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ACTIONS OF CAROTENOIDS IN BIOLOGICAL SYSTEMS

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INTRODUCTION

Carotenoids have attracted a variety of investigators since they were first isolated from carrots by Wackenroder in 1831. However, it was not until the appearance in 1952 of Trevor Goodwin's excellent book, *The Comparative Biochemistry of the Carotenoids* (32), that one could say that carotenoid biochemistry had become a legitimate field of endeavor. Goodwin's book was followed by a second edition that appeared as two volumes in 1980 (33) and 1984 (34). During the interim, Otto Isler edited a multi-author compendium, entitled *Carotenoids*, that focused primarily on carotenoid chemistry, although it did include a section devoted to carotenoid function (51). Carotenoid biochemistry is an extremely broad field: It attracts chemists intent on unraveling the structures of natural carotenoids through structural analysis and

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synthesis, taxonomists using carotenoid distribution to help deduce classification of organisms, botanists interested in the distribution of carotenoids among plants, plant physiologists studying the role of carotenoids in photosynthesis and photomovement, photobiologists studying effects of light on carotenoids, biochemists studying the metabolism of carotenoids, both with respect to interconversions and conversion to retinoids, nutritionists learning about the relative biopotency of provitamin A carotenoids and their availability from different foodstuffs, and chromatographers eager to test their skill in separating the vast panoply of carotenoids found in nature, of which there are almost 600 by the latest count (97). Members of these various specialties have interacted at triennial International Carotenoid Symposia, first held in Trondheim, Norway in 1966, and meeting again in Trondheim in 1993. During this 27-year period, representatives of all of the above groups have spoken about their fields, lobbied for attention to their discoveries, and stimulated each other to achieve more and more interesting results.

This broad circle of investigators has recently been expanded by the inclusion of clinicians. Clinical studies with carotenoids date back to two important publications. In 1970, Micheline Mathews-Roth and her associates first demonstrated that the principle of carotenoid protection against photosensitized damage in bacteria, algae, and plants (50) could be applied to humans suffering from the light-sensitive disease, erythropoietic protoporphyria (77). Since that publication, hundreds of such patients have been successfully treated, thus enabling investigators to conclude that oral β-carotene, at doses up to 180 mg/day, is a safe, nontoxic supplement for humans (7).

The second article that brought carotenoids to the attention of clinicians appeared in 1981 and raised the question, can β-carotene act as a dietary anticarcinogen (96)? The appearance of that article has led to a virtual explosion of interest in carotenoid action, in both in vitro studies and animal models, and the potential health benefits of carotenoids in humans. Over a dozen dietary intervention studies now underway will try to determine the efficacy of supplemental β-carotene in decreasing cancer mortality. In the last few years, another clinical area involving carotenoids has developed, namely, whether a causal relationship exists between carotenoid ingestion, or plasma levels, and coronary heart disease (28, 61). Investigations in this area are likely to grow as rapidly as the number of studies attempting to relate carotenoids to cancer.

Because of space limitations, this review only evaluates current work on the biological activities of carotenoids. Although it has been customary to write about and discuss the functions of carotenoids, J. A. Olson has suggested that we should differentiate between biological activities of carotenoids with respect to their functions, actions, and associations (87). The various

biological effects of carotenoids can be grouped into these three categories as follows:

1. Functions: accessory pigments in photosynthesis, via singlet excited carotenoid; protection against photosensitization, via triplet excited carotenoid; provitamin A, via central and excentric cleavage.
2. Actions: antioxidant; immunoenhancement; inhibition of mutagenesis and transformation; inhibition of premalignant lesions; screening pigment in primate fovea.
3. Associations: decreased risk of macular degeneration and cataracts; decreased risk of some cancers; decreased risk of some cardiovascular events; nonphotochemical fluorescence quenching.

Figure 1 illustrates the structures of the carotenoids that are discussed below.

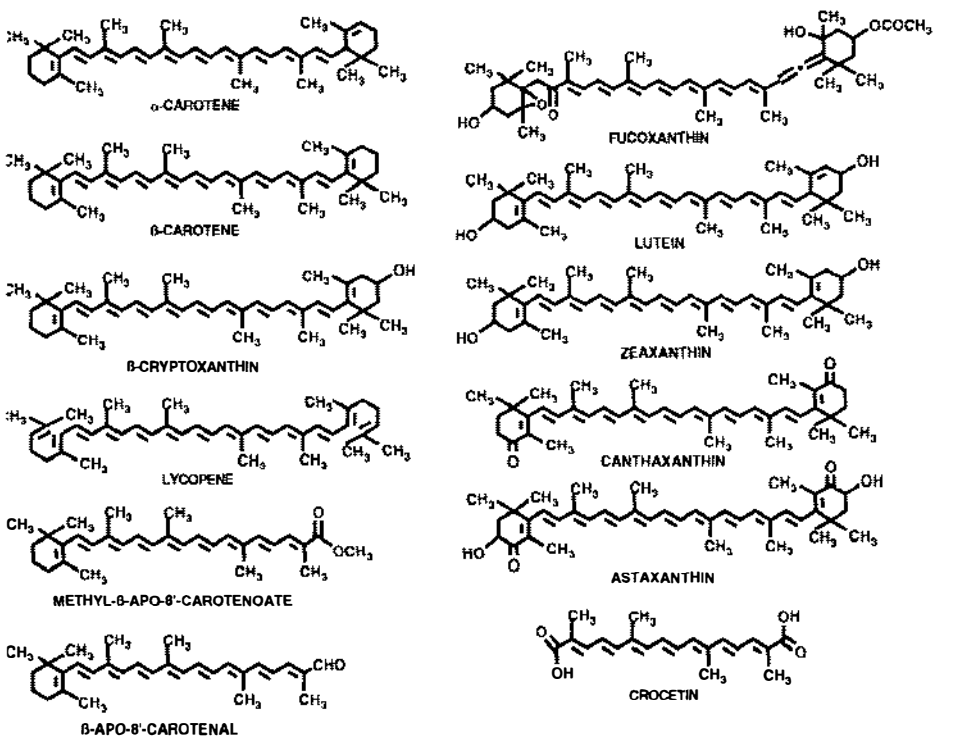

Figure 1 The structures of α-carotene, β-carotene, β-cryptoxanthin, lycopene, methyl-β-apo-8′-carotenoate, β-apo-8′-carotenal, fucoxanthin, lutein, zeaxanthin, canthaxanthin, astaxanthin, and crocetin.

CAROTENOID FUNCTIONS

Carotenoid functions are essential to the normal well-being of the organism in question. Because of their importance they are well documented and reviewed frequently. Carotenoid function as accessory pigments in photosynthetic organisms has been reviewed by Cogdell & Frank (16). Protection against light sensitization has been reviewed by Mathews-Roth (74) and by Will & Scovel (139). In the case of animals on vitamin A-deficient diets, the metabolism of the provitamin A carotenoids to retinol and retinoic acid is viewed as a function. Although we are no longer discovering new provitamin A carotenoids in nature, new information is available about the conversion of carotenoids to retinoids. A comparison of central and excentric cleavage of β-carotene has appeared recently (60)

CAROTENOID ACTIONS

The actions of carotenoids have been described as physiological or pharmacological responses to the administration of carotenoids (87) and may very well be related to the intact carotenoid molecule, rather than to a specific metabolite. This review focuses on antioxidant effects and actions associated with inhibition of mutagenesis and malignant transformation. Many of the associations have been covered in the excellent review by Byers & Perry (12).

In vitro Antioxidant Actions

Carotenoid pigments have long been considered to be antioxidants, although it is only in the last 10 years or so that investigators have begun to study their ability to interact with and quench free radical reactions either in solution (88) or in membrane systems such as liposomes (59). Many of these studies were reviewed in 1989 (53), and again in 1992 (90, 105), and included descriptions of the possible mechanisms of this antioxidant action. Almost all of these studies involve the inhibition of lipid peroxidation.

HOMOGENEOUS SOLUTIONS With the introduction (142) of lipid soluble azo-compounds such as azobisisobutyl nitrile (AIBN) to generate peroxyl radicals following thermal decomposition, many studies have been carried out evaluating the effectiveness of carotenoids as inhibitors of these reactive species. The use of homogeneous solutions for studying carotenoid protection of lipid substrates has the advantage of avoiding solubility problems, since both the carotenoid and the lipid are dissolved in organic solvents.

Work from different laboratories indicates that the antioxidant activity of these pigments in organic solution is related to the O_2 concentration, the

chemical structures of carotenoids, and the concomitant presence of other antioxidants. These three aspects are discussed below.

Oxygen Pressure Burton (10) recently reported on the effects of varying the partial pressure of O_2 (15–760 torr) and the concentration of β-carotene (0.05–5 mM) on the AIBN-induced oxidation of methyl linoleate. At low O_2 tensions (15 torr), β-carotene is an effective antioxidant, but at higher O_2 tensions (760 torr), the initial antioxidant activity is followed by an action of β-carotene that has been described as pro-oxidant (11).

Stocker et al (124) have also presented evidence supporting the increased effectiveness of β-carotene at low O_2 pressures. They compared the effect of both 20% (150 torr) and 2% (15 torr) O_2 on the antioxidant properties of β-carotene and α-tocopherol. From their data one can calculate that β-carotene was about 6% as effective as α-tocopherol at 20% O_2, but increased about 3-fold to 18% effectiveness at 2% O_2. Another example of increased antioxidant activity of β-carotene at low oxygen tensions has been reported by Vile & Winterbourn in adriamycin-treated rat liver microsomes (132). In the presence of increasing concentrations of either α-tocopherol or β-carotene (1 to 100 nmoles/mg protein), β-carotene was a better antioxidant at low pO_2 (4 mm Hg) than was α-tocopherol in terms of inhibiting malondialdehyde (MDA) formation. At a pO_2 of 8 mm Hg and above, α-tocopherol became the more effective antioxidant. Kennedy & Liebler (47) have also studied the antioxidant effectiveness of β-carotene as a function of the oxygen tension, and they conclude that it is quite effective at a physiological oxygen tension of 15 torr.

Palozza & Krinsky (89) have obtained similar results using a hexane solution of lipids isolated from rat liver microsomal membranes. Using either MDA or conjugated diene production to quantitate lipid peroxidation after AIBN treatment at 37C, they found that α-tocopherol is about 40–50 times better than β-carotene as an antioxidant in air (150 torr). However, when the pO_2 is reduced to under 20 torr, the difference in effectiveness decreases by about 40%, confirming the enhanced antioxidant activity of β-carotene at lower oxygen tensions. Since the O_2 pressures found in mammalian tissues are usually below 20 torr, these observations suggest an important role for β-carotene as an antioxidant in such environments.

Chemical structure When Krinsky & Deneke (59) first studied the antioxidant properties of β-carotene and its diketo-derivative, canthaxanthin, they did not attempt to quantitate any possible differences between these two compounds. Terao (128) was the first to report on the role of carotenoid structure in determining antioxidant activity; he compared β-carotene, canthaxanthin, astaxanthin, and zeaxanthin with respect to their ability to inhibit

the formation of hydroperoxides of methyl linoleate in a radical-initiated system. The antioxidant activities of the two keto-carotenoids, canthaxanthin and astaxanthin, were better and lasted longer than the antioxidant activites of either β-carotene or zeaxanthin. Miki (79) also observed the increased effectiveness of conjugated keto-groups in a study of β-carotene, lutein, zeaxanthin, astaxanthin, tunaxanthin, and canthaxanthin, in comparison with α-tocopherol. Using a heme-protein-Fe^{2+} as a free radical generator and measuring MDA production, he found that astaxanthin is the most efficient scavenger, with an ED_{50} of 0.2 μM, followed by zeaxanthin, canthaxanthin, lutein, tunaxanthin, and β-carotene respectively, showing an ED_{50} in the range of 0.4–1.0 μM. In contrast, the ED_{50} of α-tocopherol in this system was about 3 μM. Other studies of keto-carotenoid effectiveness in membrane systems are reported below in the section on liposomes and biological membranes.

Carotenoid-tocopherol interactions Just as the presence of δ-tocopherol enhances the protective effect of β-carotene on $^1O_2$-initiated photo-oxidation of methyl linoleate (129), so does β-carotene delay markedly the AIBN-induced loss of endogenous microsomal tocopherols (89). However, a synergistic interaction is seen in microsomes supplemented with both β-carotene and α-tocopherol, and will be discussed in the section on biological membranes (92).

LIPOSOMES Krinsky & Deneke (59) introduced the use of liposomes to study β-carotene and canthaxanthin inhibition of radical-initiated lipid peroxidation, and there are now many studies using this model membrane. Liposome preparations are amenable to manipulation of lipid composition, pH, temperature, and they avoid some of the complications associated with the introduction of carotenoids into biological membranes.

Cabrini et al (13) reported that β-carotene could inhibit liposomal autoxidation, but when 0.3 mM $FeCl_3$ was added, the protection was lost. These observations were in contrast to their findings that ubiquinone could inhibit both types of lipid oxidation. The work of Stocker et al (124) on β-carotene protection at two different oxygen tensions has already been discussed.

In agreement with the data obtained in homogeneous solutions, Kennedy & Liebler (47) reported that in liposomes, the antioxidant activity of β-carotene varies with the O_2 tension and is more effective at 15 torr than at 760 torr. They suggest that this difference could be due to the rapid formation of autoxidation products of β-carotene at high oxygen tensions, but this difference may be an example similar to the pro-oxidant effect reported at high pO_2 (11). In addition, Kennedy & Liebler suggest that α-tocopherol, by

decreasing the extent of β-carotene autoxidation, might increase the effectiveness of β-carotene as an antioxidant (47).

Terao and his associates (66) have now reported that astaxanthin, zeaxanthin, and canthaxanthin are better than β-carotene in protecting liposomes from peroxyl radical attack. A recent, preliminary publication (131) suggests that in dioleylpalmitoylcholine liposomes treated with azobis(2,4-dimethylvaleronitrile) (AMVN), β-carotene is considerably more effective than α-tocopherol in preventing a loss in the fluorescence of *cis*-parinaric acid, a method used to determine antioxidant activity.

Other workers have addressed the antioxidant properties of carotenoids in micelles. Pryor et al (100) compared the antioxidant effectiveness of various isomers of α-tocopherol in aqueous sodium dodecyl sulfate micelle solutions. Using an aqueous radical initiator, they reported that the effect of β-carotene was too small to measure. If this type of experiment was duplicated with lipid-soluble radical initiators, it would be interesting to see if the observation could be explained by the strict compartmentalization imposed on the system by the formation of micelles. In addition, Canfield et al (14) demonstrated that micellar solutions of β-carotene could inhibit soybean lipoxygenase-induced formation of conjugated dienes from linoleic acid, as well as decrease the formation of linoleic acid hydroperoxides.

LIPOPROTEINS Two factors make lipoproteins an interesting in vitro system for the elucidation of the antioxidant activities of carotenoids: the fact that the initiation of atherosclerosis has now been related to oxidative modifications of low-density lipoproteins (LDL) (119) and the well-established observation that LDL is the major carrier of β-carotene in humans (58).

Much of the work on the antioxidant role of carotenoids in LDL comes from Esterbauer's group, and this subject has been reviewed very recently (25). Human LDL, treated with Cu^{2+} as a pro-oxidant, is oxidized and the level of oxidation is highly related to the endogenous level of antioxidants. The presence of the antioxidants prolongs the lag phase that precedes the rapid oxidation of LDL, and the antioxidants are consumed in the following sequence: α-tocopherol, γ-tocopherol, lycopene, phytofluene, and β-carotene (27). Although an initial study reported a correlation between the content of endogenous tocopherols and resistance to oxidative stress in pig LDL (49), they now report that some human LDL preparations, with practically equal amounts of tocopherols, have very different lag phases (24), which indicates that factors other than the tocopherols must play a role in protecting LDL. One of these factors might be ubiquinol-10, even though it is present in much smaller amounts than the α-tocopherol. For example, Stocker et al (123) claim that ascorbate-free LDL preparations exposed to azobis-2-amidinopropane (AAPH) are first protected by ubiquinol-10, and upon destruction of this

antioxidant, lipid peroxidation begins, even though 80% of the original carotenoids and 95% of the original tocopherols are still present.

The actual role of β-carotene as an antioxidant in LDL remains controversial. Morel et al (81) reported that the addition of β-carotene to an LDL dialysate did not protect the survival of cultured human skin fibroblasts, unlike the protection offered by α-tocopherol or butylated hydroxytoluene. However, the conditions of this experiment are not clear. They claim that they used a 1.9-mM solution of β-carotene in ethanol as their stock solution and that it was added to the saline dialysate, which would have led to the precipitation of the β-carotene. In an attempt to answer directly the question of β-carotene involvement in protecting LDL against oxidation, Jialal et al (45) added β-carotene directly to LDL and initiated lipid oxidation by treatment with $CuSO_4$. Under these conditions, they report that the β-carotene was 20-fold better than an equivalent amount of added α-tocopherol in preventing lipid peroxidation. A cellular approach was used by Navab et al (84), who measured the effect of co-incubation of LDL with human aortic wall cells and measured monocyte transmigration. When the LDL was pre-treated with either β-carotene or α-tocopherol, no effect on migration was noted, but when the aortic cells were pre-treated with these two antioxidants, there was a very marked inhibition of monocyte transmigration. These findings suggest that the normal LDL oxidation brought about by the aortic cells is inhibited when the cells contain the antioxidants, in contrast to the LDL containing the antioxidants.

BIOLOGICAL MEMBRANES Studies on membrane lipid peroxidation in the presence of carotenoids are summarized in Table 1 and are briefly discussed below. Although working with membranes should represent a more physiological approach to the study of the antioxidant activity of carotenoids, the use of this model is complicated by the fact that the membranes are isolated as aqueous suspensions and the carotenoids are virtually insoluble in this medium. This fact, in addition to the limited solubility of carotenoids in organic solvents such as alcohols, may explain the very large discrepancies reported in Table 1.

Dixit et al studied the effect of β-carotene in epidermal microsomes undergoing NADPH-dependent lipid peroxidation (22). They reported that at concentrations of 5–10 μM, β-carotene inhibited MDA formation more effectively than similar concentrations of α-tocopherol. However, they would have had to have a stock solution of β-carotene in ethanol of 0.3 mM, which exceeds the solubility of the pigment in this solvent (19).

Searle & Willson (117) reported a weak inhibition of MDA formation in rat liver microsomes exposed to $FeSO_4$ and cysteine when a suspension of β-carotene, estimated to be 1–10 mM, was added. They suggested that the

Table 1 Summary of the studies on membrane lipid peroxidation in the presence of carotenoids

embrane	Pro-oxidant	Carotenoid	MDA inhibition (% of control)	Reference
at skin microsomes	1 mM NADPH	5 μM β-carotene	58%	22
		10 μM β-carotene	69%	
at liver microsomes	5μM $FeSO_4$ +	1 mM β-carotene	7%	117
	500 μM cysteine	10 mM β-carotene	13%	
ovine seminal vesicles	cyclooxygenase or	20 μM β-carotene	34%[a]	37
	lipoxygenase	40 μM β-carotene	56%[a]	
at liver microsomes	100 μM NADPH +	25 μM β-carotene	40%	132
	1.0 μM $FeCl_3$ +		70%[b]	
	30 μM adriamycin			
at liver microsomes	0.1 mM NADPH +	100 μM β-carotene, or	20%	48
	0.1 mM $FeCl_3$ +	100 μM α-carotene, or	37%	
	1.7 mM ADP	100 μM lycopene, or	42%	
		100 μM lutein	33%	
at liver microsomes	0.2 mM paraquat +	100 μM β-carotene, or	18%	48
	0.5 mM NADPH	100 μM α-carotene, or	30%	
		100 μM lycopene or lutein	33%	
at liver mitochondria	100 μM Fe^{2+}	4.2 μM astaxanthin	100%	63
at liver mitochondria	? $FeSO_4$	0.1 μM astaxanthin	100%	79
at liver microsomes	25 mM AAPH /AMVN	50 nmol β-car/mg protein	Modest	93
	0.4 μM NADPH +	10 nmol β-car/mg protein	11%	
	0.05 mM $FeCl_3$ +			
	1.7 mM ADP			
at liver microsomes	NADPH/ADP/Fe^{2+}	10 nmol β-car/mg protein	7%	92
at liver microsomes	AAPH	10 nmol β-car/mg protein	4%	
at liver microsomes	AAPH	10 nmol astax/mg protein	30%	91
at liver microsomes	AAPH	10 nmol canth/mg protein	30%	
at liver microsomes	NADPH/ADP/Fe^{2+}	10 nmol astax/mg protein	25%	

[a] Inhibition of prostanoid products.
[b] Incubation at 4 mm Hg O_2.

weak effect was due to the difficulty of dissolving the β-carotene suspension in the microsomes.

Halevy & Sklan (37) used a very different approach in assaying the antioxidant effect of β-carotene. They measured the conversion of arachidonic acid to either PGE_2 or to HETE, thus measuring either cyclooxygenase or lipoxygenase activities, and they reported that an aqueous dispersion of β-carotene at 100 μM was a more effective inhibitor than a 1-mM solution of α-tocopherol. Reports such as this one are always interesting in view of the fact that α-tocopherol is still considered to be the major lipid-soluble antioxidant in membranes (10).

As reported above, Vile & Winterbourn (132) found that β-carotene was a better antioxidant than α-tocopherol at low oxygen tensions in adriamycin-treated rat liver microsomes. The β-carotene was added to the microsomes as a concentrated chloroform solution, but it was not clear whether any attempt was made to eliminate the chloroform from the preparation.

Kim (48) has also looked at rat liver microsomal peroxidations, induced by either Fe^{+3}-ADP/NADPH or paraquat/NADPH, in the presence of several carotenoids, including α-carotene, β-carotene, lutein and lycopene, and α-tocopherol. She found that lycopene, lutein, and α-carotene were better antioxidants than β-carotene or α-tocopherol. Although the results are suggestive, it is difficult to understand how she dissolved her hydrocarbon carotenoids at 0.1 mM in absolute ethanol (19).

Recently, several publications have appeared indicating that astaxanthin is a very powerful lipid antioxidant, either equivalent to, or considerably stronger than α-tocopherol. Kurashige et al (63) reported astaxanthin protection of liver mitochondria from vitamin E-deficient rats, exposed to Fe^{2+} to initiate lipid peroxidation. The inhibitory effect of astaxanthin, dissolved in dimethylsulfoxide at concentrations ranging from 0.13 nM to 1.3 mM, on mitochondrial MDA formation was reported to be 100–500 times stronger than that of α-tocopherol. Miki (79) has reported similar results in homogenates of rat liver mitochondria exposed to Fe^{2+} and suggests that the strong antioxidant activity of astaxanthin could be related to the high affinity of this molecule for mitochondrial membranes, because of the chemical structure of this pigment. We have recently investigated the efficacy of two keto-carotenoids, astaxanthin and canthaxanthin, in inhibiting radical-initiated lipid peroxidation in rat liver microsomal membranes (91). Both of these carotenoids were found to be as effective as α-tocopherol, which is in sharp contrast to the very weak antioxidant action of β-carotene when added alone to this system (93).

A synergistic interaction of β-carotene and α-tocopherol has now been demonstrated in microsomal membranes by Palozza & Krinsky (92). These observations support earlier evidence of a synergistic effect in vivo (65) and could very well represent the inhibition, by α-tocopherol, of any pro-oxidant

effects of the β-carotene peroxyl radical (β-car-OO•), assumed to be formed during the antioxidant action of β-carotene (53). Previously, we had demonstrated that α-tocopherol protects β-carotene from autoxidation (38), although Kagan et al were not able to see this type of protection in liposomes supplemented with both α-tocopherol and β-carotene and exposed to ultraviolet (UV) light (46).

CELLS There have been very few demonstrations of carotenoids functioning as antioxidants in isolated cells. Endothelial cells of isolated, perfused rabbit cornea, treated with either β-carotene (1 μg/ml) or α-tocopherol (10 μg/ml), demonstrated extended survival times over control cells or in cells treated with either water-soluble antioxidants or the enzymes SOD or catalase (68). A good example of antioxidant behavior in cells comes from the laboratory of Bertram and his associates (144). This group added various carotenoids, as well as α-tocopherol, to C3H 10T1/2 cells and then monitored MDA production as a measure of lipid peroxidation. Under these conditions, they report that the order of effectiveness of the antioxidants is α-tocopherol > canthaxanthin = lutein = methyl bixin > β-carotene = lycopene > α-carotene. Interestingly, they do not find a relationship between the antioxidant effectiveness and the ability to enhance gap junctional communication.

In vivo Antioxidant Actions

As in the case of the in vitro studies reported on above, much of the evidence for carotenoid antioxidant effects in vivo is evaluated by the inhibition of lipid peroxidation in the presence of carotenoids. Increasing evidence that carotenoids can function as antioxidants in vivo has been reported recently in animal models in which the pigments have been injected in the animals or added directly to the diet.

Two aspects of the antioxidant activity of carotenoids seem to be particularly important in vivo. The first is the efficiency that these molecules display in directly decreasing lipid peroxidation. The second is the ability of these molecules to modulate endogenous levels of other antioxidants.

Many workers have demonstrated the antioxidant activity of carotenoid molecules in animal models. For example, Tappel and his associates reported (62) that an injection of β-carotene in mineral oil significantly blocks the increased expiration of ethane and pentane observed in ascorbate-deficient guinea pigs. They also put rats on a Se-deficient diet, and found that β-carotene, at 30 mg/day, was as effective as either supplemental Se or α-tocopherol in preventing liver damage (143). Most recently, Tappel's laboratory has demonstrated that tissue slices from animals fed various antioxidants and then treated with *t*-butylhydroperoxide can be used to

measure antioxidant effectiveness in vivo (65). Of particular interest was the finding that rats fed β-carotene showed very little protection in the tissues treated with *t*-butylhydroperoxide, but when the β-carotene was added to tissues containing either α-tocopherol, Se, or ubiquinone, it displayed a synergistic protective action.

Tan & Chu (126) have fed various palm carotenoids to rats and have measured cytochrome P450-mediated metabolism of benzo[*a*]pyrene (BP). In their system, they claim that β-carotene is much more potent than α-tocopherol in inhibiting the formation of both the 9,10-diol and the 3-OH-metabolite of the benzo[*a*]pyrene.

Another report (95) indicates that the dopaminergic neurotoxic effects of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can be prevented by subcutaneous injections of either β-carotene or α-tocopherol, as well as aqueous ascorbate and *N*-acetylcysteine, suggesting that the neurotoxin works by means of an oxidative mechanism.

A study in humans (80) who were maintained on a carotenoid-free diet for two weeks and then repleted with β-carotene demonstrated a decrease in serum lipid peroxide values, without affecting neutrophil superoxide production. This effect of β-carotene appeared to be independent of the repletion concentration, as similar effects were seen with either 15 or 120 mg/day. This study is the only example of an antioxidant effect of carotenoids in humans.

In a very preliminary report, Clausen (15) has observed a decrease in luminol-enhanced chemiluminescence from polymorphonuclear leukocytes (PMN) isolated from smokers after a 10-day treatment with various antioxidants. He reports that effectiveness is as follows: Se >> β-carotene >> ascorbic acid > α-tocopherol; ubiquinone is much less effective.

In some cases, the addition of carotenoids has not led to any enhanced antioxidant effect, and, in fact, the added carotenoid has been reported to function as a pro-oxidant. Lomnitski et al (67) reported that rats maintained on a diet deficient in α-tocopherol and supplemented with oxidized soy bean oil develop increased levels of MDA in the testes, along with increased amounts of 15-lipoxygenase activity. An additional supplementation with β-carotene at 500 mg/g resulted in increased testes MDA and 15-lipoxygenase activity. They concluded that the additional β-carotene added to an α-tocopherol deficient diet behaved like a pro-oxidant. Another example of a failure of supplemental β-carotene to prevent oxidative damage has been reported by Witt et al (140) who fed human volunteers 533 mg α-tocopherol, 1 g ascorbic acid, and 10 mg β-carotene for 1 month. These individuals were tested for exercise capacity and excretion of the oxidized RNA base, 8-hydroxyguanosine, and no difference in either parameter was detected after supplementation.

In agreement with the in vitro experiments demonstrating the antioxidant activity of astaxanthin (63, 66, 79, 91, 128), this activity has been confirmed in vivo. For example, the uncoupling of mitochondrial respiration observed

in rats deficient in α-tocopherol can be prevented by feeding the rats 1 mg/100 g astaxanthin (63). Similarly, the ability of the radical-generating system, xanthine oxidase and xanthine, to induce lysis of erythrocyte ghosts from α-tocopherol-deficient rats was also protected by the addition of astaxanthin at a level of 1 g/kg (79).

INTERACTIONS WITH OTHER ANTIOXIDANTS The work cited above indicates that carotenoid pigments can provide antioxidant activity in vivo, but it is possible that the effect is not direct. An alternative mechanism is suggested by Mayne & Parker (78), who reported that the addition of dietary canthaxanthin to chicks deficient in vitamin E and selenium increased the resistance to lipid peroxidation, primarily by increasing membrane α-tocopherol levels, and only secondarily by providing weak direct antioxidant activity.

Other interactions between antioxidants in vivo have been confirmed by Blakely and associates (9). They reported that β-carotene modulates the increase of SOD induced by peroxyl radicals produced by a high fat diet. Another interesting observation on interactions was reported by Jialal & Grundy (44), who found that added ascorbate was able to protect the endogenous tocopherols and β-carotene in human LDL oxidized with Cu^{2+}, but added probucol was without effect. In addition, Leibovitz et al (65) have reported on a clear synergistic action between β-carotene and other antioxidants in the protection of different rat organs from both spontaneous and induced lipid peroxidation.

One of the possible explanations for the lack of antioxidant activity in some of these experiments may be the poor storage of β-carotene in rat tissues or, in accord with the hypothesis of Burton & Ingold (11), the possibility that, under certain circumstances (concentration of β-carotene, oxygen tensions in the tissues, modality of administration), the action of β-carotene represents the balance between anti- and pro-oxidant properties.

Antimutagenesis

The ability of carotenoids to prevent bacterial mutagenesis has been reviewed recently (57). In summary, several investigators have clearly demonstrated antimutagenic activity in *Salmonella typhimurium* for carotenoids such as β-carotene, canthaxanthin, cryptoxanthin, β-apo-8′-carotenal, and methyl-β-apo-8′-carotenoate. A single negative study (130) has also been reported.

Protection Against Genotoxicity and Malignant Transformation

Many attempts have been made to evaluate the protection afforded cell or organ culture by the addition of carotenoids. However, to compare one experiment with another is very difficult because of the variability in methodology used by different investigators adding carotenoids to their

systems. Since most carotenoids are insoluble in aqueous environments, or poorly soluble in many polar organic solvents, a variety of techniques have been used to try to incorporate these compounds into cells. Therefore, although carotenoid concentrations are frequently reported, it is probably best not to put too much stock in the actual values. In addition, because of their hydrophobic nature, carotenoids will associate with membranes and hydrophobic portions of cells.

When this topic was first reviewed (52), only a handful of papers had demonstrated the cellular effects of carotenoids. The number of investigations has grown considerably, and Table 2 lists most of the studies that have tested carotenoids in cellular systems as protective agents. As this material has been reviewed several times recently (54, 55, 57), I only discuss some of the newer observations.

Hazuka et al (39) have added β-carotene and other antioxidants to cultures of mouse B-16 melanoma cells and have observed morphological differentiation in these cells, along with inhibition of growth and decreased survival. In addition, this treatment decreases both basal and melanocyte hormone-stimulated adenyl cyclase activity. They used β-carotene in a 1:10 mixture of DMSO:ethanol at concentrations up to 37.2 μM. Because the solvent was never above 0.4% in these cultures, their starting solutions of β-carotene were 9.3 mM in the DMSO:ethanol solution. Similar effects on growth inhibition were observed with α-carotene, retinol, and butylated hydroxyanisole, but the latter was not able to modulate the adenyl cyclase activity.

Watson and his associates have added β-carotene to either human PMN or to peripheral blood mononuclear cells (PBMC). In the former case, they report (1) secretion of a cytokine that is cytotoxic in 4 out of 6 human tumor cells lines studied and that had only low level toxicity to a normal diploid fibroblast line. Maximum secretion occurred at $0.1–1 \times 10^{-9}$ M β-carotene. In the case of the PBMC, β-carotene treatment resulted in an increase in the expression of various markers, including the interleukin-2 receptor, as well as an increase in the number of natural killer (NK) cells. Very recently, this group has used canthaxanthin added to cells, and they claim that 0.1 mM canthaxanthin inhibits 3 tumor cell lines, but stimulates the growth of 3T3 cells. The canthaxanthin is dissolved in either ethanol or DMSO as the vehicle, and since the solvent is never used above 0.1%, their original vehicle solution must have been 100 mM, i.e. it must have contained 56 mg/ml of canthaxanthin. This value is much too high for solubility in either of the organic solvents. Furthermore, they claim that the vehicle is subsequently added to their medium to yield a stock solution of 1 mM. This concentration of canthaxanthin in an aqueous medium is suspect.

Schwartz and his associates have also provided evidence that carotenoids can specifically inhibit the growth of tumor cells in culture. The addition of β-carotene or canthaxanthin at 70 μM in liposomes inhibited the proliferation

of cultured human squamous cells (SK-MES lung carcinoma or SCC-25 oral carcinoma), but there was no effect on the growth of normal human keratinocytes (115). In addition, these workers report that β-carotene, at concentrations up to 100 μM in PBS/0.1% DMSO, is toxic to SCC-25 cells in culture (116). Again, the ability to achieve such concentrations of β-carotene in an aqueous medium is difficult to accept and would certainly be accompanied by the precipitation of a large amount of this material. An additional publication (112) claims that 70 μM β-carotene or canthaxanthin is selectively toxic to 7 malignant tumor cell lines and is without effect in normal keratinocytes.

Some of the most interesting cellular effects of carotenoids have been reported by Bertram and his associates. In an early publication (101), both β-carotene and canthaxanthin, in the form of water-dispersible beadlets provided by Hoffmann-La Roche, when added to C3H/10T1/2 cells, decreased the extent of malignant transformation induced either by methylcholanthrene or by X-ray treatment. These observations have been extended to other carotenoids, for α-carotene and lycopene were also effective in inhibiting MCA-induced malignant transformation (8). In these experiments, the carotenoids were dissolved in 99.5% tetrahydrofuran to give 2-mM solutions, which are achievable in this solvent (19), and then diluted directly into the culture medium to yield solutions as high as 10^{-5} M. Although lutein was inhibitory at 10 μM, it actually increased the number of transformants at lower concentrations. α-Tocopherol also inhibited malignant transformation, but was only about 10% as active as lycopene. Recently, Bertram et al reported that these carotenoids inhibit gap junctional communication in the C3H/10T1/2 cells (144) and that apparently there is no relationship between the ability of these carotenoids to inhibit this process and their antioxidant capacity, as measured by TBARS production. Furthermore, this group has now found evidence that β-carotene, canthaxanthin, and lycopene can up-regulate the expression of the connexin43 gene, the gene responsible for the production of one of the important components of the gap junction (145). This process was not related to the antioxidant capacities of these carotenoids, and added α-tocopherol had no effect.

The above discussion would seem to indicate that distinct effects on cells have been observed when carotenoids are added, but much work needs to be carried out to see if the effects are due to the intact carotenoids to or to an enzymatic or chemical breakdown product.

Anticarcinogenesis

The preliminary reports of carotenoids inhibiting tumor formation in animals supplemented with β-carotene (70, 107) served as one of the foundations for the hypothesis that β-carotene might function as a dietary anticarcinogen in humans (96). Work of this nature has continued with animals, although with

Table 2 The effects of carotenoids on cells and cellular systems[a]

Carotenoid	Cells	Action measured	Initiated by	Observed effect	Reference
β-Carotene	Mammary; mouse	Alveolar lesions	DMBA	Inhibition	118
β-Carotene	Mammary; mouse	SCE	DMBA/MNU/DENA	Inhibition	69
β-Carotene	CHO	SCE	PMNL OR HX/XO	Reduces SCE	138
β-Carotene	Hamster bone marrow	Chromosome breaks	MMS/thiotepa/busulfan	Protection	103, 104
β-Carotene	Bone marrow; mouse	Chromosome breaks	B[a]P; mitomycin C	Protective	102
β-Carotene	CHO	SCE	MMS; 4-NQO	Protection	121
β-Carotene/α-car	Human lymphocytes	Mitogenesis; E-rosettes	Concanavalin A	Inhibits proliferation	82
β-Carotene	HCPC-1	Growth	DMBA	Inhibition	114
β-Carotene	Human PBMC	HLA-DR antigen expression	Opsonized zymosan	Prevents decrease	36
β-Carotene/canth	C3H/10T1/2	Transformation	X-rays; MCA	Canth > β-carotene	101
β-Carotene	Human PBMC	↓ Phagocytic activity	UV-B exposure	Prevention	111
α-Car/β-carotene	Neuroblastoma GOTO	Growth	Spontaneous	Inhibits; α-car > β-car	85
β-Carotene	V79	SCE	MNNG	Increases SCE	20
α-Car/β-carotene	Neuroblastoma GOTO	Growth; N-myc mRNA	Spontaneous	Inhibits/suppresses	83
β-Carotene	Human PMNL	Cytotoxic cytokine	β-Carotene	Induces secretion	1
β-Carotene/canth	Human PBMC	Expression of markers	Spontaneous	Increased expression	99
β-Carotene	Human PBMC	Expression of markers	Spontaneous	↑ NK cells/IL-2 receptor	98
β-Carotene	Human PMNL	Chemiluminescence	FMLP or luminol	↓ Chemiluminescence	5
β-Carotene/canth	Transformed C127	Chromosome instability	BPV transformation	Inhibits	122
β-Carotene	Melanoma: Mouse	Differentiation Adenylate cyclase	Spontaneous MSH, NaF	Increases Reduces	39

β-Carotene/canth	Human squamous	Growth	Spontaneous	Inhibited	115
	Normal keratinocytes	Growth	Spontaneous	No Effect	
Fucoxanthin	Neuroblastoma: GOTO	Growth	Spontaneous	Inhibited	86
Crocetin	C3H/10T1/2	Cytotoxicity; DNA adducts	Aflatoxin B1	Decreased	134, 136, 137
β-Carotene	Squamous carcinoma	Archidonic acid	Spontaneous	Increased	23
Canthaxanthin	Squamous carcinoma	metabolism to PG	Spontaneous	Decreased	
β-Carotene/α-car canth/lycopene	C3H/10T1/2	Transformation	MCA	Decreased	8
β-Carotene/α-car lycopene/lutein/canth	C3H/10T1/2	Gap junctional communication	Spontaneous	Increased	144
β-Carotene/lycopene canthaxanthin	C3H/10T1/2	Connexin43 expression	Spontaneous	Up-regulated	145
Canthaxanthin	NIH-3T3	Growth	Spontaneous	Stimulates	41
	Tumor lines (JB/MS, B16F10, PYB6)			Inhibits	
β-Carotene/canth	7 Tumor lines	Proliferation	Spontaneous	Inhibition; ↑70 kD protein	112
	Normal keratinocytes			No effect	
β-Carotene/canth	SCC-25 carcinoma line	Growth, SOD, GSH-transferase	Spontaneous	Inhibition	116
	Normal keratinocytes	Growth	Spontaneous	No effect	

[a] BPV = bovine papillomavirus, canth = canthaxanthin, α-car = α-carotene, CHO = Chinese hamster ovary cells, DENA = diethylnitrosamine, DMBA = dimethylbenzanthracene, FMLP = F-metleucylphenylalanine, HX = hypoxanthine, MCA = methylcholanthrene, MMS = methylmethanesulfonate, MNU = methylnitrosourea, MSH = melanocyte stimulating hormone, 4-NQO = 4-nitroquinoline-1-oxide, PBMC = peripheral blood mononuclear cells, PMA = phorbol myristyl acetate, PMNL = polymorphonuclear leukocytes, SCE = sister chromatid exchange, SOD = superoxide dismutase, XO = xanthine oxidase.

somewhat mixed results. A major problem has been that most of the animals tested are extremely poor absorbers of carotenoids, and therefore investigators have frequently used pharmacological doses in order to see effects. This, in turn, raises questions about the purity of the compounds used and what accompanying material might be administered at the same time as the carotenoids. The purity of the starting material is particularly important when one considers that the autoxidation of β-carotene produces breakdown products, such as retinoic acid, that have powerful biological activities (38).

ANIMAL STUDIES The anticarcinogenic properties of carotenoids have been reviewed recently by Rousseau et al (105), and in somewhat greater detail by Krinsky (55, 56). UV light (73, 75), or a combination of UV light and carcinogens such as benzo[*a*]pyrene (108, 109), dimethylbenzanthracene (DMBA) (71), or 8-methoxypsoralen (106) have been used to induce tumors in rats and mice, and carotenoids have been shown to be protective in all of the above studies. More recently, investigators have demonstrated that canthaxanthin, at 10 g/kg, can reduce the UV-induced tumor burden in mice without influencing the tumor incidence (29), and the effect is even more striking when a combination of canthaxanthin and retinyl palmitate (120 IU/g) is added to the diet.

Carotenoids have also been shown to be protective when environmental carcinogens are administered to experimental animals; positive effects have been reported against DMBA (2–4), dimethylhydrazine (DMH) (6, 127), or *N'N*-methylnitro-nitrosoguanidine (MNNG) (110). Even topical administration of β-carotene inhibited (125) or reversed (114) squamous cell carcinoma produced in the hamster buccal pouch by treatment with topical DMBA. Either β-carotene, canthaxanthin, or an algal extract containing carotenoids was reported to be effective in this assay (113). This work has now been confirmed and extended. Topically applied β-carotene not only prevents DMBA-induced cheek tumors in hamsters, but it also prevents the accompanying stomach tumors (31). In addition, these β-carotene-treated animals retained a normal SDS-polyacrylamide electrophoretic pattern of cheek pouch keratin, whereas keratin from DMBA-treated hamsters had an abnormal pattern. A similar decrease in tumor incidence in DMBA-treated hamsters, as well as a decrease in polyamine levels in erythrocytes and urine in the β-carotene-treated animals, have also been reported recently (40).

Another method for demonstrating the anticarcinogenic activity of β-carotene has been to provide combined treatment with the carcinogen DMBA as well as with the tumor promoter phorbol myristyl acetate (PMA). When Skh or Sencar mice are treated with DMBA/PMA and supplemented with 3% β-carotene in their diets, either in the form of beadlets (containing 10% β-carotene) or by adding the crystalline pigment directly to the chow, skin

tumors develop; both preparations protect Skh mice, but they do not protect the Sencar strain (64). These experiments point out the difficulty of comparing experimental results from different laboratories, for the strain differences reported above would be greatly exaggerated when one compares experiments in different species. For example, a similar protocol using DMBA/PMA-induction of skin tumors in Skh mice reported a significant decrease in the number of skin papillomas with administration of β-carotene at 2.4 mg/kg, but no effect on the ultimate development of malignant tumors (120). Based on these observations, these authors concluded that β-carotene was working during the PMA-induced promotional phase of tumor formation.

When canthaxanthin, at 1.1–3.4 mg (2–6 mmoles)/kg was fed to rats for 3 weeks prior to treatment with DMBA, a 65% decrease in the incidence of mammary tumors was observed (35).

Following an earlier report of a modest effect of crocetin in animals (72), this 20-carbon dicarboxylate carotenoid (Figure 1), along with β-carotene and lycopene, was administered by intraperitoneal injection and found to inhibit the growth of C-6 glial cells in rats (133, 135). In C3H/10T1/2 cells exposed to aflatoxin B_1 (AFB), 100 mM crocetin results in an elevation in the concentration of cytosolic GSH and an increase in the activity of GSH S-transferase and GSH peroxidase (137). These effects might explain the action of crocetin in altering the activity of microsome-activated AFB, and the resultant decrease in cytotoxicity and the decrease in AFB-DNA adducts (136).

Murakoshi et al (83) have reported that α-carotene is more effective than β-carotene in preventing spontaneous liver cancer in C3H/He male mice, and is more effective in preventing both 4-nitroquinoline-1-oxide-induced and glycerol-promoted lung cancer and DMBA-induced and PMA-promoted skin cancer in mice. The α-carotene had been prepared from palm oil carotenoids, and they also indicated that minor carotenoids of this preparation, such as γ-carotene and lycopene, may also have activity in preventing carcinogenesis.

Certainly, not all of the studies of carotenoids and cancer in animals have resulted in support for the anticarcinogenic hypothesis. As noted above, supplementation with 3% β-carotene did not significantly protect Sencar mice from DMBA/PMA-induced tumors (64), whereas SKH mice were protected. When DMH and methylnitrosourea were administered sequentially in F344 rats, β-carotene (0.2%) had only weak, organ-specific effects in preventing tumor formation (42). In addition, two reports have appeared on the effects of carotenoids on induced *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN) bladder cancer. In one case, β-carotene fed to rats at 1.6 g/kg for 42 weeks colored the organs but did not protect against tumor formation (94). In another report, β-carotene or canthaxanthin was fed at 1 g/kg for 5 weeks before and 26 weeks after treatment with OH-BBN, and only the mice

receiving the β-carotene supplement showed significant protection against the development of bladder cancer (76). The different results reported in these papers may be a result of species differences in response to either the carotenoid or the carcinogen. In addition, Colacchio et al (17, 18) have not been able to demonstrate carotenoid protection of DMH-induced colorectal cancers in rats.

Although the evidence of an association between carotenoid intake and anticarcinogenesis is not always positive and many workers have used systems unique to their laboratories, the data seem to suggest a clear anticarcinogenic role of carotenoids in animal models. However, there is still no direct evidence that any of these effects can be attributed to an antioxidant action of these carotenoids.

HUMAN STUDIES In recent years, the possibility that carotenoid pigments may exert a preventive role in some diseases in which free radicals seem to be involved has attracted increasing interest. The rationale for this interest came primarily from human epidemiological data showing that carotenoids may reduce the risk of cancer or other diseases (12). In most cases, reviews of these studies (21, 26, 30, 43) frequently attribute the effects to the antioxidant activity of these pigments. Nevertheless, we cannot as yet claim that carotenoids function as antioxidants in disease prevention in humans. In fact, consideration should be given to the single, recent report of a significant reduction in plasma α-tocopherol levels in volunteers receiving as little as 15 mg β-carotene/day for 9 months (141).

CAROTENOID ASSOCIATIONS

Many biological effects have been attributed to carotenoids, but we still lack evidence that these are anything more than associations, which may or may not be causally related. Unfortunately, with many of these associations, we are still at a very early stage in trying to understand the relationship between the structure of the carotenoid pigments, the species studied, and the observed effects. Nevertheless, the potential significance of these associations, particularly with respect to human health issues, is of such importance that it underlines the need for continued support of investigators working in this field.

FUTURE CONSIDERATIONS

Several areas of investigation require more work if we are to have a clearer understanding of carotenoid actions in biological systems. For example, better animal models should be used to test various hypotheses about possible

carotenoid action. Rats and mice are notoriously poor absorbers of carotenoids, and animals such as the ferret, which can absorb β-carotene, might yield more informative results. In particular, what is needed are clear-cut studies indicating whether or not carotenoids behave as antioxidants in vivo. We also have to pay attention to the other carotenoids in the foods that have been implicated as preventing disease. Finally, are the reported effects due to the intact carotenoid molecule or to enzymatic or nonenzymatic breakdown products? This question applies not only to β-carotene but also to the other carotenoids commonly found in the body, such as α-carotene, cryptoxanthin, lycopene, lutein, and zeaxanthin.

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