotene actually displayed prooxidant properties at high pO_2 (760 torr). Although β -carotene antioxidant efficiency in our liposome model also declined at high pO_2 (760 torr), our data indicated no difference

between β -carotene antioxidant effectiveness at 160 torr O_2 and 15 torr O_2 . We therefore conclude that β -carotene antioxidant actions are not diminished at higher physiologic pO_2 (e.g., 160 torr) and that β -carotene could be as effective an antioxidant in lung, for example, as in other tissues. The antioxidant effectiveness of β -carotene in the lung is of interest because several epidemiologic studies suggest that dietary β -carotene consumption reduces the risk of lung cancer (reviewed in Ziegler, 1991). Since carotenoids are thought to inhibit carcinogenesis through their antioxidant actions, β -carotene may reasonably be postulated to exert antioxidant effects in the lung, where the pO_2 is close to 160 torr. Although the dependence of β -carotene antioxidant chemistry on pO_2 may not greatly influence its antioxidant effects under physiologic conditions, pO_2 effects may provide valuable insight into β -carotene antioxidant chemistry (see above). Further investigations of these effects are in progress in our laboratory.

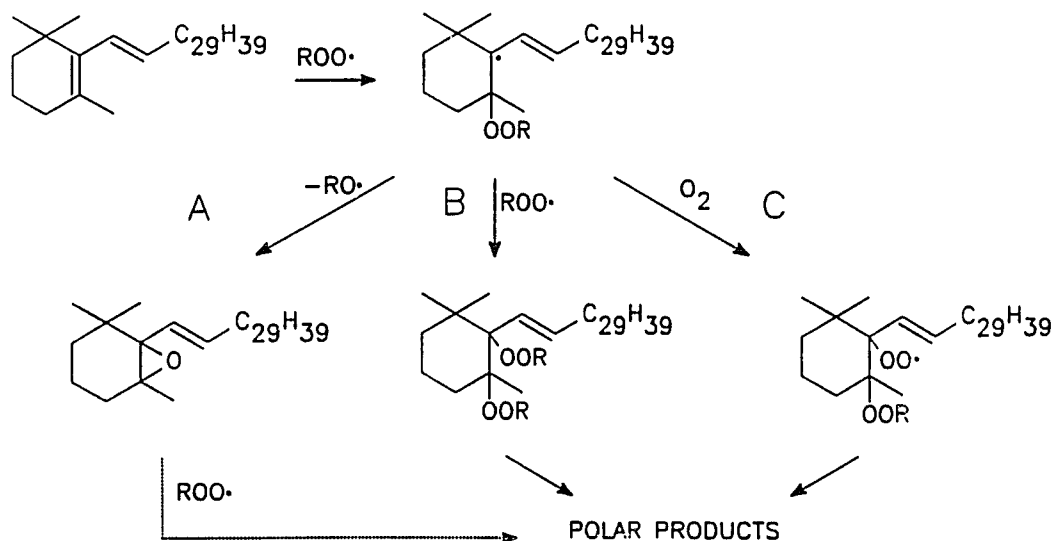


Figure 17. Proposed reaction pathways of β -carotene during peroxy radical-initiated lipid peroxidation in lipid bilayers. See text for discussion.

CHAPTER 4

INCORPORATION OF β -CAROTENE INTO MICROSOMAL MEMBRANES

INTRODUCTION

To identify β -carotene oxidation products associated with β -carotene antioxidant activity, biological membranes must be supplemented in a manner such that significant antioxidant activity is achieved with physiologically relevant concentrations of β -carotene. *In vivo* dietary supplementation requires 3-4 weeks (Shapiro et al., 1984) and is relatively inexact. *In vitro* addition of β -carotene allows a more convenient and accurate loading of biological membranes. Vile and Winterbourne examined the ability of β -carotene to protect microsomes from iron-dependent lipid peroxidation enhanced by adriamycin (Vile and Winterbourne, 1988). β -Carotene was added to microsomal membranes by homogenizing the microsomal pellet with the addition of β -carotene in chloroform. Although β -carotene inhibited lipid peroxidation under air by as much as 50 % in this system, concentrations of approximately 7.5 mol % were required. This concentration is about 40-fold greater than the concentration of β -carotene required to inhibit lipid peroxidation in liposomes challenged with AMVN-derived peroxy radicals. These differences may reflect differences in the radical challenge and/or the lipid peroxidation assay used. Also, the structure and PUFA content of the membranes may cause some differences in results. Alternatively, this procedure may not have incorporated β -carotene into microsomal

membranes in a way that allows it to effectively scavenge radicals.

The objective of these studies was to incorporate β -carotene into microsomal membranes in a way such that reasonable β -carotene concentrations (1.0 mol % or less) provide significant antioxidant protection (> 25 % inhibition). We chose tetrahydrofuran (THF) as a solvent to solubilize β -carotene in aqueous buffer because THF has proved to be an effective vehicle to supplement cultured cells with β -carotene (Bertram et al., 1991). Although a variety of physical and chemical techniques were attempted, we were unable to meet our criteria for incorporation. This may be due to the difficulty in incorporating β -carotene into microsomal membranes or to the inability of β -carotene to effectively inhibit microsomal lipid peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals. β -Carotene was purchased from Fluka (Ronkonkoma, NY) and stored at -20°C under Ar. Reagent grade THF was distilled over sodium metal to remove antioxidants. To remove residual peroxides, THF was passed over activated alumina immediately prior to use. Sodium cholate was acquired from Sigma (St. Louis, MO). 2,2'-Azo-bis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Polysciences, Inc. (Warrington, PA). All other chemicals were of the highest grade available. All procedures with β -carotene were performed under reduced light.

Microsome Preparation. Rat liver microsomes were prepared from male Sprague-Dawley rats as previously described (Guengerich, 1982), except that the antioxidant BHT was omitted from all buffers. Briefly, rats were killed by asphyxiation with CO₂ and their livers were excised and rinsed in cold 1.15 % KCl. The livers were minced with scissors, then placed in 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1 M potassium pyrophosphate and 1 mM EDTA, and homogenized. The homogenate was centrifuged at 10,000 x g for 20 min and the pellet discarded. The supernatant was centrifuged at 100,000 x g for 60 min. The resulting supernatant was decanted and frozen, and the microsomal pellet was resuspended in 0.1 M potassium pyrophosphate, pH 7.4, containing 1 mM EDTA. The suspended microsomes were recentrifuged at 100,000 x g for 60 min. The resulting microsomal pellet was homogenized in 10 mM Tris-acetate, pH 7.4, containing 1 mM EDTA and 20 % (w/v) glycerol and stored at -80°C. Microsomal protein concentration was determined with the Pierce BCA assay (Pierce, Rockford, IL). Microsomal lipid content was measured as phospholipid phosphorus after extraction of microsomal lipids with hexane:isopropanol (3:2, v/v).

Incubation of Microsomes. Rat liver microsomes were diluted to 0.5 mg/ml or 1 mg/ml protein with Chelex-treated 50 mM Tris HCl, 100 mM NaCl, pH 7.0 and incubated under air at 37°C. Lipid peroxidation was initiated by the addition of 100 mM AAPH, freshly prepared in the incubation buffer. The final AAPH concentration was 10 mM. Incubations

were carried out in pairs with and without β -carotene. Incubations without β -carotene were manipulated in an identical manner to those with β -carotene. Microsomal lipid peroxidation was determined by the thiobarbituric assay (Buege and Aust, 1978). Briefly, 200 μ l samples of microsomes were added to 0.4 ml of 10 % (w/v) trichloroacetic acid and 50 μ l of 2 % (w/v) BHT. After mixing, 0.75 ml of 0.7 % (w/v) thiobarbituric acid was added and the samples heated at 90°C in a water bath for 20 min. After cooling, the samples were centrifuged for 3 min. The absorbance of the supernatants was read at 535 nm vs. a reference wavelength of 800 nm. All samples were performed in triplicate and the results are reported as the mean \pm SD. Conjugated dienes were measured as described (Pryor and Castle, 1984; see Chapter 3).

RESULTS AND DISCUSSION

THF-Injection of β -Carotene into Microsomes

To be an effective antioxidant, β -carotene presumably must be incorporated within the lipid bilayer where the radical reactions of lipid peroxidation take place. Since microsomes are a heterogenous mixture of membranous vesicles suspended in an aqueous buffer, β -carotene must be solubilized in the aqueous phase to be delivered to the microsomal membrane. The ability of THF to serve as a vehicle to load

cells in culture with β -carotene (Bertram et al., 1991) suggested that THF may be a useful solvent for loading β -carotene into microsomes. β -Carotene (250 nmol) was dissolved in 200 μ l of THF and injected with a syringe into a rapidly stirring suspension of microsomes (0.5 mg/ml protein). The final THF concentration was 2 % (v/v). Control suspensions were injected with the same volume of THF. The final β -carotene concentration was 7.35 mol % based on phospholipid. Preliminary experiments determined that 1.0 mol % β -carotene loaded in this manner did not affect lipid peroxidation in this system (Table 5). The β -carotene concentration was increased to the levels that Vile and Winterbourne had used previously to determine if this method of loading would be effective (Vile and Winterbourne, 1988). This procedure produced no visible evidence of β -carotene crystallization. Microsomal lipid peroxidation was initiated by the addition of AAPH. At 37°C, AAPH thermolyzes in the aqueous phase to form carbon-centered radicals, which subsequently react with oxygen to form peroxy radicals. Lipid peroxidation was measured as formation of thiobarbituric acid reactive substances (TBARS). AAPH-derived peroxy radicals increased TBARS levels in a nearly linear manner (Fig. 18). β -Carotene, even at this extremely high concentration, inhibited MDA formation by only 12 % at 2 hr (Fig. 18). Similar results were observed when conjugated dienes were used as a measure of lipid peroxidation (data not shown).

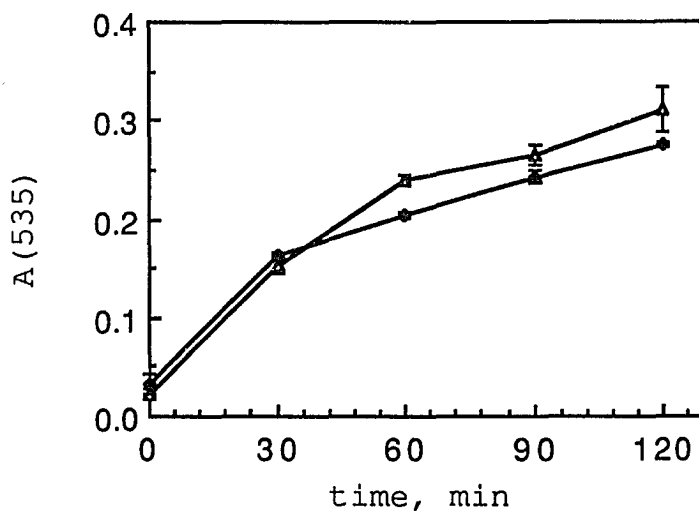


Figure 18. Effect of β -carotene on MDA formation in AAPH-challenged rat liver microsomes. Microsomes were incubated 37°C with 10 mM AAPH and either (Δ) no β -carotene or (\blacklozenge) 7.35 mol % β -carotene. THF-solubilized β -carotene was injected into microsomes as described in **Results**. Each point represents the mean \pm SD (n=3).

Homogenization of Microsomes to Incorporate β -Carotene

Because Vile and Winterbourne had observed inhibition of iron-dependent lipid peroxidation by up to 50 % by β -carotene in microsomes (Vile and Winterbourne, 1988), we examined the effect of β -carotene on iron-dependent lipid peroxidation. THF-solubilized β -carotene was added directly to undiluted microsomes or injected as described above. The mixture then was homogenized mechanically by 10 strokes in a Potter-Elvehjem homogenizer and the resulting homogenate was diluted to 1 mg/ml protein and the final β -carotene concentration was 7.35 mol %. This procedure essentially duplicates that of Vile and Winterbourne, except that THF, rather than chloroform, was used to solubilize β -carotene. Lipid peroxidation was initiated by the rapid addition of 50 mM ascorbate and 50 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. Each reagent was freshly dissolved in double distilled water. The final concentration of iron and ascorbate was 0.1 mM. In these experiments, ascorbate acts as a prooxidant by reducing iron. Although our initiating system, lipid peroxidation assay, and method for incorporating β -carotene into microsomes were similar to that of Vile and Winterbourne, we observed only between 3 and 7 % inhibition of iron-initiated lipid peroxidation at 30 min (Table 5). Such low inhibition by a very high concentration of β -carotene may represent non-specific physical effects on membrane biophysical properties, such as membrane fluidity, which affects the rate of propagation reactions within the lipid bilayer, rather than actual radical quenching. To minimize any non-specific effects, 1.0 mol

% was chosen as the maximum β -carotene concentration to be used for subsequent experiments.

Addition of Detergent to Incorporate β -Carotene

Failure of such high concentrations of β -carotene to significantly inhibit AAPH-initiated lipid peroxidation suggested that β -carotene was not incorporated within the microsomal lipid bilayer. THF appeared to completely solubilize β -carotene in the microsomal suspension as evidenced by the deep orange color of the suspensions and the lack of any visible crystalline β -carotene on any of the glassware. Based on the observations of Bertram and colleagues (discussed in Chapter 1; Bertram et al., 1991), we hypothesized that β -carotene was solubilized in the aqueous buffer by THF-solvent cages, yet was not being incorporated within the lipid bilayer. To overcome this problem, we reasoned that small amounts of detergent, which perturb membrane fluidity, may allow incorporation of THF-solubilized β -carotene within the lipid bilayer.

THF-solubilized β -carotene was injected into microsomal suspensions to a final concentration of 1.0 mol %, then sodium cholate in double distilled water was added to a concentration of 0.3 % (w/v). The same concentration of cholate also was added to control microsomes. Preliminary experiments determined that 0.1 % sodium cholate had no effect on β -carotene incorporation as determined by β -carotene antioxidant activity and that 0.5 % sodium cholate completely solubilized the microsomes. Addition of 0.3 % cholate slightly

decreased the turbidity of the suspensions. The incubation mixture was stirred overnight at 4°C in the dark. Sodium cholate had no effect on AAPH-initiated lipid peroxidation (Fig. 19). β -Carotene added in the presence of 0.3 % cholate produced only 3 % inhibition of peroxidation (Fig. 19). Other experiments determined that addition of β -carotene after cholate addition had no effect.

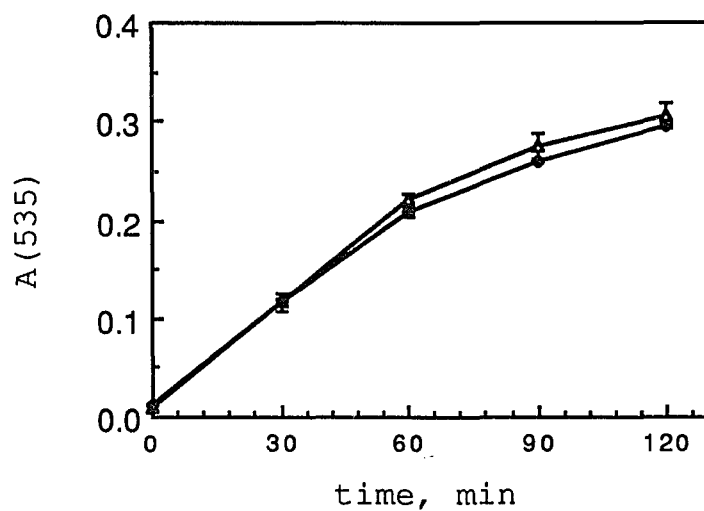


Figure 19. Effect of β -carotene on MDA formation in AAPH-challenged rat liver microsomes. Microsomes were incubated 37°C with 10 mM AAPH and either (Δ) no β -carotene or (\blacklozenge) 1.0 mol % β -carotene. THF-solubilized β -carotene was injected into microsomes as described in **Results**. β -carotene-loaded and control microsomes were incubated with 0.3 % sodium cholate overnight prior to initiation of lipid peroxidation. Each point represents the mean \pm SD (n=3).

These results suggested that a more vigorous technique may be necessary to load β -carotene into microsomes. We postulated that a transient physical disruption of the microsomal membrane may allow incorporation of β -carotene into lipid bilayers. Microsomal suspensions were sonicated for 1 min in a bath sonicator after cholate addition and immediately prior to incubation. This procedure appeared to increase β -carotene incorporation slightly. At 2 h, AAPH-initiated lipid peroxidation was inhibited by 12 % vs. the corresponding control (Fig. 20). The use of a more powerful sonicator, a cup horn sonicator, produced similar results, peroxidation was inhibited by 14 % vs. control (Table 5).

Sonication of microsomes in the presence of detergent is a vigorous treatment, which may alter the "natural" order of the lipid bilayer. Our strategy was to perturb the membrane environment enough to allow incorporation of β -carotene. Sonication of the microsomal suspension in the presence of detergent decreased the solution turbidity, which suggests that the microsomes were altered by the sonication. Yet despite this treatment, β -carotene antioxidant action improved only slightly.

One possible explanation is that β -carotene is not an effective antioxidant in this system. Both AAPH and the iron/ascorbate system generate initiating radicals in the aqueous phase. These radicals then initiate radical propagating reactions at the interface between the aqueous and lipid phases. β -Carotene may be located in the central, most lipophilic, portion of the bilayer. This location may not permit

efficient scavenging of radicals generated near the aqueous phase. AMVN is a lipid soluble analogue of AAPH. It generates radicals within the bilayer where β -carotene can efficiently inhibit their prooxidant effects (Chapter 3).

Alternatively, incorporation of β -carotene into the lipid bilayer may not increase β -carotene antioxidant activity. Although β -carotene is associated with membrane fractions *in vivo*, the actual location of β -carotene within these fractions is uncertain. Because of its extreme lipophilicity, β -carotene is often assumed to reside within the lipid bilayer. However, recent experiments have found a major fraction of β -carotene-derived radioactivity in the liver cytosol of rats administered radiolabelled β -carotene intravenously (Lakshman et al., 1989). Because this route of administration bypasses the major β -carotene metabolizing activity in the gut, the radioactivity found in the liver cytosol should be associated with β -carotene, rather than retinoids. Consistent with this assumption, 82 % of the radiolabel from total liver was recovered as β -carotene. These findings suggest the possible presence of a carotenoid binding protein that could solubilize β -carotene in cytosol. Thus, *in vivo* β -carotene may function as a cytosolic antioxidant solubilized by a binding protein.

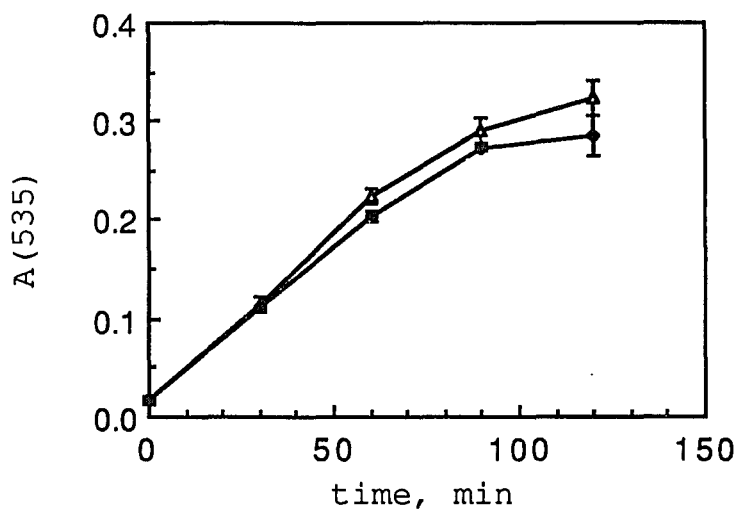


Figure 20. Effect of β -carotene on MDA formation in AAPH-challenged rat liver microsomes. Microsomes were incubated 37°C with 10 mM AAPH and either (Δ) no β -carotene or (\diamond) 1.0 mol % β -carotene. THF-solubilized β -carotene was injected into microsomes as described in **Results**. β -carotene-loaded and control microsomes, each with 0.3 % sodium cholate, were sonicated for 1 min in a bath sonicator immediately prior to initiation of lipid peroxidation. Each point represents the mean \pm SD (n=3).

Table 5. Summary of results of different methods to load β -carotene into microsomal membranes.

Initiating system	Method	[β C] ¹	Assay	%Inhibition ²
AAPH	THF injection	1.0	diene	0
Fe/Asc	THF injection	7.35	MDA	0
Fe/Asc	THF injection/ Homogenizer	7.35	MDA	5 \pm 2 ^a
AAPH	THF injection/ 0.3 % cholate/ cup horn sonicator	1.0	MDA	14

¹ β -carotene concentration in mol %.

²% Inhibition at 2 hr for incubations initiated with AAPH; at 30 min for incubations initiated with Fe/Asc.

^amean \pm SD, n=3.

CHAPTER 5

PERSPECTIVE

Despite several lines of evidence that β -carotene can prevent tumor formation, little is known about the mechanism of β -carotene anticarcinogenic effects (see Chapter 1). The ability of β -carotene to scavenge free radicals has been suggested as an explanation for this protective effect. However, it is not known if β -carotene traps free radicals as it protects against cellular transformation. Identification of products of β -carotene that are formed by radical trapping could provide a measure of β -carotene antioxidant action and therefore allow investigators to directly determine if β -carotene traps radicals during inhibition of transformation. The dissertation research presented in the previous three chapters was designed to investigate the formation of β -carotene oxidation products in model systems.

Although previous work had identified autoxidation products of β -carotene (Hunter and Krakenberger, 1947; El-Tinay and Chichester, 1970), the specific products formed by oxidation of β -carotene by peroxyl radicals had not been reported. In Chapter 2, the oxidation of β -carotene by specific peroxyl radicals in homogenous solution was discussed. The oxidation products formed in this chemically-defined system were the 5,6-epoxy- β,β -carotene, 15,15'-epoxy- β,β -carotene, and several unidentified polar products. The 15,15'-epoxide was identified here for the first time.

This initial investigation revealed important information about the peroxy radical oxidation of β -carotene. First, the mixture of products is quite complex (see Figs. 2-4 in Chapter 2), especially when compared to the peroxy radical oxidation of α -tocopherol in a similar system (Liebler et al., 1990). Second, the formation of epoxide products suggests that β -carotene oxidation is initiated by peroxy radical addition, rather than hydrogen abstraction (see Discussion in Chapter 2). Radical trapping by addition is consistent with the mechanism of β -carotene antioxidant action proposed by Burton and Ingold (1984) and contrasts with the first step of the antioxidant mechanism of α -tocopherol. Third, the formation of the 15,15'-epoxide suggests that radical attack can occur at sites other than the terminal 5,6-double bond. Previously, the 5,6-double bond was thought to be the primary site of attack. Fourth, if epoxide formation represents a significant radical trapping pathway, then the mechanism of β -carotene radical trapping is clearly distinct from that for α -tocopherol. Fifth, epoxide formation by the proposed mechanism (see Discussion in Chapter 2) apparently involves a carbon-centered radical intermediate ($\text{ROO-}\beta\text{-carotene}\cdot$). This intermediate may add oxygen to form a peroxy radical, which could mediate β -carotene autoxidation and attenuate the antioxidant effect of β -carotene at high oxygen concentration. Sixth, peroxy radical oxidation of β -carotene to polar products appears to result from at least two distinct pathways (see Discussion in Chapter 2). The epoxide-independent pathway may represent a major mechanism of β -carotene radical trapping. Also, oxidation of epoxides to polar

products may contribute to polar product formation. Finally, the epoxides may be marker products for β -carotene antioxidant activity, although results from Chapter 2 suggest that epoxides are also formed during β -carotene autoxidation.

β -Carotene has been detected in membrane fractions *in vivo* (Mayne and Parker, 1986), but no β -carotene oxidation products have been identified in a membrane system. Because the reaction environment may affect the oxidation products formed, it is important to study the peroxy radical oxidation of β -carotene in a membrane system. Further, non-peroxy lipid-derived radicals ($LO\cdot$ and $L\cdot$) formed during peroxy radical-initiated lipid peroxidation may produce different β -carotene oxidation products from those produced in homogenous solution. The use of liposomes as model membranes also allowed investigation of the effect of pO_2 on β -carotene antioxidant activity and β -carotene oxidation product formation in a biomimetic system.

Peroxy radical oxidation of β -carotene in lipid bilayers proved to be similar to oxidation in homogenous solution with one important exception. The 15,15'-epoxide was not formed in liposomes (see Fig. 13 in Chapter 3). This difference suggests that the orientation of β -carotene and the attacking radical in the lipid bilayer may limit the site of peroxy radical attack on β -carotene (see Discussion in Chapter 3). Although this result suggests that the 15,15'-epoxide may not be a marker for β -carotene antioxidant activity in membranes, it could be a selective marker for β -carotene oxidation in non-membrane environments, such as low density lipoproteins (LDL), which transport β -carotene in

the blood. Recent results suggest that β -carotene may be important in preventing LDL oxidation (Esterbauer, 1989), which is hypothesized to play a role in atherosclerosis (reviewed in Halliwell and Gutteridge, 1990).

The 5,6-epoxide and polar products were formed both in liposomes and in homogenous solution (see Fig. 13 in Chapter 3). Apparently, the terminal 5,6-double bond is accessible to attacking radicals within the lipid bilayer (see Discussion in Chapter 3). This product may be a marker for peroxy radical oxidation of β -carotene in membranes. Because the polar products have not been characterized, it is not known if the polar products produced in liposomes are the same as those formed in homogenous solution. However, it is reasonable to predict that at least some of the polar products are identical in the two systems.

The effect of pO_2 on β -carotene antioxidant activity and β -carotene oxidation product formation produced interesting results that have important implications for the effects of β -carotene *in vivo*. The antioxidant activity of β -carotene in liposomes depended on pO_2 . However, in the physiologic range of pO_2 , from 160 torr to 15 torr, there was no difference in ability of β -carotene to act as an antioxidant (see Fig. 12 in Chapter 3). These results suggest that β -carotene is an equally effective antioxidant throughout the body, regardless of tissue pO_2 . This conclusion is consistent with the antioxidant hypothesis of β -carotene anticarcinogenic activity and with epidemiologic data, which consistently suggest that β -carotene is most effective at preventing lung cancer (see Chapter 1). β -Carotene

antioxidant action decreased at 760 torr O_2 , yet a weak antioxidant effect was observed in some incubations (see Fig. 12 in Chapter 3). This result is not consistent with the proposal that β -carotene may act as a prooxidant at high pO_2 (Burton and Ingold, 1984). Their conclusion was based on rates of oxygen uptake during methyl linoleate autoxidation (see Chapter 1). Under 760 torr O_2 , in the presence of β -carotene, oxygen uptake increased after an initial lag period. This observation was interpreted as evidence of β -carotene prooxidant activity. However, even during the accelerated second phase, oxygen uptake was still less than that in the absence of β -carotene. Comparison of β -carotene antioxidant activity at different pO_2 with the corresponding rates of β -carotene oxidation and product formation suggests several important points about β -carotene antioxidant chemistry. The rate of β -carotene oxidation does not always correlate with β -carotene antioxidant effect. Therefore, the rate of β -carotene oxidation may represent the sum of both β -carotene antioxidant reactions and autoxidation reactions, which do not contribute to β -carotene antioxidant effect (see Discussion in Chapter 3). Moreover, the pO_2 of the system may govern the extent of β -carotene radical trapping vs. autoxidation (see Fig. 17 and Discussion in Chapter 3). The proposed scheme for the oxidative fate of β -carotene (Fig. 17) is consistent with previous results (see Chapter 2) and the mechanism hypothesized by Burton and Ingold (see Chapter 1 for discussion). These results also indicate that loss of β -carotene during a radical challenge does not prove that β -carotene acts as an antioxidant.

The products thus far identified may not be ideal markers of β -carotene antioxidant activity. The 5,6-epoxide is formed at the same rate whether or not β -carotene is acting as an effective antioxidant (compare Fig. 12 with Fig. 16 in Chapter 3). It represents less than 20 % of β -carotene oxidation products and may be further oxidized. However, with careful controls, the 5,6-epoxide may still be a marker of β -carotene antioxidant action at physiologic pO_2 . In homogenous solution, it is formed at much faster rates during peroxy radical oxidation of β -carotene than during β -carotene autooxidation (see Results in Chapter 2). When the rate of 5,6-epoxide formation is greater during a radical challenge than in its absence, then epoxide formation may be considered as evidence of β -carotene radical trapping. In fact, in the absence of a radicals challenge, epoxide formation may represent trapping of a low endogenous level of radicals. An ideal marker of β -carotene antioxidant activity would be formed solely during β -carotene radical trapping.

Studies on the *in vitro* supplementation of biological membranes with β -carotene illustrate the difficulties inherent in adding a lipid soluble compound to biological material in an aqueous medium. The primary obstacle is solubilization of β -carotene in aqueous solution. The second problem is incorporation of β -carotene into membrane fractions in a way that mimics the presumed *in vivo* situation. Tetrahydrofuran (THF) has proved to be an effective vehicle for the delivery of β -carotene to cells in culture. THF appears to effectively solubilize β -carotene in microsomal suspensions, yet only minimal

antioxidant effects were observed. Additional methods to incorporate THF-solubilized β -carotene into microsomal membranes resulted in mediocre improvements in β -carotene antioxidant activity. These results suggest that simple addition of β -carotene to a membrane preparation is not trivial and may produce false negative results. Previous studies with microsomal membranes achieved significant success only when extreme concentrations of β -carotene were used (greater than 7 mol %) (Vile and Winterbourne, 1988). By comparison, liver microsomes from β -carotene-fed chicks contained less than 0.01 mol % β -carotene. Similar studies with α -tocopherol found that incubation of microsomes in the presence of cytosol was required for effective supplementation *in vitro* (Robey-Bond et al., 1989).

These results raise important questions about the intracellular location of β -carotene *in vivo*. β -Carotene has been measured in membrane fractions *in vivo*, however, the exact location of the molecule has not been addressed. Often, β -carotene is assumed to reside within the lipid bilayer because of its extreme lipophilicity. Recent experiments have found large amounts of β -carotene-derived radioactivity in the cytosolic fraction of liver and lung cells (Lakshman et al., 1989). Although this radioactivity may represent some metabolism to retinoids, it also suggests the existence of a carotenoid-binding protein in these tissues. This finding also suggests that β -carotene could be associated with membranes, yet not actually incorporated within the lipid bilayer. This leads to the important question of where β -carotene is located when it is an effective antioxidant.

The results presented in Chapters 2-4 are important contributions to β -carotene antioxidant chemistry. Potential marker products of β -carotene antioxidant activity have been identified and the role of pO_2 in β -carotene antioxidant action has been better defined. Further, these studies provide models to base investigations of β -carotene antioxidant chemistry in more complex systems.

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