Carotenoids
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Chapter 12

Antioxidant/Pro-oxidant Actions of Carotenoids

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A. Introduction

In recent years numerous reviews have discussed in detail the antioxidant action of carotenoids [1-5]. The existence of an antioxidant effect has been questioned by some, however [6]. In addition, there is the complication that, under some circumstances, carotenoids exhibit a pro-oxidant effect [7,8], although some authors do not believe that this occurs *in vivo* [9]. The fundamental chemistry of carotenoid radicals and radical ions, as a basis for understanding mechanisms of antioxidant/pro-oxidant actions, is presented in *Volume 4, Chapter 7*.

Reactive oxygen species (ROS) are continuously generated by normal metabolism in the body [10] and these ROS have various physiological effects [11]. Cellular production of ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$), occurs from both enzymic and non-enzymic reactions. Mitochondria appear to be the most important subcellular site of ROS production, in particular of $O_2^{\bullet -}$ and H_2O_2 in mammalian organs. The electron transfer system of the mitochondrial inner membrane is a major source of superoxide production when molecular oxygen is reduced by a single electron. Superoxide can then dismutate to form hydrogen peroxide (H_2O_2). This species can further react to form the hydroxyl radical ($HO^{\bullet}$) and ultimately water, as shown in Scheme 1 [12].

$$O_2 \xrightarrow{1\ e^-} O_2^{\bullet -} \xrightarrow{1\ e^-} H_2O_2 \xrightarrow{1\ e^-} HO^{\bullet} \xrightarrow{1\ e^-} H_2O$$

Scheme 1

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogen-ase and tryptophan dioxygenase can generate ROS during catalytic cycling. Auto-oxidation of small molecules such as dopamine, adrenaline (epinephrine), flavins, and quinols can be an important source of intracellular ROS production as well. In most cases, the direct product of such auto-oxidation reactions is superoxide anion [13].

An imbalance between oxidant production and antioxidants may produce excess ROS that can cause oxidative damage in vulnerable targets such as unsaturated fatty acyl chains in membranes, thiol groups in proteins and nucleic acid bases in DNA [14]. Such a state of 'oxidative stress' is thought to contribute to the pathogenesis of a number of human diseases [13], but it is still not clear what kinds of ROS play a role in such pathogenesis or where the major sites of ROS action occur. There is, however, convincing evidence that lipid peroxidation is related to human pathology such as that observed in atherosclerosis [15].

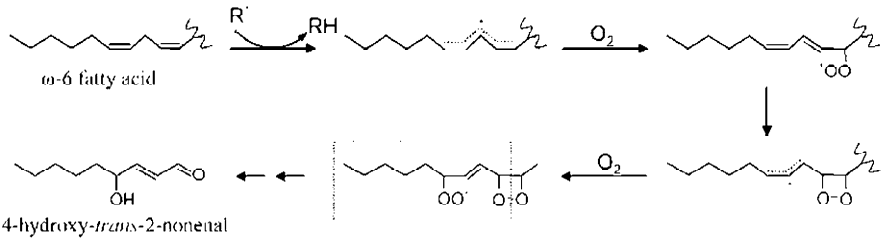


Fig. 1. Scheme for peroxidation of lipids containing ω-6 polyunsaturated fatty acid chains, illustrating the formation of 4-hydroxy-*trans*-nonenal [(*trans*)-4-hydroxynon-2-enal] (HNE).

A simplified pathway for peroxidation of lipids containing ω-6 polyunsaturated fatty acid chains (arachidonic and linoleic acid) and the subsequent formation of (*trans*)-4-hydroxynon-2-enal (HNE) is shown in Fig. 1. The ω-6 polyunsaturated acyl chains are susceptible to free-radical attack to form a free radical intermediate, which further reacts with molecular oxygen to generate first a peroxyl radical and then hydroperoxide derivatives such as (9*Z*,11*E*)-(13*S*)-13-hydroperoxyoctadeca-9,11-dienoic acid (13*S*-HPODE). The products of lipid peroxidation further react to produce HNE. It is important to underline that a peroxyl radical is capable of abstracting a H atom from another lipid molecule leading to the propagation stage of lipid peroxidation. The carbon radical formed can react with O_2 to form another peroxyl radical, and so the chain reaction of lipid peroxidation can continue [16].

The actions of antioxidants in biological systems depend on the nature of oxidants or ROS imposed on the systems, and the activities and amounts of antioxidants and their cooperative/synergistic interactions in these systems. The antioxidant actions of ascorbic acid (vitamin C) and tocopherols (vitamin A) and their interactions *in vitro* are well known [17,18],

although the biological significance of those well characterized antioxidants is still not clear [19].

This chapter will evaluate the evidence for an antioxidant action of carotenoids *in vitro*, *ex vivo* or *in vivo*, and will also consider briefly the evidence concerning pro-oxidation. Methods to determine antioxidant/prooxidant actions of carotenoids, factors that affect the efficiency of these actions, and interactions of carotenoids are also discussed.

B. Analytical Methods to Determine Antioxidant/Pro-oxidant Actions of Carotenoids in Biological Samples

Any compound that can inhibit oxidation that is induced either spontaneously or by means of external oxidants is considered to be an antioxidant. This is a relatively simple definition but, at times, it becomes very difficult to evaluate whether a compound actually has an antioxidant action, particularly *in vivo*.

Several methods to measure the antioxidant and pro-oxidant effect of carotenoids have been proposed and applied *in vitro*, *ex vivo* and *in vivo*. Some methods measure the intermediate or final products of the oxidative damage. Because carotenoids are lipid-soluble compounds and act as inhibitors of the lipid peroxidation process, many of the methods are based on the measurement of the consequences of lipid peroxidation, including intermediate (hydroperoxides, conjugated dienes) and/or final breakdown compounds, such as thiobarbituric acid reactive substances (TBARS) as shown in Section **B**.2. The methods must be sensitive and, more importantly, highly specific, because to evaluate the real antioxidant/pro-oxidant significance of carotenoids *in vivo*, they need to be applicable in complex biological matrices such as tissue preparations, plasma or cells, where the synergistic/cooperative effect of carotenoids with hydrophilic/lipophilic compounds takes place. Other methods are based on assays that measure the effect of carotenoids to modulate the oxidative resistance and total antioxidant activity of the biological matrix.

1. Total antioxidant capacity

An assay to measure total antioxidant capacity in a biological sample such as plasma must consider factors such as the heterogeneity of the sample, which consists of both hydrophilic and lipophilic compartments that contain water-soluble and fat-soluble antioxidants, respectively, as shown in Fig. 2. The cooperative/synergistic interactions among antioxidants in biological samples cannot be overlooked.

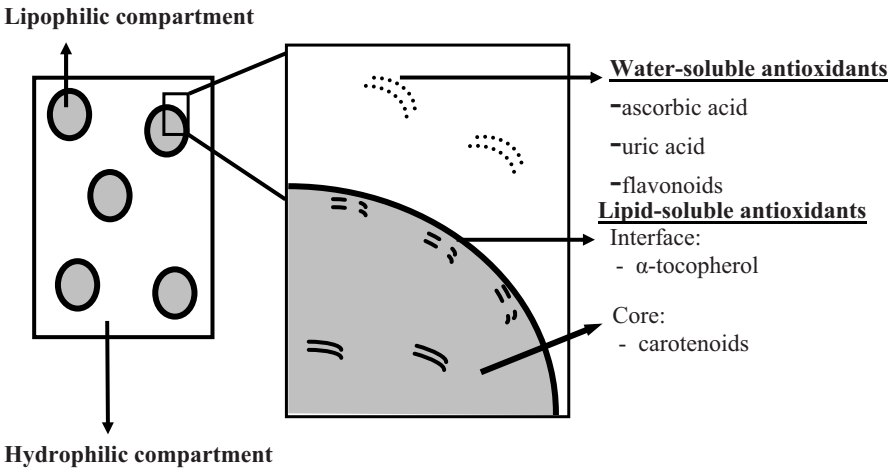


Fig. 2. Schematic representation of the hydrophilic and lipophilic compartments of plasma, indicating the antioxidants that may be present in each compartment.

Azo-initiators are a class of radical inducers (which contain the –N=N– group) widely used in experiments *in vitro* to generate radical species. The azo-initiators decompose at a temperature-controlled rate to give carbon-centred radicals which react rapidly with O_2 to give the peroxyl radical ($ROO^{\bullet}$) (Scheme 2).

$$R–N=N–R \rightarrow N_2 + 2R^{\bullet}$$
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

Scheme 2

Peroxyl radicals derived from azo-initiators can induce the lipid peroxidation cascade and can also damage proteins. Depending on the lipophilicity of the azo-initiators (AAPH is water-soluble whereas AMVN and MeO-AMVN are lipophilic), the peroxyl radicals are generated in the aqueous or lipid phase of the sample. The choice of the site of radical generation is of great importance since the activities of antioxidants present in both the lipid and aqueous compartments depend on the localization of the attacking radical species [20].

Table 1 lists assays that are used to determine antioxidant capacity in hydrophilic and lipophilic environments in biological samples such as plasma. Those assays [15,21] that use only hydrophilic radical initiators and probes are not sufficient to determine the antioxidant activity of carotenoids, which are deeply embedded in the lipoprotein core of biological samples. Attempts to determine the activity of fat-soluble antioxidants by measuring the antioxidant activity of lipid extracts dissolved in an organic solvent [22] cannot measure the

possible interactions between the fat-soluble and water-soluble antioxidants. The alternative approach of producing radicals in the lipid compartment of whole plasma and monitoring lipid peroxidation by a lipophilic probe [23] allows measurement of the actual 'total' antioxidant activity including possible interactions among antioxidants located in the hydrophilic and lipophilic compartments, because the interference of large amounts of protein in the hydrophilic compartment can be overcome by this approach.

Table 1. Assays that are used to determine antioxidant capacity in hydrophilic and lipophilic environments in plasma and other biological samples.

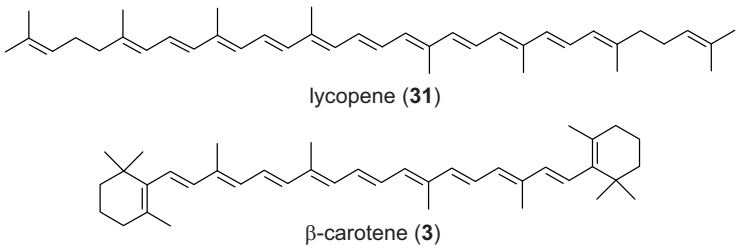
Assay	Medium	Radical inducer	Oxidizable substrate (probe)	Calculation	Ref.
Hydrophilic					
TRAP	Diluted (x250) plasma	AAPH	DCFH R-Phycoerythrin	Lag time	[15] [24]
ORAC	Diluted (x150) plasma	AAPH	R-Phycoerythrin	AUC	[25]
TEAC	Diluted plasma	ABTS		Absorbance	[26,27]
FRAP	Diluted (x100) plasma	Fe^{3+}		Absorbance	[28]
Crocin bleaching	Diluted (x250) plasma	ABAP		Absorbance	[29,30]
Lipophilic					
Lipophilic ORAC	Plasma extracts dissolved in solvent	AAPH	Fluorescein	AUC	[22]
Lipophilic antioxidant activity	Diluted (~x3) labelled plasma (requires 12 hr for labelling with DPHPC)	AAPH	DPHPC	Lag time	[31]
TAP	Diluted (x5) plasma	MeO-AMVN	BODIPY 581/591	AUC	[23]

TRAP: Total radical-trapping antioxidant parameter
ORAC: Oxygen radical-absorbing capacity
TEAC: Trolox equivalent antioxidant capacity
FRAP: Ferric-reducing ability of plasma
TAP: Total antioxidant performance
AAPH, ABAP: 2,2′-Azobis-(2-amidinopropane) dihydrochloride
ABTS: 2,2′-Azinobis-(3-ethylbenzothiazoline 6-sulphonate)
AUC: Area under the curve
MeO-AMVN: 2,2′-Azobis-(4-methoxy-2,4-dimethylvaleronitrile)
DCFH: 2′,7′-Dichlorodihydrofluorescein
DPHPC: 1-Palmitoyl-2-((2-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl-*sn*-glycero-3-phosphocholine
BODIPY 581/591: 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid

Attempts to show a direct correlation between the consumption of dietary carotenoids and subsequent changes in antioxidant capacity in humans have failed consistently [32,33]. It has even been suggested that carotenoids may not act as antioxidants *in vivo* [6]. However, these suggestions are derived from the lack of an adequate analytical method for measuring antioxidant capacity. Inasmuch as conventional methods such as TRAP, ORAC, *etc*., use primarily hydrophilic radical generators and measure primarily antioxidant capacity in the aqueous compartment of plasma, these methods are unable to determine the antioxidant capacity of the lipid compartment [34,35]. Therefore, it is not surprising that most of the methods used to measure purported 'total antioxidant capacity' of plasma show no effects of lipophilic antioxidants, such as vitamin E and carotenoids [32,33,35]. This can be explained by considering that plasma carotenoids, being deeply embedded in the core of lipoproteins, are not available for reaction with aqueous radical species or ferric complexes used in these assays. It has been reported that the activities of antioxidant nutrients present in the lipid and aqueous compartments depend on the localization of the attacking radical species, and can be increased synergistically by interactions [20].

When the hydrophilic assays were applied, the majority of the antioxidant capacity of plasma could be accounted for by protein (10-28%), uric acid (7-58%), and ascorbic acid (3-27%), whilst the effect of vitamin E (<10%) was minimal [15,28,29,36,37]. These assays measure the antioxidant capacity of the aqueous compartment only, since the radical inducers and probes are all hydrophilic. α-Tocopherol, which has its chroman head group oriented towards the lipoprotein membrane, may participate somewhat in the antioxidant action through interaction with water-soluble antioxidants such as ascorbic acid. However, it is clear that carotenoids that are deeply embedded in the lipid core could not participate in the antioxidant effect under these experimental conditions. The lack of contribution of fat-soluble antioxidants can also be ascribed to the relatively lower amount of fat-soluble antioxidants than of water-soluble antioxidants in plasma, although it should be recognized that the antioxidant activity of fat-soluble antioxidants can be greatly enhanced by synergistic interactions with water-soluble antioxidants and with other fat-soluble antioxidants.

Thus, foods such as green tea [38,39], cocoa [40], red wine [29,41], coffee [42], and strawberries [43], that contain considerable amounts of water-soluble polyphenols, significantly increase plasma antioxidant capacity as determined by these hydrophilic assays. On the other hand, diets rich in carotenoids, *e.g*. lycopene (**31**) or β-carotene (**3**) do not affect antioxidant capacity as measured by the hydrophilic TRAP, FRAP or ORAC assays [32,44,45]. In spite of the consistent failure to show the modification of antioxidant capacity by consumption of a high carotenoid diet [32] or supplementation with carotenoids in humans [20], it is noteworthy that there is considerable and consistent evidence for antioxidant actions of carotenoids [5,46], including *Z* isomers [47], tested in solvent systems *in vitro*.



2. Lipid peroxidation

In mammalian tissues, malondialdehyde (MDA) originates from the oxidative degradation of polyunsaturated fatty acids (PUFAs) with more than two unconjugated double bonds. The main precursors of MDA are arachidonic acid (20:4) and docosahexaenoic acid (22:6). In certain tissues MDA can also be formed by enzymic processes, for example by human platelet thromboxane synthase from prostaglandins (PGH_2, PGH_3 and PGG_2) or by renal polyamine oxidase from spermine [48].

The thiobarbituric acid (TBA) analysis is one of the most frequently used assays for measuring MDA in biological matrices. The reaction (Fig. 3) is carried out in acid where two moles of TBA react with one mole of MDA, to form a pink reaction product (λ_{max} = 532 nm), which is readily extractable into organic solvents such as butan-1-ol [49].

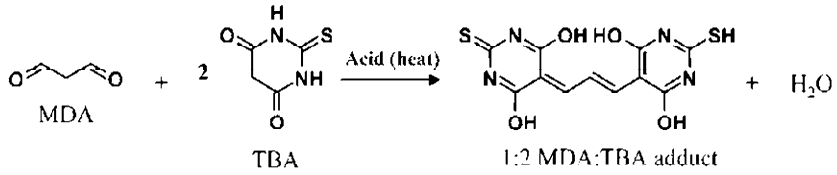


Fig. 3. The 'TBARS' reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). The 1:2 MDA:TBA adduct that is generated can be determined quantitatively by its light absorbance at 532 nm.

For many years, determination of TBARS such as MDA was assumed to be a valid measure of lipid peroxidation, but it is, in fact, a somewhat unspecific biomarker. Nevertheless, changes in MDA levels have been used to evaluate the effects of enhancing or depleting dietary or supplementary nutrients such as carotenoids in conditions where an oxidative stress might arise [50]. However, the fact that β-carotene interferes with this type of assay because its breakdown yields products that give a positive TBARS reaction should be carefully considered, especially in studies of the pro-oxidant effect of carotenoids at high concentrations [51].

Measurement of prostaglandin F_2-like compounds (F_2-isoprostanes), which are produced *in vivo* by non-enzymic free radical-catalysed peroxidation of arachidonic acid, has emerged as one of the most reliable approaches to assessing oxidative stress status [52,53]. It is generally accepted that F_2-isoprostanes more accurately reflect lipid peroxidation *in vivo* than do TBARS [54] and that F_2-isoprostane concentrations can be lowered by dietary antioxidant supplements [55]. Problems, such as the complicated technique to measure isoprostanes and their instability in biological samples, still need to be overcome, however. It has been reported that supplementary β-carotene, even when given at high doses (50 mg/day) for many years (median 4 years), does not have pro-oxidant effects in either smokers or non-smokers, as measured by urinary excretion of F_2-isoprostanes [56]. Recent efforts to determine plasma isoprostanes may help to eliminate the experimental error introduced by the complicated sample preparation steps, including derivatization, needed for the traditional GC-MS assays.

3. Oxidation of low-density lipoprotein (LDL)

The antioxidant properties of bioactive components present in food, including vitamins E and C, polyphenols and carotenoids, against low-density lipoprotein (LDL) oxidation have been reviewed extensively [57]. The general approach to measure the antioxidant capacity in the lipid compartment of plasma is to determine the susceptibility of isolated LDL to oxidation by hydrophilic radical inducers (AAPH, transition metal ions) or lipophilic radical inducers such as 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN). Lipid peroxidation can be estimated by measuring the UV absorbance of conjugated dienes at 234 nm [58], oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) or oxidation of diphenyl-1-pyrenylphosphine (DPPP) to produce the fluorescent product, DPPP oxide [59]. When the lipophilic AMVN is used as radical initiator and luminol as an oxidizable substrate, the contribution of the fat-soluble antioxidants to the antioxidant capacity of isolated LDL has been shown to be greater than 70% (tocopherol, 73%; ubiquinol-10, 2.5%) [60]. This approach is limited, however, because it does not take into account the potential interaction between water-soluble and fat-soluble antioxidants, a synergism that may greatly increase the total antioxidant activity. It is generally accepted that α-tocopherol can act as a pro-oxidant to initiate lipid peroxidation in isolated LDL [61]. This tocopherol-mediated lipid peroxidation can be prevented, however, by ascorbic acid, which can regenerate α-tocopherol from α-tocopheroxyl radical [62]. In addition, a recent report, which indicates markedly different LDL oxidation kinetics depending on the concentration of copper ion added into LDL, implies possible misinterpretation of LDL oxidation data when the LDL oxidation is calculated on the basis of the inhibition period, *i.e*. the lag-time [63].

4. DNA damage

The single-cell microgel electrophoresis technique, named the 'comet' assay, was developed to detect single or double strand breaks in DNA. The broken DNA fragments show greater migration in electrophoresis than the undegraded DNA, giving rise to a diffuse DNA substance area which, after staining, resembles a comet tail [64] as shown in Fig. 4.

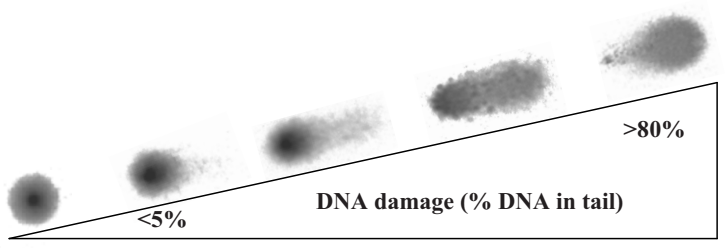


Fig. 4. Illustration of the effects of increasing damage (strand breaks) to DNA, revealed by electrophoresis in the comet assay.

Endogenous strand breaks, as well as the resistance of DNA to oxidative stress caused by treating lymphocytes with hydrogen peroxide (H_2O_2), can be evaluated by the comet assay. Much of the recent material relating to the effects of carotenoids on DNA damage, as well as effects on DNA synthesis and proliferation, has been reviewed [65], leading to the conclusion that, in cell cultures, carotenoids can inhibit DNA synthesis and proliferation, change gene expression, decrease micronucleus frequency and inhibit transformation *via* inhibition of gap-junction proteins. In humans, a diet containing carotenoid-rich foods has been shown to reduce lymphocyte DNA damage, suggesting that the carotenoids may be acting as antioxidants *in vivo* [20]. However, it is not yet known whether oxidative DNA damage in blood cells reflects similar damage in other target tissues.

The product of oxidative damage to DNA most commonly measured in urine and/or blood is 8-hydroxy-2-deoxyguanosine (8-OHdG), even though there is still some question about the validity of this marker for evaluating DNA damage, because of the lack of baseline value standardization and reliability [66].

5. Other assays for biomarkers

a) Pulse radiolysis

The effectiveness of individual carotenoids as antioxidants can be determined by pulse radiolysis. Free-radical forms of several carotenoids, if they are sufficiently long-lived for their reduction potentials to be measured, have been detected by this technique. In addition,

the rates of free radical scavenging by carotenoids have been reported [67]. Even though these experiments do not reproduce biological conditions, they contribute to better understanding of the underlying chemistry of carotenoid activity, as discussed in *Volume 4, Chapter 7*.

b) HPLC/mass spectrometry

A sensitive, selective, specific and rapid method, HPLC linked to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was developed and validated for the simultaneous determination of the Michael adducts between (*trans*)-4-hydroxynon-2-enal (HNE), one of the most reactive unsaturated aldehyde products of lipid peroxidation, and endogenous peptides containing histidine and cysteine [68]. The electrophilic nature of α,β-unsaturated aldehydes and ketones makes these compounds highly reactive with cellular nucleophiles and in particular with sulphydryl groups, the imidazolic nitrogen of histidine and the ε-amino group of lysine through the formation of Michael adducts [69]. As shown in Fig. 5 (left), C(3) of an α,β-unsaturated aldehyde is a strong electrophilic centre.

Histidine is one of the most reactive nucleophilic residues in proteins and is a primary reaction site for HNE addition [70] as shown in Fig. 5 (right).

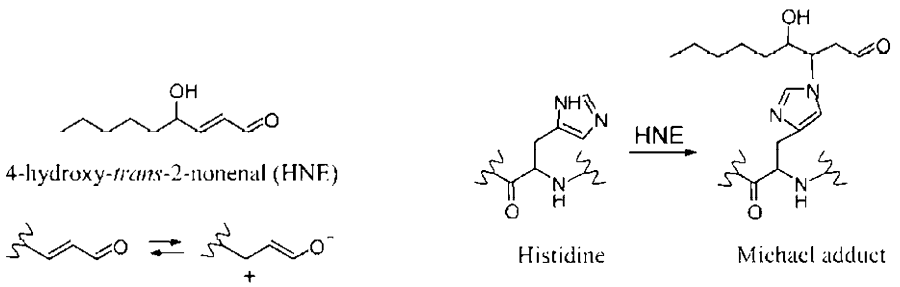
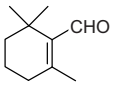


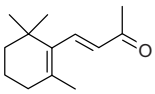
Fig. 5. Left: Tautomeric equilibrium of (*trans*)-4-hydroxynon-2-enal (HNE), illustrating the electrophilic site susceptible to nucleophilic attack by a histidine residue. Right: structure of the Michael adduct formed by reaction between HNE and histidine.

Most of the biological effects of reactive carbonyl-containing intermediate species, mainly α,β-unsaturated aldehydes, arise from the capacity of these compounds to react with the nucleophilic sites of proteins, to form advanced lipoxidation end-products [70,71], or react with DNA bases. Among the oxidation products of β-carotene, several α,β-unsaturated aldehydes and ketones such as trimethylcyclohexenone, β-cyclocitral and β-ionone, have been identified.

trimethylcyclohexenone (*1*)



β-cyclocitral (*2*)



β-ionone (*3*)

Oxidation of β-carotene gives products that react with thiobarbituric acid to give a pink condensation product, suggesting the presence of reactive dicarbonyl derivatives, structurally related to malondialdehyde, among the oxidation products [51]. It is reasonable to hypothesize that some β-carotene oxidation products containing α,β-unsaturated aldehyde or ketone groups could react with nucleophilic biological targets such as cysteine, histidine or lysine residues in proteins, or with DNA bases, to give Michael and Schiff base adducts. This could explain some of the biological effects that have been reported recently for oxidation products of β-carotene and lycopene, such as inhibition of cell proliferation [72], pro-oxidant effects [73], enzyme inhibition [74] and DNA damage [75] (see *Chapter 18*). Therefore, a specific and sensitive assay able to identify the target macromolecules of the reaction products of carotenoids would be useful to elucidate the mechanism by which carotenoids and, in particular, the corresponding oxidative breakdown products, affect the biological response.

C. Studies of Antioxidant/Pro-oxidant Actions of Carotenoids

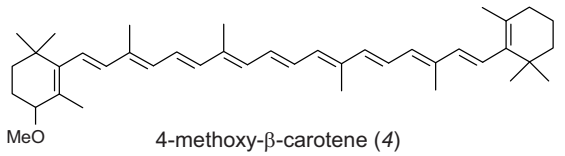
There have been many reports concerning the relative antioxidant efficacy of carotenoids, with varying results. Part of the problem is that different systems have been used to dissolve the carotenoids, initiate oxidative stress, and then evaluate efficacy. There is probably no single system that can accurately determine the antioxidant/pro-oxidant activity of carotenoids.

1. Studies *in vitro*

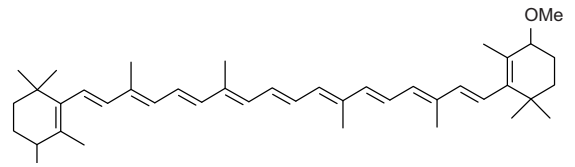
There are at least three kinds of reaction of carotenoids with radical species, namely radical addition, electron transfer to the radical, or allylic hydrogen abstraction. The radical addition/adduct formation mechanism [76] suggested that a lipid peroxyl radical ($ROO^{\bullet}$) might add to some positions on the carotenoid (CAR) polyene chain, resulting in the formation of a carbon-centred radical ($ROO\text{-}CAR^{\bullet}$). This resonance-stabilized radical would interfere with the propagating step in lipid peroxidation and would explain the many examples of the antioxidant effects reported for carotenoids in solution [5] (see Section **D**.2).

Electron transfer reactions have been reported that result in the formation of either the radical cation $CAR^{\bullet+}$, the radical anion $CAR^{\bullet-}$, or the neutral alkyl radical $CAR^{\bullet}$. The carotenoid radical cation is frequently detected by very fast spectroscopic techniques such as laser flash photolysis. This radical has been observed in studies of photosynthesis, and it has been proposed to play a role in photoprotection in photosystem 2 [77] (*Volume 4, Chapter 14*).

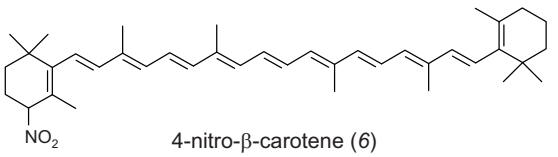
Hydrogen abstraction processes have been suggested [78] following the detection of 4-methoxy-β-carotene (*4*) and 4,4′-dimethoxy-β-carotene (*5*) when β-carotene is treated with either AIBN or AMVN in the presence of small amounts of methanol. Exposure of β-carotene to cigarette smoke resulted in the production of 4-nitro-β-carotene (*6*), also presumably *via* hydrogen abstraction at the allylic C(4) position [79].



4-methoxy-β-carotene (*4*)

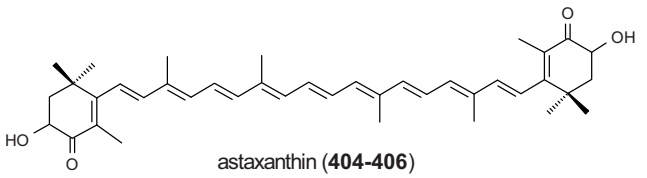


4,4'-dimethoxy-β-carotene (*5*)



4-nitro-β-carotene (*6*)

Studies on the antioxidant actions of carotenoids have been carried out in artificial membranes (liposomes, micelles) [18,80], isolated LDL [81] and tissue homogenates [82] in an attempt to mimic biological systems. It should be noted that the nature of the interaction between the carotenoids and the matrix in which they are studied dictates the effect, *e.g.* the antioxidant activity of carotenoids depends on the incorporation of carotenoids in the lipid bilayer [83]. In addition, a recent study [84] indicated that certain carotenoids such as astaxanthin (**404-406**), which can preserve membrane structure, exhibited significant antioxidant action.



astaxanthin (**404-406**)

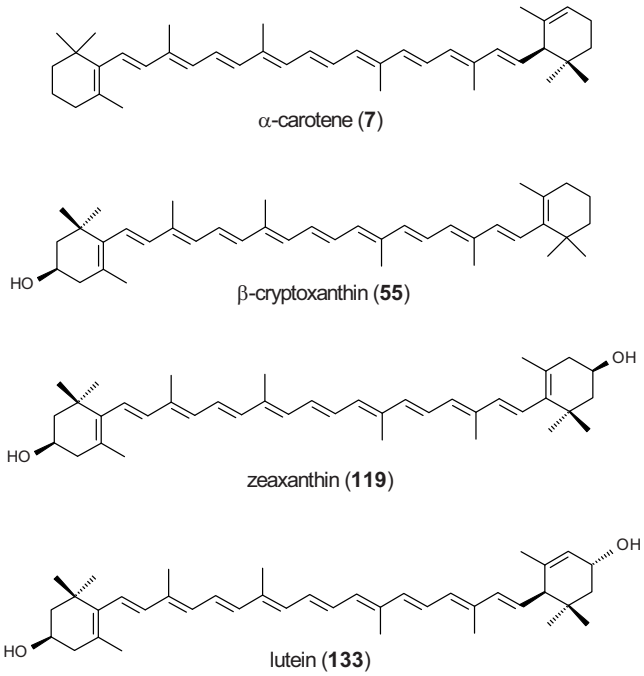
Many of the investigations *in vitro* used the development of thiobarbituric acid-related substances (TBARS) as an index of lipid peroxidation [85], but this assay is quite unspecific, as previously discussed. The oxidation of β-carotene itself by either nitrogen dioxide or oxygen results in measurable TBARS activity [51]. More direct effects have been reported; DNA in human promyelocytic leukaemia HL-60 cells exposed to a source of peroxynitrous acid was protected better by the prior administration of β-carotene than of either ascorbate or the water-soluble α-tocopherol analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [86].

2. Studies *ex vivo*

The ability to detect and/or monitor antioxidant action of any type of molecule *in vivo* is limited by the availability of adequate biomarkers. There is no single method yet that can assess the oxidant stress response or total antioxidant capacity in animals or humans.

Lipoprotein particles from animals or humans that have ingested carotenoids, either as part of their diet or *via* supplementation, can be isolated and evaluated. In the past few years, more investigators are using this approach, which is assumed to insert the carotenoids 'appropriately' in the LDL particle or target membrane. The oxidation of LDL particles was reported to be lower when they were enriched with carotenoids through dietary intervention with fruits and vegetables or by supplementation with carotenoids [87-89]. Supplementation with green vegetables did not protect LDL in either smokers or non-smokers, whereas supplementation with red vegetables was protective, but only in non-smokers [90]. Lycopene-containing, tomato-based products were reported to be effective against LDL oxidation [91] whereas pure lycopene was ineffective [87]. The variability of these results might be attributed to differences in the length of time for which the diet was supplied, in degrees of changes in the plasma carotenoid levels and, certainly, in study populations. Also, when fruits and vegetables are added to the diet, vitamin C and other potential antioxidants such as polyphenols and flavonoids may increase as well as plasma carotenoids, and could be responsible for any changes observed in LDL oxidation. Conflicting results have also been reported in studies with other carotenoids. In some cases, lycopene (**31**), α-carotene (**7**), β-cryptoxanthin (**55**), zeaxanthin (**119**) and lutein (**133**) were reported to be effective as antioxidants [92] but, in some studies in which β-carotene was effective, the addition of either lutein or lycopene actually increased LDL oxidation [93]. A 12-week period of daily supplementation with either 13 mg lycopene or 112 mg β-carotene resulted in an increase in carotenoids in LDL, but no change in LDL oxidizability [81]. Determination of antioxidant nutrient concentrations in LDL, and of LDL resistance as expressed by lag time to oxidation, led to the conclusion that LDL composition did not predict resistance to Cu-stimulated oxidation [94].

Whether dietary or supplemental carotenoids have any protective effect against LDL oxidation, therefore, remains unresolved.



3. Studies *in vivo*

The lack of appropriate biomarkers to determine oxidative stress *in vivo* makes it difficult to determine whether dietary carotenoids alter the oxidative stress in humans. Various animal species, including ferrets [95], gerbils [96] and pre-ruminant calves [97], have been used to study carotenoid absorption. Most experimental animals, however, require large, pharmacological doses of carotenoids, because their ability to absorb carotenoids is low.

For many years, the rather unspecific biomarker TBARS was used to determine lipid peroxidation. It should be noted, however, that any conjugated dialdehyde in the plasma can react with thiobarbituric acid resulting in increased TBARS values, which are usually attributed to increased MDA concentrations. Women on carotenoid-deficient diets showed increased plasma MDA levels [50] but this effect could be reversed when the diets were supplemented with a mixture of carotenoids, strongly supporting the idea that dietary carotenoids can serve to decrease oxidative stress in humans. A recent report [80] found significantly decreased plasma oxidizability in subjects given a high fruit and vegetable diet, and this was followed by a significant increase in oxidizability after transfer to a low fruit and vegetable diet. This, therefore, supports the attribution of antioxidant activity *in vivo* to some component(s) in the fruit and vegetables, which could include carotenoids.

There are a few studies on the effect of carotenoids on the enzymic antioxidant defence systems. For example, no difference was found in superoxide dismutase (SOD) activity in haemolysates of washed erythrocytes from HIV patients who had been given 30 mg/day β-carotene for 1 year [98].

When carotenoids are administered at fairly high doses, they can accumulate in the skin. This phenomenon has been the subject of many investigations to determine if this accumulation can lead to sun protection, which may or may not be related to an antioxidant action (see *Chapter 16*). When a group of volunteers were supplemented for 24 weeks with natural carotene (99% β-carotene), starting at 30 mg/day and increasing to 90 mg/day by the end of the experiment, modest protective effects against sunlight were observed, but no significant dose-dependent inhibition was seen in a commercial assay for lipid peroxidation [99]. It is not clear whether the increased tissue concentrations of carotenoids are directly associated with antioxidant activity. It has been suggested, however, that the carotenoids found in the eye, *e.g.* in the ciliary body, in the retinal pigment epithelium and the choroid, may be acting as antioxidants [100] (see *Chapter 15*).

It has been suggested that oxidative stress plays a role in the early stages of the pathophysiological processes of many chronic diseases. Significantly elevated basal DNA damage, as revealed by the comet assay, was reported in patients suffering from coronary artery disease (53 cases, 28-68 years, 42 controls, 30-67 years) [101], breast cancer (70 cases, 70 controls, 53 years) [102] (40 cases, 60 controls) [103], and head and neck squamous cell cancer (38 cases, 13-78 years, 44 controls, 44-78 years) [104] compared to the level of DNA damage in healthy subjects.

The relationship between DNA damage and the consumption of fruits and vegetables has been suggested by the observation of lower DNA damage in the summer than in the winter, corresponding to the difference in the seasonal intake of dietary antioxidants [105]. However, intervention trials involving increased fruit and vegetable intake have shown mixed results. On the one hand, decreased oxidative DNA damage has been reported [106] with 12 servings/day of fruit and vegetables for 14 days, whereas, in another study, daily consumption of 600 g of fruit and vegetables for 4 weeks showed no effect on DNA damage and repair [107], as determined by urinary and blood 8-hydroxydeoxyguanosine.

Several short-term intervention studies involving carotenoid-rich diets and assay of lymphocyte DNA damage by single-cell gel electrophoresis (comet assay) have been reported. Cross-over studies with healthy female subjects fed 600 mL/day of orange juice for 21 days (16 subjects, 20-27 years) [108], 25g of tomato puree for 14 days [109], or for 21 days (10 subjects, mean age 23.1 years) [110] and/or 150 g of spinach per day for 21 days (9 subjects, mean age 25.2 years) [111] resulted in reduced oxidative DNA damage. A similar reduction was observed in a group of 26 men (mean age 25.4 years) and women (mean age 26 years) treated with 250 mL/day of tomato extract drink for 26 days [112]. Also, a 14-day intervention with tomato juice, carrot juice or dried spinach powder (23 non-smoking male subjects, age 27-40 years) [113] or a polyphenol-rich juice [114] was reported to be beneficial

against basal DNA damage in healthy men. In addition, dietary interventions for 3 weeks with tomato sauce, providing 30 mg lycopene per day, resulted in significantly decreased oxidative DNA damage in prostate cancer patients [115,116]. On the other hand, a recent study [117] showed no difference in DNA damage between intervention groups of healthy, well-nourished non-smoking men who received two, five or eight servings/day of fruit and vegetables.

Assuming that, in humans, the bioavailabilities of lycopene and lutein supplements are similar to those of pureed and oil-containing tomato-based foods [118] and green leafy vegetables [119], respectively, (see *Chapter 7*) similar biological actions of pure forms could be expected.

Table 2. Studies that have been reported on the effects of antioxidants on the damage and repair of lymphocytes.

Subjects	Intervention	DNA damage/ repair	Ref
Male (50-59 yr) smokers (n=50) non-smokers (n=50)	Vit C 100 mg, vit E 280 mg and β-carotene 25 mg, daily for 20 wks Placebo controlled	Decreased DNA damage	[120]
Male and female (n=40, 25-45 yr)	α/β-Carotene, lutein or lycopene 15 mg, 12 weeks Placebo controlled	No effect Inverse correlation between serum carotenoids and oxidized pyrimidines	[123]
Male (n=5) female (n=3) (24-34 yr)	β-Carotene, lutein or lycopene consecutively, 15 mg/day for 7 days, with 3wk wash-out	Increased DNA repair by β-carotene and lycopene not by lutein	[124]
Female (n=37, 50-70 yr)	12 mg of either lutein, β-carotene, or lycopene or 4 mg each of lutein, β-carotene, and lycopene, daily for 57 days Placebo controlled	Decreased DNA damage	[125]
Male (n=64, 18-50 yr)	Vitamin C 60 mg daily for 21 days Placebo controlled	No effect	[127]
Male (non-smokers) (n=64, 18-50 yr)	8.2 mg β-Carotene, 3.7 mg α-carotene and 1.75 mg α-tocopherol, daily for 21 days Placebo controlled	Increased DNA repair	[127]
Male and female (n=77, age ≥40)	0, 6.5, 15, or 30 mg lycopene/day for 57 days Placebo controlled	Decreased DNA damage in 30mg/day group only	[126]

Intervention studies with a combination of antioxidant supplements have consistently shown protective effects against DNA damage (Table 2). A combination of micronutrients in a relatively high dose (*i.e.* 100 mg of vitamin C, 280 mg of vitamin E and 25 mg of β-carotene)

per day for 20 weeks resulted in a significant decrease in basal DNA damage in adult men aged 50-59 years [120]. Supplementation for 21 days with a combination of carotenoids (8 mg lycopene, 0.5 mg β-carotene) and vitamin C (11 mg) in younger females (mean age 25.2 years) [121] or a combination of carotenoids (8.2 mg β-carotene, 3.7 mg α-carotene) and α-tocopherol (1.75 mg) in males, 18-50 years, has been reported to be protective against oxidative DNA damage [122]. However, it is not clear whether there is any effect of a single carotenoid against DNA damage. No effect on endogenous DNA damage was reported following supplementation with 15 mg/day of either α-carotene and β-carotene, lutein or lycopene for 12 weeks in men and women (25-45 years) in a placebo-controlled parallel study design [123]. This study did, however, reveal an inverse correlation between total serum carotenoids and oxidized pyrimidines. On the other hand, another study using the same amount of lutein, β-carotene or lycopene supplementation (15 mg/day) successively, each for 1 week, showed significant increases in DNA repair in younger men and women (24-34 years) [124]. Further, a recent study [125] reported that there was a significant decrease in basal DNA damage after 15 days supplementation with either 12 mg of a single carotenoid or 12 mg of a combination of carotenoids (4 mg each of lutein, β-carotene and lycopene) in elderly women (50-70 years). The protective effect was maintained throughout the study period of 57 days. A dose-response study (0, 6.5, 15, or 30 mg/day lycopene) indicated that supplementation with 30mg/day lycopene for 8 weeks resulted in significantly decreased DNA damage in healthy adults [126].

In general, carotenoid-rich diets and a combination of carotenoids show a stronger protective effect against DNA damage than does a single carotenoid. Further studies are needed to establish any effect of physiological doses of carotenoids in combination with other antioxidants contained in fruits and vegetables on oxidative DNA damage, and to support the role of a diet rich in fruit and vegetables in the prevention of chronic diseases such as cardiovascular diseases and cancer.

D. Factors that Affect Antioxidant/Pro-oxidant Actions of Carotenoids

Carotenoids can exhibit pro-oxidant as well as antioxidant behaviour, as first described for the auto-oxidation of β-carotene [128].

An impetus to the study of the factors that determine whether carotenoids, in particular β-carotene, show antioxidant or pro-oxidant behaviour was the release of the findings of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) [129], an intervention trial of α-tocopherol and β-carotene for the primary prevention of lung cancer. This study showed that, in Finnish male smokers, those who received supplemental β-carotene had an 18% increase in lung cancer incidence. Then the U.S. Carotene and Retinol Efficacy Trial (CARET) was terminated nearly 2 years early because the group receiving the combination of β-carotene plus retinol had a 28% increase in lung cancer incidence [130]. Various theories

were put forward to explain why β-carotene may exhibit such pro-carcinogenic activity under these conditions. Because one of the features of both these intervention studies was the high dose of β-carotene given as supplement, several studies *in vitro* addressed the question of whether carotenoids can become harmful at high concentrations. Moreover, because the increased cancer incidence was localized in lung, a tissue exposed to a high partial pressure of oxygen (tracheal or bronchial air 150 torr, alveolar air 105 torr) relative to that of venous blood (40 torr) and the surface of the alveolar cell (20 torr), the effect of oxygen was also considered to be a parameter that could affect the behaviour of β-carotene. In a recent prospective study with 59,910 French women [131], β-carotene intake was inversely associated with the risk of tobacco-related cancers among non-smokers and there was a statistically significant dose-dependent relationship, whereas high β-carotene intake was directly associated with risk among smokers, after a median follow-up of 7.4 years. In a recent systematic review, six randomized clinical trials that examined the efficacy of β-carotene supplements and 25 prospective observational studies that assessed the associations between carotenoids and lung cancer were subjected to random-effects meta-analysis. An inverse association between carotenoids and lung cancer was detected, but the decreases in risk were generally small and not statistically significant. It was concluded that the inverse association may be due to the carotenoid measurements being a marker of a healthier lifestyle *i.e*. higher fruit and vegetable consumption, or to residual confounding by smoking [132].

Factors that may determine the switch from antioxidant to pro-oxidant behaviour are discussed in the following sections.

1. Concentration of carotenoids

The ability of supplementary carotenoids at different concentrations to protect cells against oxidatively induced DNA damage (as measured by the comet assay), and membrane integrity (as measured by ethidium bromide uptake) has been studied [133]. Either lycopene or β-carotene afforded protection against DNA damage only at relatively low concentrations (1-3 μM) whereas, at higher concentrations (4-10 μM), the ability to protect the cell against such oxidative damage was rapidly lost and, indeed, the presence of carotenoids increased the extent of the DNA damage. Similar data were obtained when protection against membrane damage was studied. An increased intracellular level of reactive oxygen species (ROS) was found in adenocarcinoma cells treated with high concentrations of β-carotene, leading to growth inhibition and apoptosis [134]. The pro-oxidant effect of β-carotene was also observed *in vivo*. Excess dietary β-carotene enhanced lipid peroxidation in animals exposed to different conditions such as methyl mercuric chloride intoxication or tocopherol depletion [8].

In recent reviews [9,135], several hypotheses have been proposed for the molecular mechanism involved in the pro-oxidant effect of high concentrations of β-carotene. The main hypotheses are that high concentrations of β-carotene can lead to one or more effects.

(i) A more favourable formation of β-carotene peroxyl radical and/or a faster rate of β-carotene auto-oxidation, leading to the formation of $O_2^{\bullet -}$.
(ii) Modification of iron concentrations, increasing the production of ROS through a Fenton reaction. This can be exacerbated by carotenoids that are characterized by a low oxidation potential (β-carotene has the lowest oxidation potential) which recycle Fe^{2+} by reducing Fe^{3+} ions, thus inducing a carotenoid-driven Fenton reaction [136].
(iii) ROS formation *via* induction of various cytochrome P450 isoforms.
(iv) Formation of aggregates that crystallize out of solution. Such carotenoid aggregates (*Volume 4, Chapter 5*) have been directly observed in membranes, and their presence is thought to have a profound effect on the properties of the membrane itself, by leading to an increase in membrane fluidity, which could result in a pro-oxidant effect.
(v) Formation of oxidation products that exert a pro-oxidant effect.

2. Oxygen tension

Oxygen tension greatly affects the switch between antioxidant and pro-oxidant effects of β-carotene [8]. The pro-oxidant effect of β-carotene induced by a high partial pressure of oxygen has been demonstrated in several experimental models such as rat liver microsomes [137], isolated DNA, and cells [138,139]. The effect of oxygen on the antioxidant/pro-oxidant character of β-carotene has been attributed mainly to a proposed equilibrium between carotenoid radicals and oxygen (Equations 1-6, Scheme 3) [2,140].

$$ROO^{\bullet} + CAR \rightarrow ROO\text{–}CAR^{\bullet} \quad (1)$$
$$ROO\text{–}CAR^{\bullet} + O_2 \leftrightarrow ROO\text{–}CAROO^{\bullet} \quad (2)$$
$$ROO\text{–}CAROO^{\bullet} + CAR \rightarrow ROO\text{–}CAROOH + CAR^{\bullet} \quad (3)$$
$$ROO\text{–}CAROO^{\bullet} + LH \rightarrow ROO\text{–}CAROOH + L^{\bullet} \quad (4)$$
$$CAR^{\bullet} + O_2 \leftrightarrow CAROO^{\bullet} \quad (5)$$
$$L^{\bullet} + O_2 \leftrightarrow LOO^{\bullet} \quad (6)$$

Scheme 3

According to this scheme, the peroxyl radical-carotenoid adduct (ROO-CAR$^{\bullet}$) is generated by reaction of a carotenoid (CAR) with peroxyl radical (Eq. 1). This adduct then reacts reversibly with oxygen to form the corresponding peroxyl radical (ROO-CAROO$^{\bullet}$) (Eq. 2). At high oxygen concentration, the equilibrium shifts in favour of formation of ROO-CAROO$^{\bullet}$, which can then either react with CAR, inducing auto-oxidation and the formation of the radical CAR$^{\bullet}$ (Eq. 3), or with lipids, thus perpetuating lipid peroxidation (Eq. 4). The resultant radicals, CAR$^{\bullet}$ and L$^{\bullet}$ can also react reversibly with O_2 to form peroxyl radicals (Eq. 5 and 6). At low oxygen concentrations, however, the equilibrium (Eq. 2) shifts toward ROO-CAR$^{\bullet}$,

which can react with another $ROO^{\bullet}$ or form an epoxide *via* alkoxyl radical elimination, thus acting as an antioxidant.

The effect of oxygen partial pressure on the antioxidant activity of β-carotene has been studied [76] by measuring the initial rate of oxidation of tetralin and methyl linoleate in chlorobenzene. This study gave evidence that β-carotene exhibits good radical-trapping antioxidant behaviour only at low partial pressures of oxygen (less than 150 torr) and that at higher oxygen pressures (760 torr), β-carotene loses its antioxidant activity and shows an autocatalytic, pro-oxidant effect, particularly at relatively high concentrations. Changes in the pO_2 alter the ability of β-carotene to inhibit lipid peroxidation in a membrane model, rat liver microsomes [137]. At 150 torr pO_2, β-carotene acted as an antioxidant, inhibiting lipid peroxidation whereas, at 760 torr pO_2, it acted as a pro-oxidant, increasing MDA formation. In cultures of human lung cells treated with AAPH to induce oxidative stress, β-carotene was found to be an antioxidant at both low (0 torr) and normal (143 torr) oxygen tension conditions but, under high oxygen conditions (722 torr; 97% oxygen), the antioxidant effects were decreased, and a pro-oxidant effect was observed, as measured by isoprostane formation [139]. More recently, the formation of lipid peroxidation products (measured as conjugated dienes and TBARS) was studied in rat lung microsomal membranes enriched *in vitro* with varying β-carotene concentrations (from 1 to 10 nmol/mg protein) and then incubated with tar (6-25 μg/ml) under different pO_2. The exposure of the microsomal membranes to tar induced a dose-dependent increment of lipid peroxidation, which progressively increased as a function of pO_2. Under a low pO_2 (15 torr), β-carotene clearly acted as an antioxidant, inhibiting tar-induced lipid peroxidation. The carotenoid progressively lost its antioxidant efficiency as pO_2 increased (50-100 torr), however, and acted as a pro-oxidant at pO_2 from 100 to 760 torr in a dose-dependent manner [141].

3. Exposure to ultraviolet light

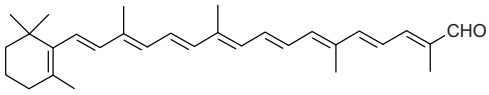
The antioxidant/pro-oxidant effect of β-carotene can also be greatly affected by exposure to UV radiation. β-Carotene is an efficient quencher of $^1O_2$, and this allows it to show protective effects against UVA-dependent matrix metalloprotease (MMP) expression in cell culture [142] and against lipid peroxidation in mouse skin [143]. The relationship between carotenoids and skin health is discussed in *Chapter 16*. Other studies, however, have demonstrated a pro-oxidant effect of β-carotene in cell cultures and a carcinogenic effect in mice on UVA exposure. In particular, a pro-oxidant effect of β-carotene in the 0.5-5 μM range was found in skin fibroblasts exposed to suberythaemal doses of UVA light (20 J/cm^2), as deduced from the induction of haem oxygenase-1 [144] and expression of interleukin-6 as markers of oxidative stress [145]. It has also been reported [146] that UVA irradiation of human skin fibroblasts led to a 10-fold to 15-fold rise in matrix metalloprotease-1 (MMP-1) mRNA, and that this increase was suppressed in the presence of low μM concentrations of

vitamin E, vitamin C, or carnosic acid but not by β-carotene or lycopene (prepared in a special nanoparticle formulation). Indeed, in the presence of 0.5-1.0 μM β-carotene or lycopene, the UVA-induced MMP-1 mRNA was further increased by 1.5 to 2-fold. This increase was totally suppressed when vitamin E was included in the nanoparticle formulation.

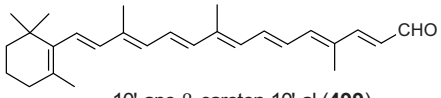
In human dermal fibroblasts, the presence of β-carotene or lycopene (0.5-1.0 μM) led to a 1.5-fold rise in the UVA-induced haem oxygenase-1 mRNA levels. A pro-oxidant effect of β-carotene was reported in mouse fibroblasts under UVA irradiation; β-carotene (20 μM) enhanced DNA strand breaks, an effect which was significantly suppressed by co-incubation with flavonoids such as naringenin, rutin or quercetin [147]. Relatively stable cyclic mono- and diendoperoxides have been identified as first products of the reaction of β-carotene with singlet oxygen [148]. These products remain reactive in the dark and cause auto-oxidation of β-carotene. The cyclic endoperoxides have been considered as candidates to explain the unforeseen pro-oxidant activity of β-carotene.

4. Oxidative stress

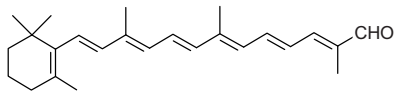
Recently an interesting hypothesis has been proposed for the mechanism responsible for the switching between antioxidant/pro-oxidant effects of β-carotene [73,149,150]. According to this, under conditions of moderate oxidative stress, the antioxidant effects of β-carotene predominate, but under heavy oxidative stress, β-carotene undergoes an oxidative breakdown leading to the formation of reactive breakdown products which are responsible for the pro-oxidant activity and harmful effects of β-carotene. These β-carotene breakdown products include reactive aldehydes such as 8′-apo-β-caroten-8′-al (**482**), 10′-apo-β-caroten-10′-al (**499**), 12′-apo-β-caroten-12′-al (**507**), 14′-apo-β-caroten-14′-al (**513**), retinal (*7*), and short-chain products such as β-cyclocitral (*2*), β-ionone (*3*), 5,6-epoxy-β-ionone (*8*) and 4-oxo-β-ionone (*9*) [149].



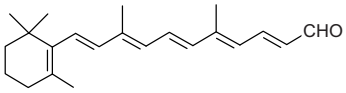
8'-apo-β-caroten-8'-al (**482**)



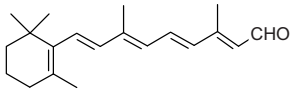
10'-apo-β-caroten-10'-al (**499**)



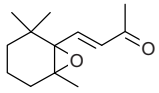
12'-apo-β-caroten-12'-al (**507**)



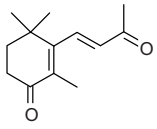
14'-apo-β-caroten-14'-al (**513**)



retinal (*7*)



5,6-epoxy-β-ionone (*8*)



4-oxo-β-ionone (*9*)

The chemical reactivity of most of these compounds with other biomolecules is still unknown although it has been proposed that the reactivity and biological effects may be similar to those induced by the reactive aldehydes from lipid peroxidation, such as 4-hydroxynonenal (HNE) and MDA [149]. These β-carotene breakdown products were found to exert several damaging effects such as (i) inhibiting state 3 respiration in isolated rat liver mitochondria, which is accompanied by increased oxidative stress in the mitochondria, as reflected by a decrease in glutathione and protein SH groups and an increase of MDA, and (ii) genotoxic effects (micronuclei and chromosomal aberrations) at sub-micromolar concentrations [151]. This hypothesis could explain the pro-oxidant effects induced by a series of different factors such as O_2, UV, and general oxidants including smoke or hypochlorous acid (HOCl), that are known to switch on the pro-oxidant effects of β-carotene and are characterized by the induction of oxidative breakdown of carotenoids. The cytotoxic effects of aldehydes derived from breakdown of β-carotene, lutein and zeaxanthin on human retinal pigment epithelial cells (ARPE-19) have been examined. A significant increase of oxidative stress and ROS generation accompanied by an increased number of apoptotic cells was observed following treatment with the aldehydes [152]. A mixture of β-carotene breakdown products in primary hepatocytes showed a genotoxic potential at concentrations in the range100 nM and 1 μM, [153] and significantly enhanced the genotoxic effects of oxidative stress exposure [154].

The question of whether some of the reported beneficial effects of carotenoids may, in fact, be mediated by some of these oxidation products is treated in *Chapter 18*.

5. Interaction with membranes

Studies with model membranes enriched with polyunsaturated fatty acids have indicated that interaction with the membrane is a critical influence on the antioxidant/pro-oxidant activity of carotenoids. Differential effects of carotenoids on lipid peroxidation rates were partially attributed to their orientation and location, as determined by small angle X-ray diffraction (see *Volume 4, Chapters 5* and *10*). The apolar carotenoids lycopene and β-carotene, which disorder the membrane bilayer, show a potent pro-oxidant effect whereas astaxanthin preserves membrane structure and exhibits significant antioxidant activity [84,155].

6. Up-regulation of the receptor for advanced glycation endproducts (RAGE)

Receptors for advanced glycation endproducts (RAGE) have recently been implicated as promoters and/or amplifiers of oxidant-mediated cell death induced by diverse agents. Increased RAGE expression is observed in conditions that are associated with unbalanced production of reactive species, such as atherosclerosis and neurodegeneration. It was proposed that supplementation with retinol increases RAGE protein expression in cultured Sertoli cells, and that co-treatment with antioxidant reversed this effect. Moreover, the retinol-increased RAGE expression was observed only at concentrations that induce production of intracellular reactive species, as assessed by the DCFH assay [156].

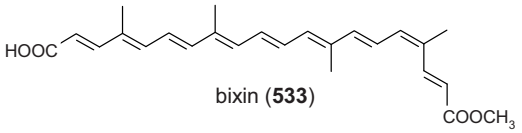
E. Interactions of Carotenoids

The synergistic/cooperative interactions of hydrophilic and lipophilic antioxidants have been studied *in vitro* in various systems such as homogeneous phase solvent systems [17], liposomes [18,80], micelles, isolated LDL [157], cells [158] and tissue preparations [82].

1. Interactions between carotenoids

Possible interactions between carotenoids in terms of competition for incorporation into micelles, carotenoid exchange between lipoproteins, and inhibition of provitamin A cleavage have been reviewed [159]. It has been reported that β-carotene supplementation, which results in a moderate increase in the serum β-carotene concentration, does not significantly affect the serum concentrations of other carotenoids [160]. In the past few years, intervention studies with a combination of carotenoids have been reported. Supplementation with 24 mg/day of β-carotene for 12 weeks or an equal amount of a carotenoid mixture, containing lutein, β-carotene and lycopene, ameliorated UV-induced erythaema in humans [161]. In addition, a protective effect of a mixed carotenoid supplement [β-carotene, α-carotene, lycopene, lutein, bixin (**533**) and mixed paprika carotenoids] against LDL oxidation induced by fish oil, has

been reported [162]. Furthermore, a recent study comparing the effect of individual carotenoids (12 mg each of lutein, β-carotene or lycopene) with that of an equal amount of mixed carotenoids (4 mg each of lutein, β-carotene and lycopene) against lymphocyte DNA damage clearly indicated synergistic interaction between carotenoids *in vivo* [105].



bixin (**533**)

2. Interactions of carotenoids with other antioxidants

Interactions of different antioxidants in plasma have been studied extensively in the past decade. In particular, work has focused both on the interactions between hydrophilic and lipophilic antioxidants, such as ascorbic acid and α-tocopherol [163], or carotenoids and ascorbic acid [164] and between lipophilic antioxidants (carotenoids and α-tocopherol) [82,165].

It has been reported that β-carotene, which is located in the lipophilic core of the membrane bilayer, can directly interact with water-soluble antioxidants. By scavenging radical species in a heterogeneous micellar environment, β-carotene can be converted into its radical cation $CAR^{\bullet+}$ or peroxyl radical cation $CAR\text{-}OO^{\bullet+}$ [166], which are more polar than β-carotene itself, and can be reoriented towards the hydrophilic compartment, allowing ascorbic acid to repair the β-carotene radical [167]. Other work [164] has also shown an interaction between β-carotene radical cations and ascorbic acid.

The combination of α-tocopherol and β-carotene has been reported to act cooperatively to slow down MDA formation initiated by the aqueous peroxyl radical generator, AAPH, in a liver microsomal membrane preparation [82]. β-Carotene added to preformed lipid bilayers produced much less of an antioxidant effect than β-carotene incorporated in the liposomes during bilayer formation [83]. It is possible that α-tocopherol reduces β-carotene peroxyl radicals ($LOO\text{-}\beta\text{-}C\text{-}OO^{\bullet}$) as well as β-carotene radical cations ($\beta\text{-}C^{\bullet+}$), as has been shown in a homogeneous solution [165]. In addition, β-carotene may recycle α-tocopherol from the α-tocopheroxyl radical ($\alpha\text{-}TO^{\bullet}$) through electron transfer [168], although this possible mechanism of action should be studied further, because the reduction potential of β-carotene is reported to be lower than that of α-tocopherol [169,170]. In addition, a synergistic antioxidant activity of lycopene in combination with vitamin E in a liposome system has been reported [171].

It is interesting to note that daily supplementation with moderate doses of combined antioxidants (100 mg vitamin C, 100 mg vitamin E, 6 mg β-carotene and 50 μg selenium) significantly increased plasma antioxidant capacity and decreased chromosome aberrations in

lymphocytes [172]. On the other hand, a meta-analysis of randomized trials with antioxidant supplements suggested that high doses of β-carotene [173] or α-tocopherol [174] led to significant increases in mortality due to all causes and no effect against coronary heart disease risk [175,176].

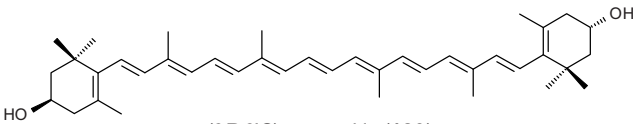
It is likely that physiological doses of a combination of water-soluble and fat-soluble antioxidant nutrients are required to establish an effective antioxidant network *in vivo*.

F. Conclusions: Possible Biological Relevance of Antioxidant/Pro-oxidant Actions of Carotenoids

Epidemiological evidence (see *Chapter 10*) continues to accumulate that diets high in fruits and vegetables [177-181] and carotenoids [182,183] are associated with a reduced risk of chronic diseases such as cardiovascular disease (*Chapter 14*). The evidence for a protective effect against cancer (*Chapter 13*) is much less clear [178], possibly, in part, because of measurement error [184]. Carotenoids may be among the group of antioxidants in fruits and vegetables that help to prevent damage caused by harmful reactive oxygen species, which are continuously produced in the body during normal cellular functioning and are introduced from exogenous sources [10]. It is believed that dietary supplementation with antioxidants, including carotenoids, can be a part of a protective strategy to minimize the oxidative damage in vulnerable populations, such as the elderly. Carotenoids have antioxidant activity *in vitro* at physiological oxygen tensions [139]. However the significance of the antioxidant effect of carotenoids *in vivo* remains controversial and difficult to demonstrate [122,185]. It should be pointed out that the metabolism and functions of carotenoids *in vivo* and *in vitro* may not be the same. For example, antioxidant nutrients can interact with each other during gastrointestinal absorption and metabolism [186-189]. Considering that the antioxidant system *in vivo*, which is finely balanced, and requires the right amounts, possibly an optimal range, of both the hydrophilic and lipophilic antioxidants, then carotenoids located in the core of a lipophilic compartment may be necessary for the antioxidant network to function properly in biological systems.

The benefits of carotenoids for eye health have been of great interest recently (see *Chapter 15*). It is generally accepted that lutein and zeaxanthin are associated with protection of the retina and retinal pigment epithelium from damage by light and oxygen [190,191]. For example, individuals (n = 356 Age-related Macular Degeneration (AMD) cases, n = 520 controls) in the highest quintile of carotenoid intake had a 43% lower risk for AMD compared with those in the lowest quintile [192], and among the specific carotenoids, lutein and zeaxanthin were most strongly associated with a reduced risk for AMD ($p = 0.001$). Laboratory studies, which had identified the macular pigments as lutein and zeaxanthin, are supportive of the epidemiological observations [193-195]. A recent prospective 18-year follow-up study of 71,494 women and 41,564 men, aged ≥50 and with no diagnosis of AMD,

showed no association between lutein/zeaxanthin intake and neovascular AMD risk [196]. Further, a double blind randomized study indicated no evidence of an effect of 9 or 18 months of daily supplementation with a lutein-based nutritional supplement on visual function in healthy subjects [197].



(3*R*,3'*S*)-zeaxanthin (**120**)

In a high fruit and vegetable diet, the intake of lutein is more than five times higher than that of zeaxanthin. It has been proposed that lutein and zeaxanthin in blood are taken up by the retina, where some of the lutein is then converted into (3*R*,3'*S*)-zeaxanthin (*meso*-zeaxanthin, **120**) [198]. It is possible that zeaxanthin may be more effective than lutein as an antioxidant in the central macula [193]. Lutein circulates in the blood at higher concentrations than zeaxanthin, but the concentration of zeaxanthin in the central macula is higher than that of lutein. However, increased consumption of dietary sources of lutein and zeaxanthin has been shown to increase macular pigment in some, but not all individuals. It has been reported that there was 27% prevalence of non-responders in terms of macular pigment density after the consumption of lutein/zeaxanthin-rich foods such as spinach [199]. It is not yet known what factors affect individual responses to lutein supplementation. Further research is required in an effort to determine the biological function of lutein and zeaxanthin in relation to eye health. A recent prospective study of 39,876 female health professionals [200] and a cross-sectional study (n = 1443) conducted in North India [201] reported that higher dietary intakes of lutein/zeaxanthin and vitamin E from food and supplements were significantly associated with decreased risks of cataract. In addition, astaxanthin has been reported to protect porcine lens crystallins from oxidative damage [202]. Even though its antioxidant activity [203] is still questionable [204], the beneficial effect of astaxanthin to improve vascular elastin and arterial wall thickness in hypertensive rats by modulating oxidative conditions has been reported [205].

The antioxidant property of lycopene may be one of the mechanisms for its putative effect against coronary heart disease (*Chapter 14*). It has been reported that lycopene inhibits LDL oxidation synergistically in combination with vitamin E or flavonoids [206]. In addition, in healthy women, increased tomato consumption resulted in reduced susceptibility of LDL to oxidation [207]. However, knowledge of the mechanism of action of lycopene as well as well-designed clinical studies are required to provide stronger evidence for a direct role of lycopene in coronary heart disease prevention [208].

In addition to its anti-atherogenic effect, the possible anti-carcinogenic effect of lycopene has been studied. Increased lycopene consumption resulted in significantly reduced leukocyte

DNA damage in prostate cancer patients [116]. Recently, in a pilot study involving supplementation with 15 mg lycopene/day for 6 months [209], patients with histologically proven benign prostate hyperplasia but free of prostate cancer showed significantly decreased prostate-specific antigen (PSA) levels and no change in the prostate, whereas the placebo group showed progression of prostate enlargement. An anti-carcinogenic property of lycopene has been demonstrated at the molecular level in an animal model, the ferret [210-212]. It has been suggested that lycopene may have protective effects against smoke-induced lung carcinogenesis through up-regulating insulin-like growth factor binding protein-3 (IGFBP-3) [213] as well as against smoke-induced gastric carcinogenesis through changing the gastric mucosal p53 phosphorylation [212] in ferrets (see *Chapter 11*). Whether this is related to any antioxidant action of lycopene remains to be determined.

Evidence has accumulated that high fruit and vegetable intake is associated with lower risk of chronic diseases such as cardiovascular diseases and age-related macular degeneration. It is possible that antioxidant nutrients such as carotenoids in fruits and vegetables can prevent or reduce damage from harmful free radicals that are produced in the body. However, intervention studies have failed to show a consistent beneficial effect of high doses of antioxidant supplementation against chronic diseases. One possible explanation for these apparently contradictory results between observational studies and intervention trials is that the antioxidant system *in vivo*, which is finely balanced, requires the right amount, possibly an optimal range, of both the hydrophilic and lipophilic antioxidants to be working properly. The optimal ranges of antioxidants might be achieved best by a balanced dietary fruit and vegetable intake but not by a high dose of only one or a limited mixture of antioxidant supplements, which could cause an imbalance of the antioxidant machinery leading in some cases to a pro-oxidant effect. In addition, other phytonutrients abundant in fruits and vegetables may not only exert unique biological functions but may also interact synergistically with well recognized antioxidants to promote antioxidant effects.

Various biomarkers have been developed to determine the biological functions of carotenoids and effects on genomic stability. It seems, however, that there is no single system that accurately determines the biological actions of carotenoids, due to the limitations of analytical techniques in relation to the lipophilicity of carotenoids and the model systems used to evaluate the effects. In addition, there are various factors such as carotenoid concentration, oxygen tension, UV exposure and oxidative stress that can affect the antioxidant activity of carotenoids. A synergistic/cooperative interaction between carotenoids and with other antioxidants such as tocopherols, ascorbic acid, and flavonoids, appears to play an important role in the biological antioxidant network. Therefore, an important future direction of research is to elucidate how best to improve our body defence systems against oxidative damage, which in turn might reduce the risk of chronic diseases, by means of dietary modification rather than by taking large amounts of antioxidant supplements.

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