Katri Haila

Effects of Carotenoids and Carotenoid-Tocopherol Interaction on Lipid Oxidation *In Vitro*

1) Scavenging of Free Radicals

2) Formation and Decomposition of Hydroperoxides

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium XII, University Main Building, on April 24th 1999, at 10 o'clock.

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Abstract

This thesis dealed with antioxidant/pro-oxidant action of carotenoids in *in vitro* lipid systems. The review of literature described the antioxidant/pro-oxidant chemistry of carotenoids and summarized the available data on the antioxidant/pro-oxidant action of carotenoids in different lipid systems. The experimental part evaluated the effects of carotenoids and carotenoid-tocopherol interaction on lipid oxidation based on free radical scavenger properties by electron spin resonance (ESR) study and the formation and decomposition of hydroperoxides.

In ESR spin-trapping experiments, radicals were thermally generated from (2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) and trapped by N-*tert*-butyl-α-phenylnitrone (PBN). In acetone, all carotenoids (0.17 mM) except β-carotene diminished the formation of the amount of spin adducts. β-Carotene was a pro-oxidant at higher concentration (0.90 mM). In toluene, all carotenoids except lutein, bixin and a combination of lutein and β-carotene diminsihed the amount of spin adducts. The differences in free radical scavenging between eight carotenoids including β-carotene, astaxanthin, canthaxanthin, zeaxanthin, lutein, cryptoxanthin, lycopene and bixin were small.

The effects of carotenoids on the formation of hydroperoxides was examined in three different lipid models *in vitro*: triacylglycerol fraction of rapeseed oil (RSO TAGS), emulsified RSO TAGS and methyl linoleate. The decomposition of hydroperoxides was examined in emulsified RSO TAGS. Based on promotion of hydroperoxide formation, β-carotene (20 μg/g), lutein (5-40 μg/g) and lycopene (20 μg/g) were pro-oxidants in autoxidized RSO TAGS. Based on both formation and decomposition of hydroperoxides, β-carotene (0.45-20 μg/g) was a pro-oxidant in emulsified RSO TAGS. In methyl linoleate, both β-carotene (5-200 μg/g) and its oxidative breakdown product, retinal (7-360 μg/g), promoted the formation of hydroperoxides. The pro-oxidant effect was stronger with higher concentrations of the carotenoid. This thesis raised the question of the effect of carotenoid breakdown products on pro-oxidant role of carotenoids as demonstrated with retinal. The loss of yellow colour indicates the pro-oxidant action of carotenoids in lipids. On the other hand, even a minor amount of tocopherol protected carotenoids from destruction and thus from pro-oxidant effect. In RSO TAGS and emulsified RSO TAGS, a combination of carotenoid and tocopherol at certain proportion was a more efficient antioxidant than tocopherol alone.

In conclusion, the present results demonstrated that carotenoids are *in vitro* pro-oxidants in lipid environment and may act as free radical scavenging antioxidants in chemical solutions.

Preface

Colour is essential to the full enjoyment of our food. Yellow, orange and red coloured carotenoids are much more than pigments. For this reason the multidisciplinary area of carotenoid anti- and pro-oxidant chemistry is not only important, but also attractive. I have experienced the moments of scientific work challenging and educational.

The present PhD thesis was carried out at the University of Helsinki, Department of Applied Chemistry and Microbiology, Food Chemistry, Viikki, Finland and at the Royal Veterinary and Agricultural University (KVL), Food Chemistry, Frederiksberg, Denmark. This academic dissertation is done under the 'Applied Bioscience -Bioengineering, Food & Nutrition, Environment' (ABS) program of the Finnish Graduate School. My warm thanks go to all my colleagues who have provided me with advice in their particular area of expertise. Not forgetting those who helped me with minor or major practical questions, I would particularly like to acknowledge the following.

In Viikki, I thank most sincerely Professor Vieno Piironen for thorough reading of the thesis manuscript. Vieno's attitude and support have had a fundamental importance in completing this thesis. I would like to thank Professor emeritus Pekka Koivistoinen for introducing me to the field of food chemistry and for advice during my first steps as a PhD student. I am grateful to Marina Heinonen, PhD, Anu Hopia, PhD, Anna-Maija Lampi, LicSc, Satu Lievonen, MSc, Marjukka Mäkinen, MSc, Leena Kataja, MSc, and Vieno Piironen, for sharing the experimental part of the projects and for contribution in writing the papers. I thank Marina for introducing me to the field of carotenoids and supervising the projects. The discussions concerning the chemistry of antioxidants and lipid oxidation with Anu were stimulating. My warm thanks go to Marjukka and Satu for sharing good moments in and out of the lab.

I was privileged to visit KVL and share the positive atmosphere at the Department of Food Chemistry during this study. I am grateful to Professor Leif Skibsted and Dr. Bo Rud Nielsen for all the advice and help during the ESR-project. I also warmly thank Leif for continuous encouragement and support.

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I appreciate Dr. Marja Mutanen's interest in my writing of the thesis manuscript. The discussions concerning the manuscript as well as scientific work in general with Maisa were interesting and valuable.

Participation in international scientific meetings and discussions with colleagues have provided me an important source of inspiration and current knowledge during the PhD studies. I am glad for the experience at the Nordic (NorFA) network on lipid oxidation, the European Nutrition Leadership Programme and other scientific meetings in the area of free radicals and antioxidants.

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This work would not have become a reality without genuine support of many wonderful people. I would like to express my deepest gratitude to my closest network. This strong net, including parents, brother, grandparents, cousins and other relatives, goddaughters, bird group and friends near and far, has always been present whenever needed. I have enjoyed not only good food and wine, but also long and interesting discussions concerning many aspects of life. It is chemistry.

Helsinki, March 1999

Katri Haila

List of Original Publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Haila, K., Nielsen, B., Heinonen, M. & Skibsted, L. 1997. Carotenoid reaction with free radicals in acetone and toluene at different oxygen partial pressures. An ESR spin-trapping study of structure-activity relationships. Z. Lebensm. Unters. Forsch. A/Food Res. Technol. 204: 81-87.

II Haila, K., Hopia, A. & Heinonen, M. 1999. Effects of β-carotene and retinal on the formation and isomer distribution of methyl linoleate hydroperoxides. Submitted.

III Haila, K., Lievonen, S. & Heinonen, M. 1996. Effects of lutein, lycopene, annatto, and •-tocopherol on autoxidation of triglycerides. J. Agric. Food Chem. 44: 2096-2100.

IV Haila, K. & Heinonen, M. 1994. Action of β–carotene on purified rapeseed oil during light storage. Lebensm.-Wiss.u.-Technol./Food Sci. Technol. 27: 573-577.

V Heinonen, M., Haila, K., Lampi, A.-M. & Piironen, V. 1997. Inhibition of oxidation in 10% oil-in-water emulsions by β-carotene with α- and γ-tocopherols. J. Am. Oil Chem. Soc. 74: 1047-1052.

Contents

List of Abbreviations

AAPH	2,2’-azobis(2-amidinopropane)
ABTS$^{\bullet+}$	2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt)
AIBN	azo-*bis*-isobutyronitrile
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
A-N=N-A	azo-initiator e.g. AMVN
AnV	anisidine value
BHT	2,6-di-tert-butyl-4-methylphenol
BO	butter oil
CAR	carotenoid
CAR$^{\bullet}$	carotenoid radical
CAR$^{\bullet-}$	carotenoid radical anion
CAR$^{\bullet+}$	carotenoid radical cation
$CCl_3OO^{\bullet}$	trichloromethylperoxyl radical
CD	conjugated dienes
$DNPO_2$	3,3’-(1,4-naphthylene) dipropionate
ESR	electron spin resonance
HPLC	high performance liquid chromatography
LDL	low density lipoprotein
MDA	malondialdehyde
$NO_2^{\bullet}$	nitrogen dioxide radical
$^1O_2$	singlet oxygen
$[O_2]$	oxygen consumption
PBN	N-*tert*-butyl-α-phenylnitrone
PBNO$^{\bullet}$	N-benzoyl-N-*tert*-butylnitroxide
PC	phosphatidylcholine
R$^{\bullet}$	alkyl radical
RH	unsaturated fatty acid
RO$^{\bullet}$	alkoxyl radical
ROO$^{\bullet}$	alkylperoxyl radical (used for lipid ROO$^{\bullet}$ unless otherwise mentioned)
ROOH	lipid hydroperoxide
RS$^{\bullet}$	thiyl radical
$RSO_2^{\bullet}$	sulfonyl radical
RSO	low erucic acid rapeseed oil
RSO TAGS	triacylglycerols purified from low-erucic acid rapeseed oil (see 4.1)
PV	peroxide value
SO	soybean oil
SO TAGS	soybean oil triacylglycerols
TAGS	triacylglycerols
TBA	thiobarbituric acid
TBARS	thibarbituric acid reactive substances
UV	ultraviolet

1. Introduction

In nature, carotenoids are mainly responsible for red, yellow and orange colours. However, in green plant tissues, the colour of carotenoids is masked by the more dominant pigment, chlorophyll, and becomes evident only during the degradation of chlorophyll. This phenomenon can be seen during the ripening of fruits as well as in autumn leaves. In foods, in addition to their function as the natural pigments and provitamin A precursor role of certain carotenoids, these compounds can be used as food additives for colouring (European Parliament and Council Directive, 1994). The natural characteristic of carotenoid colour is significantly present in fruits and vegetables. Furthermore, diets rich in fruits and vegetables containing carotenoids have been of interest because of their potential health benefit against chronic diseases.

Since 1981, when Peto et al. raised the idea dietary β-carotene from fruits and vegetables as a protective agent against cancer, carotenoids have received wide research interest as potential antioxidants. According to Krinsky (1993), the appearance of Peto's article was the start of a growing interest in carotenoid action, in both *in vitro* studies and animal models, and intervention studies. In the beginning of the 1980's, the scientific knowledge of the antioxidant action of β-carotene was mainly based on observational studies which widely reported that a higher consumption of vegetables and fruits rich in β-carotene and other carotenoids was associated with a lower risk of cancer and cardiovascular disease. In the 1990's, the large intervention trials on supplemental β–carotene have reported unexpected results. Contrary to observational studies, the major intervention trials on β-carotene supplementation have reported a lack of protection against cancer and cardiovascular disease. In Finland, the ATBC study reported that supplemental β-carotene may in fact have harmful effects, whereas dietary β-carotene had an adverse protective effect, among smokers (The Alpha-Tocopherol, Beta Carotene Cancer [ATBC] Prevention Study Group, 1994; Rapola, 1998). In addition, the supplementation of β-carotene did not protect smokers and workers exposed to asbestos (Omenn et al., 1996) or healthy men (Hennekens et al.,1996). On the other hand, the supplementation with the combination of β–carotene, vitamin E and selenium may inhibit cancer development. The Linxian trial observed a significant reduction in total mortality, due mostly to a lowered risk of cancer, among general adult population receiving the combination of β-carotene, vitamin E and selenium (Blot et al., 1993). Many questions have remained unanswered after the results of antioxidant boom. Several authors e.g. Krinsky (1993), Mayne (1996) and Omaye et al. (1997), have recently stated that more evidence is needed to understand many of the associations between carotenoids and the observed effects in risks of disease. Perhaps the bright orange colour of β-carotene was partly a misleading mask in fruits and vegetables when β-carotene, a single pigment, was picked up as the potential protective agent from thousands of components in fruits and vegetables. As Mayne (1996) commented, it is difficult to interpret whether the apparent benefits are due to antioxidant vitamins, nutrients, dietary habits or other nondietary lifestyle characteristics. Still, much more experimentation is needed to establish the

important dietary antioxidants and their optimal intake (Halliwell, 1997; Osborne et al., 1997).

In the beginning of the 1980's, in addition to the provitamin A activity of some carotenoids, the possible beneficial effects of carotenoids on health were linked to their role as antioxidants. According to Omaye et al. (1997), much of the evidence has supported the hypothesis that lipid oxidation or oxidative stress may be the underlying mechanism in chronic diseases and that β-carotene would act as an antioxidant *in vivo*. Furthermore, Burton and Ingold suggested in their pioneering work that β-carotene would be an unsual type of lipid antioxidant working at low oxygen concentrations (Burton and Ingold, 1984). In all, carotenoids have been considered as antioxidants, rather than pro-oxidants based on experimental evidence *in vitro* (Astorg, 1997; Burton, 1989; 1993; Edge et al., 1997; Handelman, 1996; Krinsky, 1989; 1993; Sies et al., 1992). It is known that carotenoids may act as antioxidants by quenching singlet oxygen or by reacting with free radicals. Moreover, the antioxidant/pro-oxidant properties of carotenoids are affected by the concentration of carotenoid, oxygen partial pressure and the nature of the environment. The mechanisms of reactions between carotenoids and radical species may involve radical addition, hydrogen abstraction and electron transfer, but the precise antioxidant/pro-oxidant mechanisms remain unclear (Britton, 1995; Tsuchihashi et al., 1995; Liebler and McClure, 1996; Edge et al., 1997). Further chemically based understanding on antioxidant/pro-oxidant action of carotenoids is necessary.

The antioxidant/pro-oxidant action of carotenoids on lipid oxidation have been of interest in food lipids as well as in biological membrane lipids. Since the major constituents of biological membranes are lipid and protein, lipid oxidation can damage membrane lipids (Halliwell and Gutteridge, 1995, p. 188). Most previous research on the effects of carotenoids on lipid oxidation have reported antioxidant activity in membrane lipid models *in vitro* (recently reviewed e.g. by Krinsky, 1993; Handelman, 1996). In foods, extensive investigation has been focused on lipid oxidation, which is of importance by leading in rancidity (Labuza, 1971). Although carotenoids have not been considered as food antioxidants (Löliger, 1991), some authors have proposed antioxidant role for carotenoids in food lipids. In different experimental conditions, the discrepant data has shown both antioxidant (e.g. Kiritsakis and Dugan, 1985; Fakourelis et al.,1987; Lee and Min, 1988; 1990; Min and Lee, 1989; Jung and Min, 1991) and pro-oxidant activity in food lipids (e.g. Olcovich and Mattill, 1931; Olcott, 1934; Terao et al., 1980; Faria and Mukai, 1983; Suzuki et al., 1989; Warner and Frankel, 1987). In all, β-carotene has been the prototype for examining antioxidant action of carotenoids in different *in vitro* lipid models. Thus, there has been a clear need for information on antioxidant/pro-oxidant effects of carotenoids other than β-carotene as well as carotenoid-tocopherol interaction.

This thesis deals with antioxidant/pro-oxidant action of carotenoids, with emphasis on the effects on lipid oxidation *in vitro*. The review of literature describes the antioxidant/pro-oxidant chemistry of carotenoids as well as summarizes previous studies on the antioxidant/pro-oxidant action of carotenoids in different lipid systems. The review of the experimental part of the thesis, which refers to five original articles, summarizes studies on the effects of carotenoids and carotenoid-tocopherol interaction on autoxidized lipids based on formation and decomposition of hydroperoxides as well as an electron spin resonance study on the free radical scavenger properties of carotenoids.

2. Review of the Literature

2.1 Basic concepts

Oxidation
Oxidation can be defined as: loss of electrons by an atom or molecule and the adverse reaction reduction as: gain of electrons by an atom or molecule (Halliwell and Gutteridge 1995, p. 12).

Free radical
The term *free radical* can be defined as any species capable of independent existence that contains one or more unpaired electrons. Ground-state O_2 ($^3O_2$) has two unpaired electrons each located in a different antibonding orbital. An oxidizing agent, such as O_2, is good at absorbing electrons from the molecule it oxidizes (Halliwell and Gutteridge, 1995, p. 10). The collective terms reactive oxygen species (Halliwell et al., 1995) or active oxygen species (Handelman, 1996) have been applied for a variety of free radicals and non-radical intermediates such as singlet oxygen or hydrogen peroxide involved in oxidative stress.

Lipid oxidation
This thesis deals with non-enzymic lipid oxidation and uses the term *lipid oxidation*. The literature dealing with living systems on the other hand often uses the term *lipid peroxidation* (e.g. Halliwell and Gutteridge, 1995) for the same phenomenon. Photosensitized lipid oxidation, with added photosensitizers, is discussed in 2.5.2. For a thorough discussion of lipid oxidation see e.g., Chan (1987), Frankel (1991), Gardner (1989), Halliwell and Gutteridge (1995, p. 188), Labuza (1971) and Porter et al. (1995). In brief, a chemical reaction that usually takes place at ambient temperatures between atmospheric oxygen and organic compound is generally referred to as *autoxidation*. An important feature of autoxidation is that it is autocatalytic. The rate of spontaneous oxidation is slow at the beginning and increases as the reaction progresses (Chan, 1987). Autoxidation is a free radical chain reaction consisting of initiation, propagation and termination steps (*reactions 1-4*).

Initiation:	$X^{\bullet} + RH$	$\rightarrow$	$R^{\bullet} + XH$			(1)
Propagation:	$R^{\bullet} + O_2$	$\rightarrow$	$ROO^{\bullet}$			(2)
	$ROO^{\bullet} + RH$	$\rightarrow$	$ROOH + R^{\bullet}$			(3)
Termination:	$ROO^{\bullet} + ROO^{\bullet}$	$\rightarrow$	[ROOOOR]	$\rightarrow$	nonradical products	(4)

In the initiation step, polyunsaturated lipids (RH) may form alkyl radicals ($R^{\bullet}$) which react very rapidly with oxygen to form peroxyl radicals ($ROO^{\bullet}$). In the

propagation step, a chain reaction with more lipids produces hydroperoxides (ROOH), i.e. primary oxidation products. These hydroperoxides decompose in the presence of metals to produce alkoxyl radicals ($RO^{\bullet}$), which cleave into a complex mixture of aldehydes and other products, i.e. secondary oxidation products (Frankel, 1995).

According to Chan (1987) the initiation step of autoxidation can be difficult to define because of the low concentration of radicals and the likelihood of there being more than one process. The most probable initiation processes in autoxidation of unsaturated lipids are metal-catalyzed reactions (Chan, 1987). On the other hand, most available data on the antioxidant effects of carotenoids on lipid oxidation have focused on the free radical initiated, i.e. with added initiators, oxidation of different lipid models. For example, lipid soluble azo initiator 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) (paper I) and water soluble 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) decompose at a temperature-controlled rate to give a known flux of radicals. Thus, the use of added radical initiators has been chosen by many authors. AMVN and AAPH (A-N=N-A) form carbon-centred radicals (*reaction* 5, p. 13) that react with O_2 to yield peroxyl radicals (*reaction* 6, p. 13) capable of abstracting hydrogen from lipid in solution (Halliwell and Gutteridge, 1995, p. 211).

$$\text{A-N=N-A} \rightarrow N_2 + A^{\bullet} + A^{\bullet} \quad \text{carbon-centred radicals} \qquad (5)$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \quad \text{peroxyl radical} \qquad (6)$$

Antioxidants may inhibit oxidation by scavenging free radicals at various steps of oxidation. Lipid oxidation can be inhibited first, by reacting with $ROO^{\bullet}$ stops chain propagation and inhibit the formation of ROOH. Secondly, by reacting with alkoxyl radicals $RO^{\bullet}$ decreases the decomposition of hydroperoxides and the formation of aldehydes. In the evaluation of antioxidants, varied results can be obtained by methods which measure products at different stages of lipid oxidation. Thus, it is important that more than one method is used (Frankel, 1995; Lampi et al., 1997b). In brief, the most common methods to measure lipid hydroperoxides are peroxide value (PV) and conjugated dienes (CD). The aldehydes can be measured by anisidine value (AnV) analysis and thiobarbituric acid (TBA) test. The volatile aldehydes can be determined by head-space gas chromatography (GC) or as derivatives by high-performance liquid chromatography (HPLC). The above mentioned methods for measurement of lipid oxidation products have been widely applied to follow autoxidation of lipids as well as free radical initiated lipid oxidation in the presence of carotenoids.

Inhibition of the early phases of oxidation by antioxidants can also be measured by detecting the free radicals themselves by using, for example, the electron spin resonance (ESR) spectroscopy, which was applied in the present thesis (I). ESR detects the presence of unpaired electrons, which have a spin of either + ½ or - ½ and behaves as a small magnet. When a magnetic field is applied to a sample, the electron spin will be absorbed and used to move the electron from the lower energy level to the upper one. Thus an absorption spectrum, ESR signal, is obtained, usually in the microwave region of the electromagnetic spectrum. To be able to detect very unstable radicals, by the spin trapping technique a highly reactive radical is allowed to react with a compound to produce a long-lived radical. For example,

reaction of nitroso compounds or nitrones, such as α–phenyl-*tert*-butylnitrone (PBN), with radicals produces nitroxide radicals that have a long lifetime (Halliwell and Gutteridge, 1995, p. 47).

Antioxidant

The following examples emphasize that the term *antioxidant* is used and defined differently by different authors in the free radical literature. The available definitions are based both on exact chemical terminology or terminology based on phenomena. In more precise chemical terms, Britton (1995) defined that to be an effective antioxidant, a molecule such as a carotenoid would have to remove these radicals from the system either by reacting with them to yield harmless products or by disrupting free radical chain reactions. Tsuchihashi et al. (1995) proposed that the antioxidant potency is determined by several factors such as intrinsic chemical reactivity of the antioxidant toward the radical, site of generation and reactivity of the radicals, site of antioxidant, concentration and mobility of the antioxidant at the microenvironment, stability and fate of antioxidant-derived radical, and interaction with other antioxidants. Classically lipid antioxidants have been divided into two groups: primary or chain-breaking antioxidants, and secondary or preventive antioxidants (in Halliwell and Gutteridge, 1995, p. 236). In this thesis, the term antioxidant means a compound inhibiting oxidation based on scavenging of free radicals (I) and formation and decomposition of hydroperoxides (II-V).

In broader terms, Halliwell and Gutteridge (1995, p. 236) defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation". This emphasizes the source of oxidative damage in the characterization of an antioxidant (Halliwell et al., 1995). Krinsky (1992) defined biological antioxidants broadly as "compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations". In general, oxidizable substrates include lipids, proteins, carbohydrates and DNA. In addition, some antioxidants, such as vitamins E and C, are known to have synergistic interactions through their recycling mechanisms, whereby the combination of compounds has a better antioxidant activity than the sum of separate activities (Niki, 1987).

Pro-oxidant

The term *pro-oxidant* is used in different connections in the literature. Pro-oxidant can be defined as a component, such as a metal ion, able to lower the activation energy for the initiation of lipid oxidation (Labuza, 1971). In addition, added radical initiators have been named as pro-oxidants in reviews such as those by Krinsky (1993) and Palozza and Krinsky (1992a). In this thesis, the term pro-oxidant means a compound promoting oxidation, based on scavenging of free radicals (I) and formation and decomposition of lipid hydroperoxides, in comparison to a control sample (II-V). It should be stressed that it is possible for a compound to show both pro- and antioxidant properties depending on concentrations, experimental conditions, stage of oxidation and the presence of reaction partner. For example, Aruoma (1991) noted that lipid antioxidants may exert adverse pro-oxidant activity in non-lipid systems.

2.2 Carotenoids: general characteristics

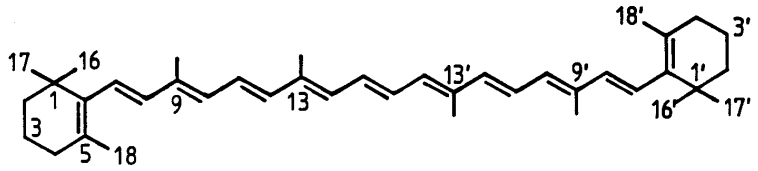
The electron rich conjugated double bond structure is primarily responsible for the excellent ability of β-carotene to physically quench singlet oxygen without degradation and for the chemical reactivity of β-carotene with free radicals, and for its instability toward oxidation (Britton, 1995; Krinsky, 1994). The structures of carotenoids and tocopherols are presented in Figure 1, p. 17 (modified from Straub, 1987). This section summarizes the general characteristics of carotenoids, whereas for further reading on chemistry of carotenoids see e.g. Belitz and Grosch (1987, p. 189), Britton (1995), Elbe and Schwartz (1996), Krinsky (1994) and Straub (1987).

Carotenoids are isoprenoid compounds, biosynthesized by tail-to-tail linkage of two C_{20} geranylgeranyl diphosphate molecules. This produces the parent C_{40} carbon skeleton from which all the individual variations are derived. This skeleton can be modified 1) by cyclization at one end or both ends of the molecule to give different end groups, 2) by changes in hydrogenation level and 3) by addition of oxygen-containing functional groups. Carotenoids that contain one or more oxygen atoms are known as xanthophylls, the parent hydrocarbons as carotenes. For clarity, and to avoid confusion in nomenclature, the use of both end-group prefixes for a carotene is now recommended. For example, β-carotene is referred to as β,β-carotene (in review by Britton, 1995).

The most characteristic feature of the carotenoid structure is the long system of alternating double and single bonds that forms the central part of the molecule (Figure 1). This constitutes a conjugated system in which the π-electrons are effectively delocalised over the entire length of the polyene chain. This feature is responsible for the molecular shape, chemical reactivity and light-absorbing properties, and hence colour, of carotenoids (reviewed by Britton, 1995). Handelman (1996) suggested that the following structural properties could contribute to antioxidant functions of carotenoids: 1) A multiplicity of closely spaced energy levels between the excited state and ground state of the carotenoid, such that the carotenoid can dissipate excited state energy via small collisional exchanges with the solvent, 2) minimal tendency for the excited-state carotenoid to sensitize other molecules, 3) resonance states in the excited state carotenoid, allowing delocalisation and stabilisation of the excited state and 4) multiple potential sites on the carotenoid for attack by active oxygen.

Each double bond in the polyene chain of a carotenoid can exist in two configurations, *trans* or *cis* geometrical isomers. The presence of a *cis* double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups, so that *cis* isomers are generally less stable thermodynamically than the *trans* form. Most carotenoids occur in nature predominantly or entirely in the all-*trans* form (in review by Britton, 1995).

I



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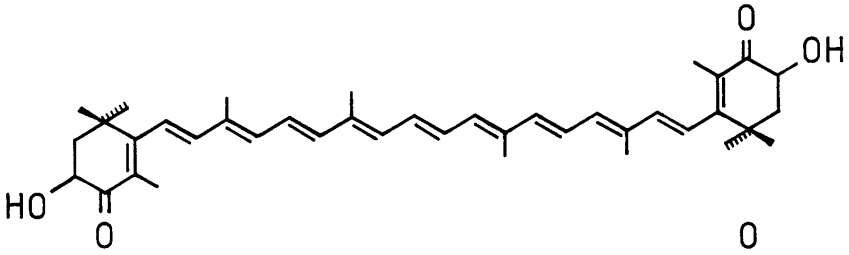

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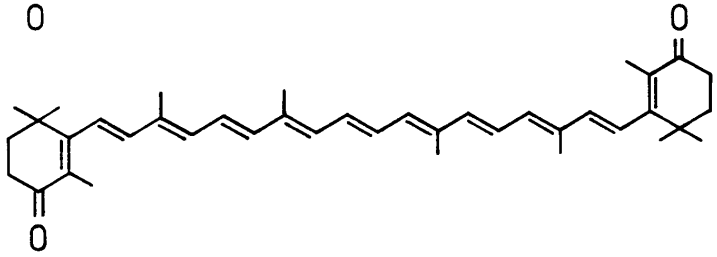

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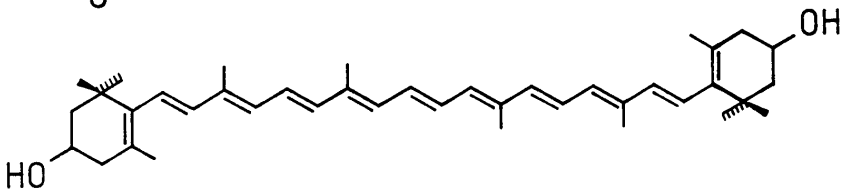

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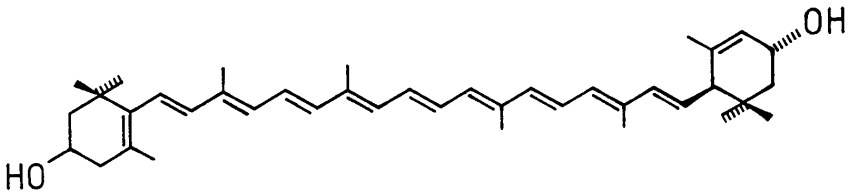
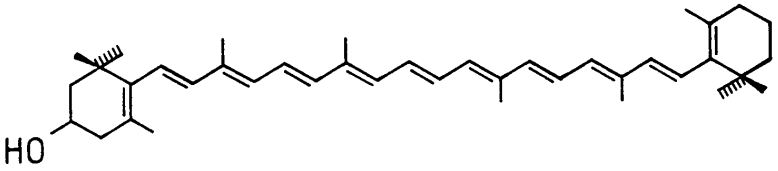


VI



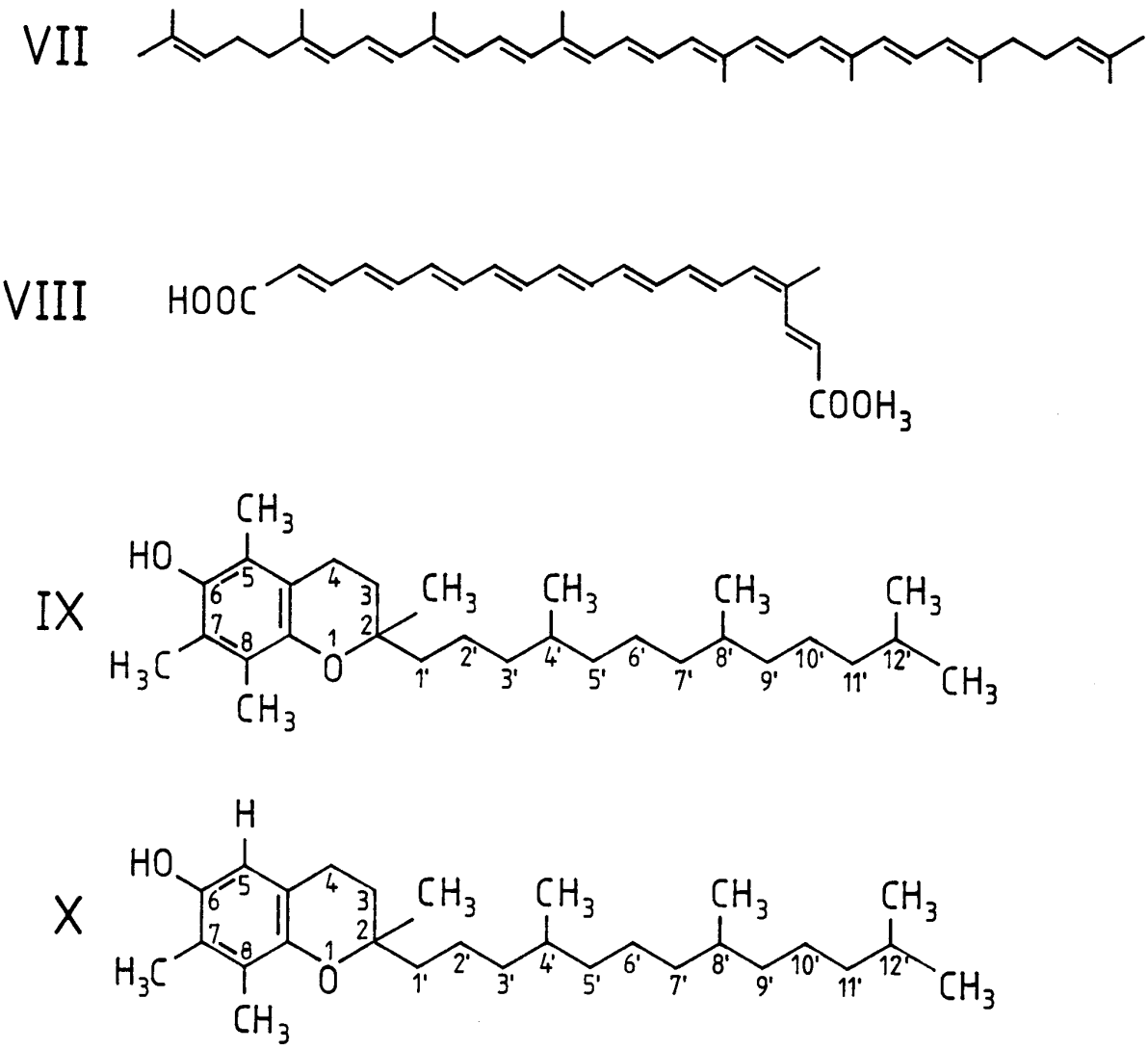


Figure 1. Structures of carotenoids and tocopherols. β-Carotene (I), astaxanthin (II), canthaxanthin (III), zeaxanthin (IV), lutein (V), β-cryptoxanthin (VI), lycopene (VII), bixin (VIII), α-tocopherol (IX) and γ-tocopherol (X) (modified from Straub, 1987).

Pure carotenoids are unstable in the presence of oxygen. The usual indication of carotenoid breakdown is bleaching. Importantly, carotenoids *in vivo* are much more stable than when they are isolated and in an organic solution. *In vivo*, carotenoids are commonly located in membranes where they constitute an integral part of the complex membrane structure. Moreover, the physical and chemical properties of a carotenoid are influenced by interactions with other molecules e.g. lipids and proteins in the membranes. Particularly important in relation to the functioning of carotenoids is the role of proteins in maintaining the correct position of the carotenoid with respect to other molecules (in review by Britton, 1995).

2.3 Quenching of singlet oxygen ($^1O_2$) by carotenoids

Carotenoids may act as antioxidants by physical or chemical $^1O_2$ quenching or by reacting with a variety of free radicals. The benefit of physical $^1O_2$ quenching is that carotenoids may act as antioxidants without loosing its own structure. Quenching of $^1O_2$ mainly leads to energy dissipation as heat, whereas the reactions between carotenoids and free radicals, such as lipid oxidation, lead to electron transfer or further radical reactions. This section discusses the antioxidant role of carotenoids as $^1O_2$ quenchers in brief, for review in detail see e.g. Edge et al. (1997), Krinsky (1979; 1989) and Stahl & Sies (1993). The effects of carotenoids on photosensitized lipid oxidation will be discussed below in 2.5.2.

2.3.1 Mechanisms of physical quenching of triplet sensitizer (^3S) and $^1O_2$

Triplet sensitizer is formed by the excitation of the sensitizer to the singlet state (*reaction 7*), followed by intersystem crossing to the formation of triplet state (*reaction 8*). The energy is transferred from triplet sensitizer to oxygen to give singlet oxygen (*reaction 9*). The singlet oxygen state, in which both electrons are paired in a single orbital leaving the other vacant, has an energy only 22 kcal above that of the ground state. As with most sensitizers, the reaction proceeds best with visible light (Foote, 1968; Frank and Cogdell, 1993).

$$S \rightarrow {}^1S^* \quad (7)$$

$$^1S^* \rightarrow {}^3S^* \quad (8)$$

$$^3S^* + {}^3O_2 \rightarrow S + {}^1O_2^* \quad (9)$$

Triplet sensitizer (^3S) can initiate both type I and type II photosensitized reactions. Type I reactions involve hydrogen or electron abstraction resulting in the production of radical species that can lead to further radical-catalyzed damage. In the type II reaction, ^3S reacts directly with ground state O_2 generating the highly reactive $^1O_2$ (*reaction* 9). For example, traces of the sensitizer chlorophyll in vegetable oils would tend to promote photosensitized oxidation by a type II pathway (reviewed by Krinsky, 1979; Krinsky, 1989; Bradley and Min, 1992).

As originally discovered by Foote and Denny (1968), the basis for carotenoid protection against photosensitized reactions is the ability of these pigments to quench, by an energy transfer process, either triplet sensitizers or $^1O_2$. In both *reactions (10) and (11)*, the carotenoid triplet that is formed can readily loose its energy to the environment and return to its original form, as seen in *reaction 12*. This characteristic makes carotenoids efficient against photosensitized reactions (discussed by Frank and Cogdell, 1993; Krinsky, 1979; Krinsky, 1989; Palozza and Krinsky 1992a). The energy difference between $^1O_2$ and $^3O_2$ is 94 kJ mol^{-1}. Thus, the quenching of $^1O_2$ by carotenoids can proceed efficiently only if the triplet energy level of the carotenoid molecule is < 94 kJ mol^{-1}. For example, C_{50} and C_{60} carotenoids quench $^1O_2$ faster than the C_{40} series, which suggests that the energy

transfer from $^1O_2$ to C_{50} and C_{60} is more exothermic than that involving the C_{40} series (Conn et al., 1991). The ability of carotenoids to quench $^1O_2$ is related to the number of conjugated double bonds, with a maximum protection shown by pigments having nine or more conjugated double bonds, and to end groups in the structure of pigments. Thus it is not only the triplet energy level which is responsible for the quenching; charge-transfer interactions may also be involved (Edge et al., 1997).

$$^3S^* + {}^1CAR \rightarrow S + {}^3CAR^* \quad (10)$$

$$^1O_2{}^* + {}^1CAR \rightarrow {}^3O_2 + {}^3CAR^* \quad (11)$$

$$^3CAR^* \rightarrow CAR + \text{heat} \quad (12)$$

2.3.2 Rate constants for physical $^1O_2$ quenching by carotenoids

The quenching rate constant (k_q) for $^1O_2$ quenching by carotenoids is very high, close to the diffusion controlled rate, approximately 10^{10} $M^{-1}s^{-1}$ (Di Mascio et al., 1989; Conn et al.,1991; Edge et al., 1997). Carotenoids have been examined for their ability to quench $^1O_2$ generated by the thermodissociation of the endoperoxide of 3,3'-(1,4-naphthylene) dipropionate ($DNPO_2$) by monitoring infrared chemiluminescence at 1270 nm (*reaction 13*)(Di Mascio et al., 1989; Conn et al., 1991; Devasagayam et al., 1992; Di Mascio et al., 1992). Much of this work has been carried out in benzene, toluene, chloroform and mixed solvents. The $^1O_2$ k_q of carotenoids may have significant variations due to differencies in techniques, solvents, solubilities (Di Mascio et al., 1992; Edge et al., 1997) and temperatures (Conn et al., 1991). In comparison, a near-infrared steady-state luminescence study by Oliveros et al. (1992) determined that the $^1O_2$ k_q of canthaxanthin and β-carotene were 10-12 x 10^9 and 1.4-6.3 x 10^9 $M^{-1}s^{-1}$, respectively.

$$^1O_2 \rightarrow {}^3O_2 + h\nu\ (1270\ \text{nm}) \quad (13)$$

In summary, carotenoids are highly efficient quenchers of $^1O_2$. Lycopene is known to be the most efficient biological carotenoid $^1O_2$ quencher (Di Mascio et al., 1989; 1992; Conn et al., 1991; Stahl et al., 1997). For example, Di Mascio et al. (1989) compared that $^1O_2$ k_q of lycopene was 100-fold higher than $^1O_2$ k_q of α–tocopherol.

2.3.3 Chemical reactions of carotenoid-$^1O_2$ interaction

For protection against $^1O_2$ by the carotenoid it is essential that chemical quenching is only a very minor side reaction (Edge et al., 1997). Thus, the antioxidant impact of this chemical reaction is negligible. The reaction is of potential interest because it may generate specific marker products for β-carotene-$^1O_2$ interactions (Liebler, 1993; Stratton et al., 1993). Stratton et al. (1993) identified $^1O_2$ oxidation products

of β–carotene by reverse-phase HPLC, UV-VIS spectrophotometry and MS. Their study demonstrated that β-carotene-5,8-endoperoxide appeared to be uniquely formed by $^1O_2$ in a solution of β–carotene and photosensitizer rose bengal. Further studies are needed to resolve markers for $^1O_2$ quenching in biological systems.

2.3.4 Summary of $^1O_2$ quenching by carotenoids

Carotenoids have shown relevance as $^1O_2$ physical quenching antioxidants in photobiology and photochemistry (Frank and Cogdell, 1993). The advantage of the available data is that $^1O_2$ quenching rate constants have been compared between a large number of carotenoids such as in Stahl et al. (1997) and in review by Edge et al. (1997). However, as Edge et al. (1997) noted, although lycopene quenches $^1O_2$ more efficiently than β-carotene, does not necessarily mean that it is a better antioxidant since lycopene is more susceptible to oxidation than β-carotene and may therefore be consumed more quickly. On the other hand, the physical $^1O_2$ quenching abilities of carotenoids have been reported in simple chemical systems, which are difficult to compare with more complex and general biological environments.

2.4 Antioxidant/pro-oxidant chemistry of carotenoids in free radical reactions

2.4.1 Carotenoid-radical interaction mechanisms and rate constants

The experimental data on chemical parameters of carotenoid-free radical interaction is limited. In addition to rate constants in Table 1, further knowledge is needed on the one-electron redox potentials of the carotenoids and the lifetimes and reactivity of carotenoid radicals in biological environments (Edge et al., 1997). However, Hill et al. (1995) suggested that carotenoids exhibit marked differences in their redox potentials.

Presently available data have proposed 1) electron transfer, 2) addition reaction and 3) hydrogen abstraction as mechanisms for reactions between carotenoids and a range of free radicals. In addition, according to Simic (1992) and Britton (1995), the addition of one electron to the carotenoid molecule would give the radical anion as $CAR + e^- \rightarrow CAR^{\bullet-}$ by reduction. In the carotenoid radicals, the unpaired electron is highly delocalised over the conjugated polyene chromophore. This has a stabilising effect and allows further reactions, e.g. additions, to take place in many parts of the molecule. In the following, a summary of the proposed antioxidant mechanisms of carotenoids is presented.

1) Electron transfer forming carotenoid radical cation. Oxidizing radicals with high redox potential can remove one electron from the carotenoid molecule to give the radical cation ($CAR^{\bullet+}$)(*reaction* 14). As will be discussed in 2.6, several electron withdrawing radicals have been shown to react by this mechanism: trichloromethylperoxyl radical ($CCl_3OO^{\bullet}$), sulfonyl radical ($RSO_2^{\bullet}$), nitrogen dioxide radi-

cal ($NO_2^{\bullet}$) and tocopheroxyl radical cation $TO^{\bullet+}$ (Edge et al., 1997). In addition, Edge et al. (1997) suggested that $CAR^{\bullet+}$ could be generated electrochemically in equilibrium with carotenoid dications and carotene itself, i.e. $CAR^{2+} + CAR \leftrightarrow 2CAR^{\bullet+}$.

$$R^{\bullet} + CAR \quad \leftrightarrow \quad R^{-} + CAR^{\bullet+} \qquad (14)$$

2) Addition reactions forming a carotenoid-adduct radical, which reacts further to form a nonradical product as demonstrated for thiyl radical ($RS^{\bullet}$) (*reactions* 15,16) (see 2.6). As noted by Edge et al. (1997), the radical cation forming process may often arise via an addition complex e.g. $CAR + CCl_3OO^{\bullet} \rightarrow [CAR - CCl_3OO]^{\bullet} \rightarrow CAR^{\bullet+} + O_2CCl_3^{-}$.

$$ROO^{\bullet} + CAR \quad \rightarrow \quad ROO\text{-}CAR^{\bullet} \qquad (15)$$

$$ROO\text{-}CAR^{\bullet} + ROO^{\bullet} \quad \rightarrow \quad ROO\text{-}CAR\text{-}ROO \qquad (16)$$

3) Hydrogen abstraction forming the neutral carotenoid radical, $CAR^{\bullet}$ (*reaction* 17, p. 21). For example, $CAR + CCl_4 \rightarrow CAR^{\bullet} + HCl + CCl_3^{\bullet}$

$$R^{\bullet} + CAR(H) \quad \leftrightarrow \quad RH + CAR^{\bullet} \qquad (17)$$

The scavenging rate constants are the kinetic determinants for antioxidative capacity. There may be multiple sites for attack on the carotenoid, each with its own rate constant (Handelman,1996). Table 1 demonstrates some of the rate constants in reactions between β-carotene and radicals and α-tocopherol.

Table 1.Examples of the rate constants in reactions between β-carotene and radicals, and between $CAR^{\bullet+}$ and α-tocopherol.

Radical	Rate constant [$M^{-1}s^{-1}$]	Reference
$ROO^{\bullet}$	$< 10^6$	Mortensen and Skibsted, 1998
α–tocopherol	1.7×10^7	Mortensen et al., 1998
$TO^{\bullet}$	10×10^9	Böhm et al., 1997
$RS^{\bullet}$	10^6-10^9	Everett et al., 1996
$NO_2^{\bullet}$	1.1×10^8	Everett et al., 1996
$GS^{\bullet}$	2.2×10^8	Everett et al., 1996

2.4.2 Proposed antioxidant/pro-oxidant mechanisms of carotenoids in lipid oxidation

Proposed antioxidant mechanisms

The very recent and growing body of evidence shows that scavenging of lipid $ROO^{\bullet}$ by β-carotene may not to proceed via electron transfer, but rather by adduct formation and/or hydrogen abstraction. However, Mortensen and Skibsted (1998) demonstrated by laser flash photolysis experiments that β-carotene is a poor direct scavenger of $ROO^{\bullet}$ because the second-order rate constant was estimated to be less than 10^6 $M^{-1}s^{-1}$.

Based on studies using a radical initiator to induce a free radical chain oxidation of lipid in solution, the addition mechanism yielding a nonradical product terminating the chain reaction (*reactions 15,16,* p. 21) has been widely hypothesized as the antioxidant mechanism of β-carotene on lipid oxidation (Burton and Ingold, 1984; Jørgensen and Skibsted, 1993; Kennedy and Liebler, 1991; 1992; Liebler, 1993; Palozza and Krinsky, 1992; Terao et al., 1992; Tsuchihashi et al., 1995; Yamauchi et al., 1993). According to Liebler (1993), ROO-CAR-ROO formed in the *reaction* 16, p. 21, could alternatively: 1) decompose to yield carotenoid-epoxide and $RO^{\bullet}$ (*reaction 21*, p. 23), 2) react further to form a *bis*-peroxyladduct. The decomposition of this *bis*-adduct would yield either carbonyl products and two $RO^{\bullet}$ or non-radical polar compounds and 3) react directly with $^3O_2$ (*reaction 22,* p. 23). The experimental conditions, such as pO_2, may determine which of these simultaneous reaction pathways would be the primary one (see also below pro-oxidant mechanisms).

Based on product analysis by mass spectroscopy of reaction between β-carotene with radicals resulting from the thermal decomposition of AMVN (Liebler and McClure, 1996) and a steady-state photolysis study (Mortensen and Skibsted, 1998), β-carotene has been proposed to react with $R^{\bullet}$, $RO^{\bullet}$ and $ROO^{\bullet}$ by hydrogen transfer (*reaction 17,* p. 21) or radical addition (*reactions 15,16,* p. 21). The bond dissociation energy of the most labile hydrogen in β-carotene is reported as 309 kJ/mol (Luo and Holmes, 1994), whereas the bond dissociation energy of LOO-H is around 370-380 kJ/mol. Thus, hydrogen transfer from β-carotene to $ROO^{\bullet}$ is thermodynamically feasible (Mortensen and Skibsted, 1998). This mechanism yields a neutral, resonance stabilized $CAR^{\bullet}$ (*reaction 17,* p. 21), which then combines with a second radical to form a substitution product (*reaction 18*)(Liebler and McClure, 1996).

$$CAR^{\bullet} + R(O)^{\bullet} \rightarrow CAR\text{-}O(R) \qquad (18)$$

Proposed pro-oxidant mechanisms

At higher pO_2, a carotenoid radical, $CAR^{\bullet}$, could react with oxygen to generate a carotenoid-peroxyl radical, $CAR\text{-}OO^{\bullet}$ (*reaction* 19, p. 23). This is an autoxidation process and $CAR\text{-}OO^{\bullet}$ could act as a pro-oxidant by promoting oxidation of unsaturated lipid (RH)(*reaction* 20, p. 23)(Britton, 1995). Furthermore, the reactions

of adduct formed in addition process (*reactions 15, 16*, p. 21) may lead to a pro-oxidant effect (*reactions* 21, 22, p. 23)(Liebler, 1993; Liebler and McClure, 1996).

$CAR^{\bullet} + {}^{3}O_2$	$\rightarrow$	$CAR\text{-}OO^{\bullet}$	(19)
$CAR\text{-}OO^{\bullet} + RH$	$\rightarrow$	$CAR\text{-}OOH + R^{\bullet}$	(20)
$ROO\text{-}CAR^{\bullet}$	$\rightarrow$	$CAR\text{-epoxide} + RO^{\bullet}$	(21)
$ROO\text{-}CAR^{\bullet} + {}^{3}O_2$	$\leftrightarrow$	$ROO\text{-}CAR\text{-}OO^{\bullet}$	(22)

Summary

Information is not available on antioxidant/pro-oxidant mechanisms of carotenoids with structures different from β-carotene. The functional groups of carotenoid structure may affect the mechanism of action on lipid oxidation. The mechanistic studies on scavenging of lipid $ROO^{\bullet}$ by carotenoids are rare: only very recently Mortensen and Skibsted (1998) have estimated the rate constant of β–carotene towards $ROO^{\bullet}$. It would be of interest to extend this study to other carotenoids. Further mechanistic investigation on the fundamental chemistry of carotenoid action on lipid oxidation is clearly indicated.

2.4.3 Oxidation products of β-carotene

The available data on carotenoid oxidation products have provided important information on markers for radical scavenging mechanisms and antioxidant chemistry. These studies have primarily used azo-initiator to accelerate the formation of oxidation products of β-carotene in solvent systems measured by different methods including HPLC, ultraviolet (UV) spectrometry, gas chromatography-mass spectrometry, high-performance liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy (reviewed by Handelman, 1996). The carotenoid oxidation products are a complex mixture of products with epoxy, hydroxy and carbonyl groups (see e.g. Figure 2, p. 24, adapted from Handelman et al., 1991)(El-Tinay and Chichester, 1970; Handelman, 1996; Handelman et al., 1991; Kennedy and Liebler, 1991; 1992; Liebler and Kennedy, 1992; Liebler, 1993; Mordi et al., 1991; 1993; Yamauchi et al., 1993). The major β-carotene oxidation products formed in free radical reactions are carbonyl products. Epoxides appear to be formed by the decomposition of the $ROO\text{-}CAR^{\bullet}$ adduct resulting in a release of $RO^{\bullet}$ (*reaction 21*, p. 23). Thus, oxidation of β-carotene to epoxides would not be expected to produce an antioxidant effect.

Furthermore, Kigoshi and Niki (1992) and Tsuchihashi et al. (1995) observed by gel permeation chromatography that polymeric products were formed from β-carotene incubated in benzene in the presence of AMVN and methyl linoleate. Interestingly, Handelman et al. (1991) demonstrated by HPLC analysis that the breakdown products formed from β-carotene during autoxidation in toluene solution with 100% oxygen at 60 °C and during azo-*bis*-isobutyronitrile (AIBN) initiated oxidation under air at 37 °C were completely identical.

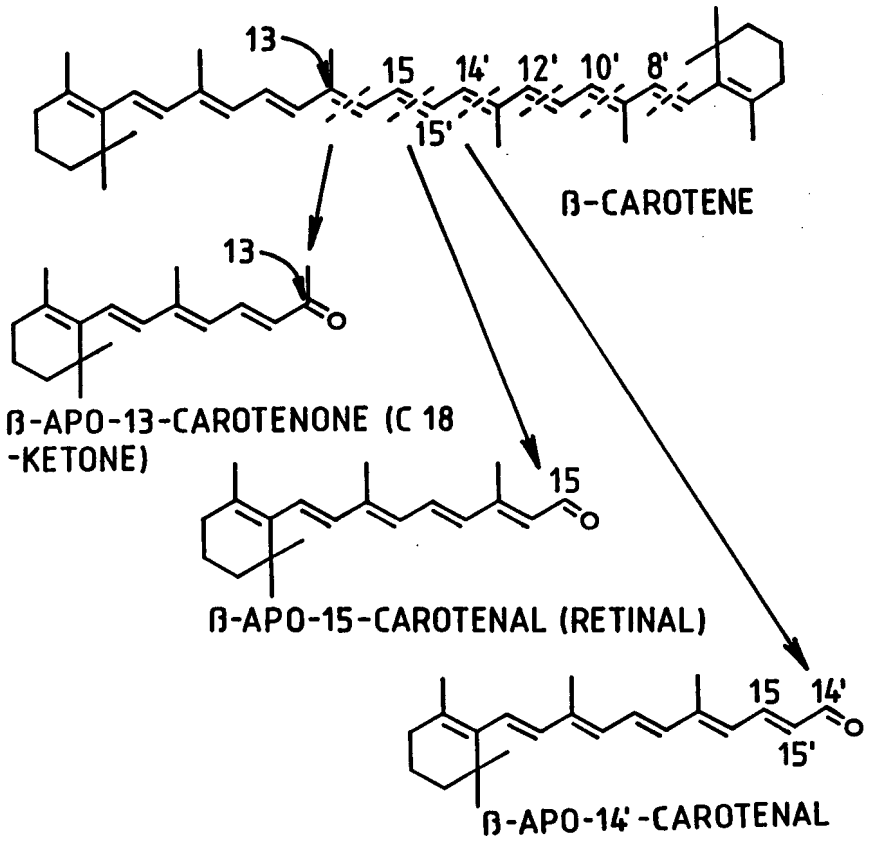


Figure 2. Breakdown of β-carotene, yelding β-apo-13-carotene, retinal (β-apo-15-carotenal) and β-apo-14'-carotenal (modified from Handelman et al., 1991).

2.4.4 Effect of oxygen partial pressure (pO_2) on antioxidant/pro-oxidant action of carotenoids

Antioxidant/pro-oxidant properties of β-carotene have classically been linked to the effect of pO_2 (Burton and Ingold, 1984). In addition, pO_2 may affect the antioxidant/pro-oxidant mechanism of carotenoids (see above 2.4.2). The data obtained both by methyl linoleate in solutions (Burton and Ingold, 1984; Jørgensen and Skibsted, 1993; Kigoshi and Niki, 1992) and liposomal or microsomal lipids (Kennedy and Liebler, 1992; Palozza and Krinsky, 1991; Vile and Winterbourn, 1988) have demonstrated an increasing antioxidative effect of β-carotene with decreasing pO_2. Jørgensen and Skibsted (1993) also reported similar results for astaxanthin, canthaxanthin and zeaxanthin, which have chemical structures with keto or hydroxyl groups. These data with the exception of the work by Kigoshi and Niki (1992) showed antioxidant effect of carotenoids at atmospheric pO_2. Burton and Ingold (1984) proposed that β-carotene (> 5 mM) may act as a pro-oxidant at high pO_2 (> 20% O_2), whereas Jørgensen and Skibsted (1993) and Kennedy and Liebler (1992) reported antioxidant action at even 50% and 100% O_2, respectively. However, at 100% O_2, β-carotene was a pro-oxidant in rat liver microsomes (Palozza et al., 1995) and normal and tumor cells (Palozza et al., 1997). In all, these experi-

ments support the hypothesis by Burton and Ingold (1984) that antioxidant activity of carotenoids would be enhanced at low pO_2.

2.5 Antioxidant/pro-oxidant action of carotenoids in different lipid systems

Most available research on the effects of carotenoids on lipid oxidation has focused on a variety of biological model systems *in vitro* with radical initiators in organic solution. On the other hand, the studies on spontaneous lipid autoxidation as well as photosensitized lipid oxidation in solution have focused on food lipids. The following overview in this section summarizes this literature excluding *in vivo* studies.

2.5.1 Effects of carotenoids on autoxidation of lipids

Table 2 summarizes major studies on the effects of carotenoids on spontaneously autoxidized lipids that is without added radical initiators in solutions. As seen in Table 2, the data on the effect of β–carotene on lipid autoxidation in the 1980's is conflicting, whereas early works in 1930's already reported a pro-oxidant action of carotenoids. A disadvantage of the literature is that it is limited to only β-carotene with the exception of the study by Olcott (1934). The discrepancies between studies may be due to differencies in lipid systems, the presence of other antioxidants in natural food lipids, concentration of carotenoids, experimental conditions and methods including PV, $[O_2]$ and volatiles to follow oxidation (see 6.1).

Table 2. Summary of major studies on the effect of carotenoids on autoxidation in the dark/light of different lipid models.

Lipid	Carotenoid	Experimental conditions	Method	Effect	Reference
Lard 5 g + 10 drops cod liver oil	carotene	a	induction period	pro-oxidant	Olcovich and Mattill, 1931
Lard	carotene, lycopene, xanthophyll	75 °C	$[O_2]$	pro-oxidant	Olcott, 1934
Linoleic acid	β-carotene	40 °C, light, UV 315-380 nm, 20 W	$[O_2]$	pro-oxidant	Faria and Mukai, 1983
Soybean oil	β-carotene 1-20 μg/g	60 °C, dark	PV	pro-oxidant	Warner and Frankel, 1987
Soybean oil	β–carotene 1-20 μg/g	25 °C, light, 7535 lx	PV volatiles	antioxidant	Warner and Frankel, 1987
Soybean triglyceride	β-carotene 100 μg/g	30 °C, dark	PV	antioxidant	Suzuki et al., 1989
Methyl linoleate	β-carotene 1-5000 μg/g	30 °C, dark	PV	pro-oxidant	Suzuki et al., 1989
Methyl linoleate	β-carotene 1-5000 μg/g	30 °C, light	PV	antioxidant	Suzuki et al., 1989
Refined rapeseed oil	β–carotene 10 μg/g	30 °C, light, 4.5 W/m^2	PV	antioxidant	Suzuki et al., 1989
Refined rapeseed oil	β-carotene 5 μg/g	30 °C, dark	PV	antioxidant	Suzuki et al., 1989

[a] not reported
PV = peroxide value
$[O_2]$ = oxygen consumption

2.5.2 Effects of carotenoids on photosensitized lipid oxidation

Due to the high efficiency of physical $^1O_2$ quenching by carotenoids (2.3), it has been of considerable interest to examine the effects of carotenoids on photosensitized lipid oxidation. These studies have mainly focused on food lipids in solution. Natural pigments present in foods, such as chlorophyll, are known to be efficient photosensitizers (Bradley and Min, 1992). Bradley and Min (1992) have suggested that the addition of effective $^1O_2$ quenchers, such as carotenoids, in foods containing unsaturated oils could improve their shelf-life.

For reactions of $^1O_2$ and photosensitizers in detail see 2.3.1 above. The reaction mechanism of photosensitized lipid oxidation differs from autoxidation as $^1O_2$ reacts with lipids through ”ene” reaction (Chan, 1987; Halliwell and Gutteridge, 1995, p. 214). However, by using conventional methods, such as PV, to follow oxidation, it is impossible to know whether carotenoids act as $^1O_2$ quenchers in the beginning of the reaction or as radical scavengers in the following free radical chain reactions.

As seen in Table 3, the studies have most commonly added chlorophyll as a photosensitizer. On the basis of photosensitized oxidation of purified soybean oil in methylene chloride, Min and Lee (1989) and Lee and Min (1990) reported that the $^1O_2$ quenching rate constant of astaxanthin was highest, 9.88×10^9 and 9.79×10^9 $M^{-1}s^{-1}$, respectively, among tested carotenoids. Furthermore, Jung and Min (1991)

determined that the $^1O_2$ quenching rate constant of canthaxanthin was highest, 1.12 x 10^{10} $M^{-1}s^{-1}$, among tested carotenoids. A study by Hirayama et al. (1994) compared the structure and $^1O_2$ quenching abilities of 19 carotenoids by measuring the toluidine blue-sensitized photo-oxidation of linoleic acid. The $^1O_2$ quenching increased as the number of conjugated double bonds of carotenoids increased. Furthermore, acyclic carotenoids enhanced quenching more than cyclic carotenoids. In contrast to the above antioxidant results, Terao et al. (1980) and Matsushita and Terao et al. (1980) reported that β-carotene may also act as a pro-oxidant. β-Carotene inhibited the formation of methyl linoleate hydroperoxides in the beginning, but oxidation occurred rapidly as β-carotene disappeared.

To summarize from the above section, the available data (Table 3) have reported that carotenoids act both as anti- and pro-oxidants on photosensitized lipid oxidation in solutions. However, it is apparent that photosensitized lipid oxidation does not continue for long periods of irradiation because chlorophyll decreases during the reaction (Terao et al., 1980). Consequently $^1O_2$ stimulates further radical reactions of lipid oxidation (Bradley and Min, 1992). Therefore, it is possible that these studies have reported the effect of carotenoids during the free radical process. A disadvantage of these data is that the content of remaining tocopherols in purified oil was defined as not present, although the determination limit was not reported (Jung and Min, 1991; Lee and Min, 1988, 1990; Min and Lee, 1989). In conclusion, it is difficult to interpret the available data because carotenoid actions have been investigated by studies differing in lipid systems, experimental conditions and methods including CD, PV and [O_2] to follow oxidation (see 6.1).

Table 3. Some studies reporting antioxidant effect of carotenoids on photosensitized oxidation of different lipi d models in solutions (modified from Lievonen, 1996).

Lipid model	Sensitizer	Carotenoids	Light, °C	Method	Reference
Soybean oil in chloroform	chlorophyll 0.005 wt%	β-carotene 0.1%, 0.5% (w/v), 0.1 wt%	10 mW/cm^2 20 °C	PV	Terao et al., 1980 Matsushita and Terao, 1980
Olive oil TAGS[a]	chlorophyll 4 and 6 μg/g	α-, β-carotene 4 and 6 μg/g	4500 lx	PV CD	Kiritsakis and Dugan, 1985
Soybean oil TAGS in methylene chloride	chlorophyll 4 μg/g	β-carotene 5,10 and 20 μg/g	4000 lx 10 °C	[O_2]	Lee and Min, 1988
Soybean oil TAGS in methylene chloride	chlorophyll 4 μg/g	astaxanthin, isozeaxanthin, zeaxanthin, lycopene, lutein 10, 20 and 30 μg/g	4000 lx 20 °C	PV [O_2]	Min and Lee, 1989
Soybean oil TAGS in methylene chloride	chlorophyll 4.4 x 10^{-9} M	astaxanthin, isozeaxanthin, zeaxanthin, lycopene, lutein 1.75, 3.50 and 5.0 x10^{-5} M	4000 lx 20 °C	PV [O_2]	Lee and Min, 1990
Soybean oil TAGS in methylene choride	chlorophyll 3.3 x10^{-9} M	β–apo-8'-carotenal, β-carotene, canthaxanthin 1.0, 2.5 and 5.0 x 10^{-5} M	4000 lx 25 °C	PV CD	Jung and Min, 1991
Linoleic acid in hexane/ethanol 60 mM	toluidine blue 0.05 mM	19 different carotenoids 15 mM	11 000 lx 30 °C	[O_2] CD	Hirayama et al., 1994

[a] TAGS = triacylglycerols
PV = peroxide value
[O_2] = oxygen uptake
CD = conjugated dienes

2.5.3 Effects of carotenoids on free radical initiated lipid oxidation

Most available literature on antioxidant action of carotenoids have applied microsomal or liposomal membranes as lipid models since carotenoids *in vivo* are commonly located in membranes. Furthermore, most of the data reporting that carotenoids are capable of producing antioxidant effects comes from *in vitro* studies using microsomal or liposomal membranes in organic solution in the presence of radical initiators to induce lipid oxidation (*reactions 5,6*, p.13). Several authors e.g. Handelman (1996), Krinsky (1989; 1993) and Palozza and Krinsky (1992a) have reviewed these results carefully, whereas this section focuses on a summary of the available data.

The data on microsomal membranes such as rat liver microsomes, rat liver mitochondria and rat skin microsomes have demonstrated antioxidant action of carotenoids including β-carotene (Kim, 1990; Palozza and Krinsky, 1991; 1992b; 1992c; Vile and Winterbourn, 1988), α-carotene, lycopene, lutein (Kim, 1990) astaxanthin and canthaxanthin (Miki, 1991; Palozza and Krinsky, 1991,1992c). Kim (1990) proposed that lycopene, lutein and α–carotene were better antioxidants than β-carotene or α-tocopherol. However, the solubility of carotenes at 0.1 mM in absolute ethanol is unclear (Krinsky, 1993). The inhibition of malondialdehyde (MDA) formation by carotenoids have varied between studies even from 4% to 100% of control. Commenting on these data, Krinsky (1993) explained the variation by the limited insolubility of carotenoids in the aqueous suspensions that membranes are isolated. In summary, these studies demonstrated antioxidant activity despite the differencies in the levels of carotenoids (0.1 μM-10 mM or 10-50 nmol/protein), type of radical initiators and models of microsomal membranes.

Studies using liposomes as lipid model systems have demonstrated that carotenoids are lipid antioxidants. Recently, Stahl et al. (1998) reported that in egg yolk phosphatidylcholine (PC) liposomes with AMVN as a radical initiator, antioxidant activity of carotenoids was in the ranking: lycopene > α-tocopherol > α–carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein. The synergistic effect was most pronounced in the presence of lycopene and lutein. Lim (1990) showed that zeaxanthin and astaxanthin were more efficient antioxidants than canthaxanthin or β-carotene, with and without hydroxyl groups in structure, respectively. At different levels, β-carotene inhibited the oxidation egg-PC liposomes induced by ferrous salts or UV radiation (254 nm)(Krinsky and Deneke, 1982), egg-PC liposomes in the presence of AMVN (Lim et al., 1992), soybean PC in the presence of AMVN (Kennedy and Liebler, 1992; Stocker et al., 1987; Tsuchihashi et al., 1995) and dimyristoyl PC liposomes in the presence of AAPH or AMVN (Tsuchihashi et al., 1995). Oxidation of liposomes was followed by measuring TBARS (Krinsky and Deneke, 1982; Stahl et al., 1998), CD (Kennedy and Liebler, 1992; Liebler et al., 1997) and PC-OOH by HPLC (Kigoshi and Niki, 1992; Lim et al., 1992; Stocker et al., 1987; Tsuchihashi et al., 1995).

In contrast to the above results, the recent work by Liebler et al. (1997) indicated that β-carotene is a relatively ineffective antioxidant in biological membranes. Based on the measurement of TBARS, the supplementation by β-carotene *in vitro* did not inhibit AAPH-initiated oxidation of rat liver microsomes at atmospheric or 3.8 torr O_2. In PC liposomes, in which β-carotene was incorporated into the bilayer during liposome preparation, the carotenoid inhibited the AAPH-initiated lipid oxidation based on CD analysis. In contrast, β-carotene added to pre-

formed liposomes provided essentially no antioxidant activity. The content of β-carotene (0.35 mol%) was similar in both liposome preparations, which was also in agreement with the concentration (0.38 mol%) of the previous study by authors (Kennedy and Liebler, 1992). Thus, the manner of carotenoid incorporation into a membrane may affect the results of antioxidant activity. Liebler and co-workers noted that the previous antioxidant effects in membrane models were seen at carotenoid levels well above those achieved through dietary supplementation.

The interest of using of LDL as an *in vitro* lipid model for the evaluation of antioxidant activities of carotenoids is due to the well-established observation that LDL is the major carrier of β-carotene in humans as well as the relation between oxidative modification of LDL and atherosclerosis (Krinsky, 1993). In brief, the data on the effects of carotenoids on LDL oxidation is controversial, and thus the evidence of antioxidant action remains weak. Recently, several authors e.g. Gaziano et al. (1995), Handelman (1996), Jialal et al. (1991), Krinsky (1993), Packer (1993) and Reaven et al. (1993; 1994) have discussed in detail the antioxidant/pro-oxidant role of β-carotene in LDL.

The use of homogeneous solutions as lipid models avoids solubility problems since both the carotenoid and methyl linoleate are dissolved in organic solvents (Krinsky, 1993). Based on the measurement of the formation of hydroperoxides, β-carotene inhibited the oxidation of methyl linoleate induced by AIBN in chlorobenzene (Burton and Ingold, 1984), AIBN in chloroform or metmyoglobin in heterogeneous lipid-water system (Jørgensen and Skibsted, 1993), AMVN in a mixture of hexane-isopropanol-tetrahydrofuran (Terao, 1989) and AMVN in benzene (Tsuchihashi et al., 1995). In addition, canthaxanthin and astaxanthin were more effective antioxidants than β-carotene and zeaxanthin (Jørgensen and Skibsted, 1993; Terao, 1989), whereas in heterogenous system the antioxidant effect showed little dependence on the structure of the carotenoid (Jørgensen and Skibsted, 1993). Furthermore, Tsuchihashi et al. (1995) estimated that β-carotene was 32 times less reactive than α-tocopherol toward $ROO^{\bullet}$ and approximately as reactive as 2,6-di-*tert*-butyl-4-methylphenol (BHT) in benzene solution. In summary, the antioxidant results of carotenoids using methyl linoleate in solution as a lipid model have been obtained in studies differing in concentrations of carotenoids (from 0-8.8 μM by Jørgensen and Skibsted,1993 to 0.05-5 mM by Burton and Ingold, 1984), solvents and type of radical initiators.

In contrast to the above results in methyl linoleate the works by Niki and co-workers demonstrated that in the same experimental conditions β-carotene at higher level, 0.1% (0.53 mM/453 mM methyl linoleate), showed a pro-oxidant effect (Kigoshi and Niki, 1992), whereas at lower level, 10.7-64.3 μM (/453 mM methyl linoleate), β-carotene showed an antioxidant effect (Tsuchihashi et al., 1995) on AMVN initiated oxidation of methyl linoleate in benzene. This example demonstrates that the concentration of β-carotene may highly affect its antioxidant/pro-oxidant properties.

In linoleic acid (19.3 mM), Li et al. (1995) demonstrated that β-carotene (11.2 μM) inhibited the AIBN (6.09 mM) inititated oxidation in *tert*-butyl alcohol. In this study, the thermal decomposition of azo-initiator was performed at 50 °C, which is higher than the usual 37 °C.

In summary, the studies discussed in this section have primarily used natural membrane models in order to evaluate the action of carotenoids in a more physiological environment. Many different carotenoids have been compared and the oxidation reactions have been carried out at similar temperature (37 °C). Although the

liposomal or microsomal lipid models are biologically a more relevant environment, it is difficult to evaluate the antioxidant actions of carotenoids in biological systems such as membranes as it may be difficult to distinguish the contributions of carotenoids and other antioxidants naturally present to the observed antioxidant effect. A disadvantage is that there is often lack of information on the contents of biological membrane model. Furthermore, the presence of radical initiators to induce lipid oxidation in solution as well as the presence of proteins in membranes have an important role in the functioning of carotenoids, and may in part explain the differencies of results between membrane models in solution and autoxidized lipid models (2.5.1).

2.6 Mechanisms of carotenoid-tocopherol interaction and other reactions between carotenoids and free radicals studied by fast reaction techniques

The most recent contribution to understanding of mechanisms between carotenoids and free radicals has come from the studies using fast reaction techniques, such as pulse radiolysis, laser flash photolysis and stopped-flow methods. These techniques can be used to measure the reaction rates for radical reactions and thus have been applied in mechanistic studies. Recently, Edge et al. (1997) and Handelman (1996) have carefully reviewed these studies including radical species e.g. superoxide anion ($O_2^{\bullet-}$)(Conn et al. 1992) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt ($ABTS^{\bullet+}$) radical cation (Miller et al., 1996), but these studies have been excluded here as beyond the scope of this thesis. This section focuses on the most recent data related to the area of this thesis.

Regarding the discussion (see 6.3) of mechanisms of carotenoid-tocopherol interaction, the available data on reactions between carotenoids and tocopheroxyl and phenoxyl radicals are of interest in this thesis. Recently, Mortensen et al. (1998) showed that α-tocopherol (TOH) scavenged $CAR^{\bullet+}$ generating α-tocopheroxyl radical (α-$TO^{\bullet}$) via *reaction 23*. Furthermore, Mortensen and Skibsted (1997a) studied by laser flash photolysis of $CAR^{\bullet+}$ and $TO^{\bullet}$ formed in chloroform and found that α-, β-, and γ-TOH reduce $CAR^{\bullet+}$ whereas the δ- $TO^{\bullet}$ can be reduced by lycopene and β-carotene.

$$CAR^{\bullet+} + TOH \rightarrow CAR + TO^{\bullet} + H^{+} \quad (23)$$

In contrast, Böhm et al. (1997) proposed in a pulse radiolysis study that lutein, canthaxanthin, β–carotene, septapreno-β-carotene, lycopene, 7,7'-dihydro-β-carotene and zeaxanthin, but not astaxanthin, could reduce $TO^{\bullet}$ to TOH (*reaction 24*). In addition, Mortensen and Skibsted (1996a) found that carotenoids may reduce the phenoxyl radical to phenol.

$$TO^{\bullet} + CAR + H^{+} \rightarrow TOH + CAR^{\bullet+} \quad (24)$$

As discussed above (2.1), the peroxyl radicals ($ROO^{\bullet}$) are formed in the propagation step of lipid oxidation (*reaction* 2, p. 21). As model for lipid $ROO^{\bullet}$, Mortensen & Skibsted (1998) investigated by laser flash and steady-state photolysis the ability of β-carotene to scavenge cyclohexylperoxyl, tetrahydrofuranperoxyl and tert-butanolperoxyl radicals, in homogeneous solutions including cyclohexane, tetrahydrofuran and *tert*-butanol/water. This method based on photolysis of di-*tert*-butylperoxide at 266 nm which lead to the generation of *tert*-butoxyl radical.

$$(CH_3)COOC(CH_3)_3 \rightarrow 2(CH_3)_3CO^{\bullet} \quad (25)$$

This tert-butoxyl radical abstracts hydrogen from cyclohexane, tetrahydrofuran or *tert*-butanol.

$$(CH_3)_3CO^{\bullet} + RH \rightarrow (CH_3)_3COH + R^{\bullet} \quad (26)$$

$ROO^{\bullet}$ will be formed in the presence of oxygen (*reaction* 2, p.12). These results of laser flash analysis showed that β-carotene was a poor scavenger of $ROO^{\bullet}$, whereas steady-state photolysis generating $ROO^{\bullet}$ showed an increased bleaching of β-carotene. The authors proposed that the mechanism of $ROO^{\bullet}$ scavenging was adduct formation (*reactions 15, 16,* p. 21) and/or hydrogen abstraction (*reaction 17,* p. 21).

In a pulse radiolysis study, Hill et al. (1995) reported that β-carotene, septapreno-β-carotene, canthaxanthin, astaxanthin, zeaxanthin and lutein reacted with substances such as trichloromethylperoxyl radical($CCl_3OO^{\bullet}$), a halogenated peroxyl radical, both by forming the $CAR^{\bullet+}$ (*reaction 14,* p. 21) and an addition radical. However, as $CCl_3OO^{\bullet}$ is an electron-seeking species with high reduction potential, it behaves differently from $ROO^{\bullet}$. Thus, e.g. Tsuchihashi et al. (1995) and Mortensen and Skibsted (1998) stated that it is not relevant as a lipid model.

The mechanism and rate of scavenging depends more on the nature of the radical than the carotenoid structure. Mortensen et al. (1997) found in a recent pulse radiolysis study that the rate constants for radical scavenging by carotenoids followed the sequence 2-mercaptoethanol thiyl radical ($HOCH_2CH_2S^{\bullet}$) > sulfonyl radical ($RSO_2^{\bullet}$) > thiyl radicals ($GS^{\bullet}$) > nitrogen dioxide radical ($NO_2^{\bullet}$). Furthermore, carotenoids scavenged both ($HOCH_2CH_2S^{\bullet}$) and glutathione ($GS^{\bullet}$) by adduct formation $[RS\text{-}CAR]^{\bullet}$. Astaxanthin, canthaxanthin, zeaxanthin and lycopene all reacted at the same rate with $HOCH_2CH_2S^{\bullet}$, whereas lutein, having one less conjugated double bond, reacted somewhat more slowly ($k=10^9\ M^{-1}s$). In addition, carotenoids exhibited a narrow range of reactivity toward ($NO_2^{\bullet}$) at the rate of 1.2-2.1 x $10^7\ M^{-1}s^{-1}$. Lycopene, lutein, zeaxanthin, astaxanthin and canthaxanthin all reacted with $NO_2^{\bullet}$ via an electron transfer process to generate $CAR^{\bullet+}$ without any intermediate radical adduct formation. The reaction between $RSO_2^{\bullet}$ and carotenoids resulted in both radical addition $[RSO_2\text{-}CAR]^{\bullet}$ and electron transfer ($CAR^{\bullet+}$).

To summarise from the above section, these very recent findings have increased a chemically based understanding of the interaction between carotenoids and free radicals. Although these data have given evidence that carotenoids scavenge a variety of radical species in simple chemical models, in different solvents, it is difficult to estimate the antioxidant activity of carotenoids seen in these models into a more complex and general biological environment.

3. Objectives of the Present Study

This thesis will evaluate the antioxidant/pro-oxidant action of carotenoids on lipid oxidation. The studies were initiated to investigate the effects of carotenoids and carotenoid-tocopherol interaction on spontaneously autoxidized lipids. The study on free radical scavenger properties of carotenoids focused on the early stages of oxidation.

The main objectives were:

1 To compare the free radical scavenging effects of naturally occurring carotenoids (I).

2 To study the effects of carotenoids on lipid oxidation:
   a) formation of hydroperoxides in three different lipid models; triacylglycerols, emulsified triacylglycerols and methyl linoleate (II-V),
   b) the effect of β-carotene on the isomerization (II) and decomposition of hydroperoxides (V) and
   c) the effect of β-carotene breakdown product on lipid oxidation (II).

3 To study the effect of carotenoid-tocopherol interaction on lipid oxidation:
   a) the formation of hydroperoxides in triacylglycerols and emulsified triacylglycerols (III-V) and
   b) the consumption of carotenoids and tocopherols during lipid oxidation (III-IV).

4. Materials And Methods

The experimental design is presented in Scheme 1. Materials and methods are described in detail in original papers (I-V) and evaluated in 6.1.

Scheme 1. Experimental design.

Focus on stages of lipid oxidation

(see for detailed lipid oxidation *reactions* 1-6, p.12)

RH	→ R•	→ ROO•	→ ROOH	→ decomposition products of ROOH
	↑		↑	↑
	Study I		*Studies II-V*	*Study V*

Study	**Carotenoid Tocopherol (concentration)**	**Model**	**Method**
I	β-carotene astaxanthin canthaxanthin zeaxanthin lutein cryptoxanthin lycopene bixin (0.17 mM, 0.90 mM)	chemical solutions (acetone, toluene) and methyl linoleate in toluene in the presence of radical initiator 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN)	electron spin resonance (ESR) spin-trapping technique
II	β–carotene (5-200 μg/g) retinal (7-360 μg/g)	methyl linoleate	formation and isomerization of lipid hydroperoxides i.e. ROOH by high-performance liquid chromatography (HPLC)
III	β–carotene (20 μg/g) lutein (5-40 μg/g) lycopene (20 μg/g) annatto (bixin 30-60 μg/g) γ-tocopherol (10-15 μg/g)	triacylglycerol fraction of purified rapeseed oil i.e. RSO TAGS	formation of ROOH as peroxide value (PV), consumption of carotenoids spectrophotometrically and γ-tocopherol by HPLC
IV	β-carotene (20 μg/g) γ-tocopherol (20-50 μg/g)	RSO TAGS	formation of ROOH by PV, consumption of β-carotene spectrophotometrically and γ-tocopherol by HPLC
V	β-carotene (0.45-20 μg/g) γ- and α-tocopherol (0.25-30μg/g)	10% oil-in-water emulsion i.e. emulsified RSO TAGS	formation of ROOH by PV, volatile decomposition products of ROOH by HPLC as di-nitrophenylhydrazine (DNPH)-derivatives

4.1 Materials

4.1.1 Carotenoids, tocopherols, radical initiator, spin trap and other chemicals

Figure 1, p. 17, presents the structures of carotenoids and tocopherols. The structure of retinal is presented in figure 2, p. 24. All studies (papers I-V) used synthetic carotenoids (all-*trans*-β-carotene, i.e.β,β-carotene; astaxanthin, (3S, 3'S)-3,3'-dihydroxy-β,β-carotene-4,4'-dione; canthaxanthin, β,β-carotene-4,4'-dione; zeaxanthin, (3R,3'R)-β,β-caroten-3,3'-diol; lutein, (3R,3'R,6'R)-β–ε-carotene-3,3'-diol; β-cryptoxanthin, (3R)-β,β-caroten-3-ol; lycopene, ψ,ψ-carotene; bixin, methyl hydrogen 9'-cis-6,6'-diapocarotene-6,6'-dioate) provided by Roche Oy (Espoo, Finland). Paper IV used also all-*trans*–β–carotene by Sigma Chemical Co. (St. Louis, MO, USA). In paper III, a natural annatto containing 3.8% bixin was provided by Chr. Hansen's Lab. (Denmark). Carotenoids are sensitive to light, heat, oxygen, acids and alkali. These factors were avoided as far as possible during the sample preparation. Thus, samples were stored under nitrogen, solvent was evaporated by nitrogen and sample preparations were performed under reduced light. α-Tocopherol and γ–tocopherol were from E. Merck (Darmstadt, Germany). In paper I, the radical initiator 2,2'-azo*bis*(2,4-dimethyl-valeronitrile) (AMVN) was from Wako Pure Chemical (Osaka, Japan) and the spin trap N-*tert*-butyl-α-phenyl-nitrone (PBN) was from Aldrich (Milwaukee, WI, USA). For levels of added carotenoids and tocopherols see Scheme 1. Other chemicals such as solvents are described in the original papers (I-V).

4.1.2 Lipid models

This thesis was based on three different lipid models; triacylglycerols (III-IV), emulsified triacylglycerols (V) and methyl linoleate(I-II). The triacylglycerol fraction (RSO TAGS) was purified from commercial low-erucic acid rapeseed oil (RSO). This low-erucic acid rapeseed (*Brassica rapa subsp. oleifera*) oil is comparable to turnip rapeseed oil and canola oil. Each study (III-V) obtained fresh RSO from Van den Bergh Foods, Helsinki, Finland. A new bottle of RSO was opened for every purification procedure. The oil was stored under dark below 8 °C before its use. The RSO contained initially 36 mg/100 g γ-tocopherol, 16 mg/100g α-tocopherol, 6 mg/100 g β-tocopherol and 1 mg/100 g δ-tocopherol (unpublished data). The polyunsaturated acid content of RSO TAGS was 0.1% 14:0, 3.7% 16:0, 0.2% 17:1, 1.4% 18:0, 53.8% 18:1n-9, 2.4% 18:1n-7, 22.1% 18:2n-6, 10.9% 18:3n-3, 0.4% 20:0, 1.3% 20:1n-9, 0.2% 22:0 and 0.7% 22:1n-9 (Hyvönen et al., 1993).

The purified rapeseed oil triacylglycerols (RSO TAGS) were prepared by a multilayer chromatographic method, as described by Lampi et al. (1992). This purification method removed tocopherols and metals. RSO TAGS were characterized by its γ-tocopherol content and peroxide value (PV). Unless otherwise reported, the content of γ-tocopherol of purified RSO TAGS was <1 ug/g, which was the determination limit of tocopherols (described in IV). γ-Tocopherol was the only tocopherol, which was detectable in purified RSO TAGS. Paper V described a lipid model

of 10% oil-in-water emulsion, emulsified RSO TAGS, which was prepared from RSO TAGS. In papers I and II, methyl linoleate was from Nu-Chek-Prep, Inc. (Elysian, MN, USA).

The carotenoids and tocopherols were added to purified RSO TAGS, emulsified RSO TAGS and methyl linoleate in solution, of which solvent was evaporated by nitrogen. The antioxidant or pro-oxidant activity of carotenoids and tocopherols was based on the effect compared to a control sample without an added carotenoid and tocopherol, respectively (see original articles I-V).

4.2 Methods

Electron spin resonance (ESR) study (I)

The ESR study was performed at the Royal Veterinary and Agricultural University, Denmark. These ESR-spin trapping experiments, which focused on the early stages of oxidation, describe an experimental system with a radical initiator in solution. The radicals were generated from thermal (37 °C) cleavage of AMVN (see *reactions* 5 and 6, p. 13) and trapped by PBN, and the production of PBN spin adducts was detected as described in detail in paper I. The statistical methods used in ESR study are described in detail in paper I. See also p. 13.

Autoxidation of lipids (II-V)

Studies II-V were performed at the University of Helsinki, Finland. As described in papers II-V, samples were autoxidized in a light cabinet with white fluorescent lamps at 25 °C or under darkness at 40 °C. All autoxidation experiments were done under air at atmospheric oxygen conditions. In experiments with light, the light power and light intensity were 15-20 W/m^2 and 10 000 lux, respectively (for experimental conditions detailed see original papers II-V).

Photosensitized lipid oxidation (IV)

In paper IV, in order to evaluate the effect of β-carotene and γ-tocopherol on photosensitized lipid oxidation, chlorophyll b was added as a photosensitizer to RSO TAGS.

Consumption of carotenoids and tocopherols (III, IV)

Carotenoids were determined spectrophotometrically at 453 nm. Tocopherols were determined by HPLC as described in paper IV.

Formation of hydroperoxides (II-V)

The formation of hydroperoxides was determined as peroxide value (PV) by the spectrophotometric ferric thiocyanate method (FID-IDF, 1974; Ueda et al., 1986)

(III-V). PV was expressed as milli-equivalents of oxygen per kilogram of fat. The reproducibility of the PV measurement was as following: 0.4 ± 0.21, 1.5 ± 0.09, 2.6 ± 0.17, 4.7 ± 0.32, 14.8 ± 0.37, 56.9 ± 0.62 and 86.1 ± 0.82 (unpublished data from Mäkinen et al., 1995). In paper II, the formation of the four isomers of methyl linoleate hydroperoxides and their isomerization were analyzed by normal-phase HPLC as described by Hopia et al. (1996a). In papers III and V, the results were calculated by one-way analysis of variance.

Decomposition of hydroperoxides (V)

In paper V, the decomposition of hydroperoxides into volatile aldehydes was analyzed as dinitrophenylhydrazine (DNPH) derivatives. This method is described in detail in paper V, Lampi (1994) and Lampi et al. (1996).

5. Results

This section summarizes the results of this thesis and refers to data presented in tables and figures in original papers I-V (see appendix).

5.1 Free radical scavenger properties of eight carotenoids in acetone and toluene solutions (I)

The ESR spin-trapping study compared free radical scavenger properties of the following eight carotenoids: β-carotene, astaxanthin, canthaxanthin, zeaxanthin, lutein, cryptoxanthin, lycopene and bixin (I; Table 2). This ESR technique detected alkoxyl radical adducts of PBN. In polar solvent acetone under air, each of the carotenoids (0.17 mM) except β-carotene diminished the amount of PBN spin adducts by 20%, i.e. showed an antioxidant effect. The effect of β-carotene was significantly ($p<0.05$) different from all other carotenoids, but not from control. In contrast, β-carotene at a higher concentration (0.90 mM) showed a pro-oxidant effect by increasing the number of PBN spin adducts in this chemical environment.

In the less polar solvent toluene under air, β-carotene, astaxanthin, canthaxanthin, zeaxanthin, cryptoxanthin, lycopene, β-carotene plus astaxanthin and β-carotene plus canthaxanthin (0.17 mM) diminished the formation of PBN spin adducts. In contrast, the effect of bixin, lutein and β-carotene plus lutein did not differ from control. There were no other differences between individual carotenoids. Also, the effect of carotenoid mixtures was not different from individual ones, and thus no synergy was observed. In toluene under 2% O_2, either with or without added methyl linoleate, the carotenoids did not affect the formation PBN spin adducts. However, in toluene under 2% O_2, astaxanthin, canthaxanthin and lycopene, suppressed the formation of N-benzoyl-N-*tert*-butylnitroxide ($PBNO^{\bullet}$), an oxidation product of PBN.

5.2 Effects of β-carotene, lycopene, lutein, retinal and annatto on hydroperoxide formation of methyl linoleate (II), autoxidized RSO TAGS (III,IV) and emulsified RSO TAGS (V)

The experiments in three different tocopherol-free (< 1 μg/g) lipid models, methyl linoleate, RSO TAGS and emulsified RSO TAGS, showed constantly pro-oxidant activity of β-carotene based on formation of hydroperoxides (II-V). In methyl linoleate, oxidized at 40 °C under dark, β-carotene at concentrations of 5, 20 and 200 μg/g was a pro-oxidant by promoting the formation of hydroperoxides, which were analyzed by HPLC. The higher the concentration of b-carotene was, the more

hydroperoxides were formed (II). In RSO TAGS, oxidized at 25 °C under light, β-carotene at concentration of 20 μg/g promoted the formation of hydroperoxides of RSO TAGS (III; Figure 6b, IV; Figures 1a,2,3b). In addition, the effect of β-carotene on chlorophyll sensitized oxidation of RSO TAGS (unpublished data from IV) did not differ from the pro-oxidant results in autoxidized RSO TAGS without chlorophyll (IV). In 10% oil-in-water emulsion, emulsified RSO TAGS, β-carotene at concentrations of 0.45, 2 and 20 μg/g promoted the formation of hydroperoxides at 25 °C under dark (V; Figures 1a,2a,3,4).

The higher the concentration of the carotenoid lutein was, the stronger was the pro-oxidant effect (III; Figure 2a). Lutein promoted the formation of hydroperoxides at concentrations of 5, 20, 30, and 40 μg/g both under dark and light, at 40 °C and 25 °C, respectively (V; Figures 2a,3a). In addition, at 25 °C under light, lycopene at 20 μg/g promoted the formation of hydroperoxides of RSO TAGS (III; Figure 5a).

Retinal, a shorter chain oxidative breakdown product of β-carotene, showed a pro-oxidant effect in methyl linoleate. At 40 °C under air in dark, the formation of methyl linoleate hydroperoxides was higher in the presence of retinal at concentration of 7, 18, 180, 360 μg/g during the first 96 hours of incubation. The higher the concentration of retinal was, the more hydroperoxides were formed. After 168 hours of incubation, the content of hydroperoxides was highest in the control sample, and it was increasing in the presence of retinal at concentrations of 7 and 360 μg/g. Instead, the content of of hydroperoxides decreased rapidly after 144 hours in the presence of retinal at 18 and 180 μg/g, which indicated the effect of retinal on the decomposition of hydroperoxides (II).

In contrast to single carotenoids, the natural food colour annatto, containing bixin as a coloring pigment, was an effective antioxidant in RSO TAGS. At 25 °C under light, annatto containing 30 and 60 μg/g of bixin inhibited the formation of hydroperoxides in RSO TAGS, whereas the inhibition at 20 μg/g of bixin was not significant (III; Figure 6a,6b). In addition, the annatto product contained an unidentified fluorescent component coeluting in HPLC analysis of tocopherols, which may have contributed to the effect of annatto.

5.3 Effect of β-carotene on decomposition of hydroperoxides of emulsified RSO TAGS (V) and isomerization of methyl linoleate hydroperoxides (II)

In emulsified RSO TAGS, 10% oil-in-water emulsion, which was oxidized at 25 °C under dark, the pro-oxidant effect of β-carotene based on both the formation of hydroperoxides and decomposition of hydroperoxides into volatile aldehydes including hexanal, 2-heptenal and 2,4-heptadienal. β-Carotene at concentrations of 0.45 μg/g and 2 μg/g promoted the formation hexanal (V; Figures 1b,2b). At 2 μg/g, β-carotene promoted the formation of 2-heptenal, whereas the effect on the formation of 2,4-heptadienal was not significantly different from the control (V; Figure 2c,2d).

In methyl linoleate, β-carotene at concentrations of 5 and 20 μg/g did not affect the ratio of *cis,trans*-form to *trans,trans*-form of conjugated diene hydroperoxides.

At the endpoint of incubation (168 hours), the % *cis,trans*-form was lower in the presence of β-carotene at 200 μg/g (41.8%) than in the control sample without an added carotenoid (44.4%). In the presence of retinal at concentrations of 18, 180 and 360 μg/g, the % *cis,trans*-form was decreasing, thus % *trans,trans*-form was increasing during the incubation period. At the endpoint of incubation (168 hours), the % *cis,trans*-form was as following: 43.6 (control, 0 μg/g retinal), 41.7 (7 μg/g retinal), 42.0 (18 μg/g retinal), 41.5 (180 μg/g retinal) and 41.3 (360 μg/g retinal).

5.4 Effects of carotenoid-tocopherol interaction and tocopherols on autoxidation of RSO TAGS and emulsified RSO TAGS (III-V)

The combinations of different proportions of added carotenoid and α- or γ-tocopherol as well as combinations of added carotenoid and remaining γ-tocopherol after purification of RSO TAGS resulted in an antioxidant effect. Already very low amounts of the remaining γ-tocopherol (determination limit 1 μg/g) in purified RSO TAGS showed a crucial effect on the results. Under light at 25 °C, the presence of lutein at 20 μg/g + remaining γ-tocopherol at 3 μg/g inhibited the formation of hydroperoxides of RSO TAGS (III; Figure 4a). In addition, the presence of remaining γ-tocopherol at 8 μg/g + β-carotene at 20 μg/g, inhibited the formation of hydroperoxides of RSO TAGS (IV; Figures 1a,1b).

In RSO TAGS, the combinations of added β-carotene and γ-tocopherol at 20 μg/g + 20 μg/g, lutein and γ-tocopherol at 20 μg/g + 15 μg/g, and lycopene and γ-tocopherol at 10 μg/g + 20 μg/g, respectively, inhibited the formation of hydroperoxides under light at 25 °C (IV; Figure 4, III; Figure 3a, III; Figure 4a, respectively). In addition, in the presence of remaining γ-tocopherol at 8 μg/g, the two different combinations of β-carotene and γ-tocopherol at 20 μg/g + 50 μg/g and 20 μg/g + 33 μg/g, respectively, were antioxidants (IV; Figures 3a,3b).

In emulsified RSO TAGS, the combination of β-carotene and α-tocopherol at 2 μg/g + 1.5 μg/g, respectively, inhibited the formation of hydroperoxides under dark at 25 °C (V; Figure 3). At the same concentrations (2 μg/g + 1.5 μg/g), the combination of β-carotene and γ-tocopherol inhibited both the formation of hydroperoxides and decomposition of hydroperoxides into hexanal, 2,4-heptadienal and 2-heptenal (V; Figures 2a, 2b,2c,2d). In addition, the antioxidant effect of the combination of β-carotene and γ–tocopherol at 0.45 μg/g + 3 μg/g, respectively, was based on both the formation of hydroperoxides and the decomposition of hydroperoxides into hexanal (V; Figures 1a,1b).

The combinations of carotenoid and tocopherol at certain proportions were even more effective antioxidants than tocopherol alone. In RSO TAGS, the following combinations were more efficient antioxidants than γ-tocopherol alone: β-carotene and γ-tocopherol (20 μg/g + 20 μg/g) > γ-tocopherol (20 μg/g), lutein and γ-tocopherol (20 μg/g + 15 μg/g; molar ratio 1:1) > γ-tocopherol (15 μg/g) and lutein and γ–tocopherol (20 μg/g + 10 μg/g; molar ratio 1.5:1) > γ-tocopherol (III,IV). In emulsified RSO TAGS, the combination of β-carotene and α–tocopherol (2 μg/g + 1.5 μg/g), was more efficient than α-tocopherol (1.5 μg/g) alone in inhibiting the formation of hydroperoxides (V).

The experiments with individually added α– or γ–tocopherols were studied for comparison of the effects of a combination of carotenoid and tocopherol as well as an individual carotenoid. In RSO TAGS, added γ-tocopherols showed an antioxidant effect by inhibiting the formation of hydroperoxides at levels 10 μg/g (III; Figure 4a), 15 μg/g (III; Figure 3a) and 20 μg/g (IV; Figures 2,4). In addition, both α- and γ-tocopherols inhibited both the formation and decomposition of hydroperoxides in emulsified RSO TAGS (V).

5.5 Consumption of added carotenoids and tocopherols during lipid oxidation (III, IV)

In RSO TAGS, the concentration of lutein was 89.5% in the presence of added γ-tocopherol and only 60.0% without added γ–tocopherol after 24 h storage under light at 25 °C (III; Figure 3b). In comparison, the concentration of lycopene was 82.6% in the presence of added γ-tocopherol and only 45.7.% without added γ-tocopherol after 26 h storage under light at 25 C (III; Figure 5b). The consumption of γ-tocopherol was not seen to be affected by the presence of carotenoids (III; Figures 3b, 4b).

6. DISCUSSION

In summary, the results demonstrated that carotenoids acted as free radical scavenging antioxidants in chemical solutions (I), whereas the experiments with spontaneously autoxidized lipids resulted in pro-oxidant action of carotenoids (II-V). An important finding of this thesis was that the breakdown of carotenoids, hence the loss of colour, demonstrated the simultaneous pro-oxidant action of carotenoids in autoxidized lipids. On the other hand, even a minor amount of tocopherol protected carotenoids from destruction and thus inhibited the pro-oxidant action of carotenoids. Based on promotion of hydroperoxide formation, the first experiments on spontaneously autoxidized RSO TAGS with β-carotene showed a pro-oxidant effect (IV), which was further demonstrated with other carotenoids including lutein and lycopene, both under the dark and light, at 40 °C and 25 °C, respectively (III). Based on both the formation and decomposition of hydroperoxides, β-carotene was a pro-oxidant in emulsified RSO TAGS and methyl linoleate (II,V). On the other hand, in RSO TAGS and emulsified RSO TAGS, the certain proportion of carotenoid and tocopherol in combination was a more efficient antioxidant than tocopherol alone (III,V).

Regarding the discussion on the *in vitro* antioxidant or pro-oxidant role of carotenoids, the present results suggest that attention should be paid to the experimental model system and the possible presence of other antioxidants in lipid models.

6.1 Experimental models and methods in antioxidant/pro-oxidant studies of carotenoids on lipid oxidation

In this thesis, the comparison between experiments in chemical solution in the presence of radical initiator (I) and the lipid environment (II-V) showed that the antioxidant/pro-oxidant activity of carotenoids may critically depend on the choice of model system. Many factors such as the differencies in methodology, the lipid model and solution involved, the presence of other antioxidants, the concentration of carotenoids and other experimental conditions such as pO_2 complicate the interpretation of results.

Lipid models

To understand the action of carotenoids on lipid oxidation, three different lipid models were evaluated as an oxidizing substrate in the following chronological order: RSO TAGS (III-IV), emulsified RSO TAGS (V) and methyl linoleate (I, II).

Fats are mainly in the form of triacylglycerols, such as RSO TAGS, many of which are of importance to rancidity (Labuza,1971). Thus, natural TAGS have been widely used as food lipid models in oxidation studies. In this thesis (III-V),

the purification of natural RSO by the method by Lampi et al. (1992) provided the possibility to examine the reaction between lipid and carotenoid without disturbing pro- or antioxidants. This was an important advantage of using natural RSO TAGS as a lipid model for autoxidation studies (III-V). On the other hand, the variation of remaining γ–tocopherol after purification of RSO may be a source of discrepancies between the experiments. Since a very low amount of γ-tocopherol affected the results obtained with carotenoids (III,IV), a natural lipid model such as RSO TAGS may not be the best choice for mechanistic investigation in case of added carotenoids. A crucial observation was that a trace amount such as 3 μg/g of remaining γ-tocopherol in RSO TAGS affected the action of carotenoid and thus resulted in an antioxidant effect of a combination of carotenoid and γ-tocopherol (III). This is in agreement with the very recent data by Lampi et al. (1997a) who found that minor amounts of γ-tocopherol inhibited oxidation of TAGS of RSO and butter oils (BO). In 50% RSO/BO TAG mixture, γ-tocopherol was a significant antioxidant even at 3 μg/g. The results of this thesis support the conclusion by Lampi et al. (1997a) that the characterization of the natural lipid model used for antioxidant studies is very important.

The presence of other antioxidants, such as tocopherols, may also affect the action of carotenoids in other natural lipid models than TAGS. Most of the carotenoid antioxidant studies have been performed using natural membranes as lipid models *in vitro* (see above 2.5.3). For example, Palozza et al. (1995) reported that microsomes, common membrane models, have an endogenous content of α-tocopherol 0.824 nmol/mg protein. Liebler et al. (1997) reported that in rat liver microsomes β-carotene was not depleted during the oxidation, whereas endogeneous α-tocopherol of 0.16 nmol was depleted, which suggests that β-carotene may not affect the oxidation in rat liver microsomes. Thus, carotenoid was neither anti- or pro-oxidant in this system. It is known that proteins and other naturally present compounds stabilize β-carotene in biological environment (Britton, 1995). Proteins may thus protect carotenoids from destruction and possible pro-oxidant effect on lipid oxidation in its natural environments such as in membranes and in muscle based foods. For example, this situation exists in salmon where the orange colour of astaxanthin is present at significant amounts (Andersen et al., 1990; Christophersen et al., 1991).

Emulsified RSO TAGS, 10% oil-in-water emulsion, was a model of hydrophilic lipid system (V). In emulsified lipids, the presence of the droplet membrane, the interactions between ingredients, and the partitioning of ingredients between the oil, affect the oxidation, which differs from that of bulk lipids (Coupland and McClements, 1996). In comparison with RSO TAGS, the pro-oxidant effect of β-carotene was weaker in emulsified RSO TAGS (III-V). Furthermore, in emulsions, depending on their hydrophilic and hydrophobic characteristics, antioxidants are distributed between the water and oil phases in different proportions (Hopia et al., 1996b; Huang et al., 1996). Polarity and solubility of antioxidants determine their concentrations in different locations in multiphase systems. Frankel (1995) stated that in heterogeneous food systems, such as emulsions, interfacial phenomena including the physical properties such as lipophilicity, solubility and partition between the aqueous and lipid phases may be important in determining antioxidant activity. In case of added β-carotene in emulsified RSO TAGS, it is likely that β-carotene without hydroxyl groups is located in lipids. On the other hand, the situation may be different with other carotenoids with different structures, which may affect further the antioxidant/pro-oxidant role of carotenoids. In comparison, mem-

brane models, which most of the carotenoid antioxidant studies have applied for an oxidizing substrate, represent a heterogeneous lipid system as well. In membranes, the balance between hydrophilic groups and the hydrophobic polyene skeleton is important for incorporation in the bilayer, and the presence of hydroxyl and carbonyl groups as in astaxanthin, helps to anchor the carotenoid at the water/lipid interface (Jørgensen and Skibsted, 1993). Tsuchihashi et al. (1995) suggested that β-carotene may scavenge lipophilic radicals present in membranes, whereas α-tocopherol may scavenge aqueous radicals in liposomal membranes. Although the physical manner of β-carotene association with the lipid bilayers is not known, it appeared that β-carotene incorporated during vesicle formation was more available for antioxidant action and therefore may be more uniformly dispersed throughout the lipid.

Simple predictive lipid models are suitable for mechanistic studies (Fuster et al., 1998). In study II, methyl linoleate, a simple lipid, was chosen as an oxidizing substrate due to the knowledge that methyl linoleate has been a convenient substrate to study the mechanisms of antioxidants. It is known that methyl linoleate produces four kinds of conjugated diene hydroperoxides quantitatively (Porter et al., 1995; Tsuchihashi et al., 1995; Hopia et al., 1996a). On the other hand, Frankel (1993), Huang et al. (1996) and Hopia et al. (1996b) have suggested that the use of free fatty acids, such as linoleic acid, may be irrelevant to most food or biological systems. The majority of fatty acids in these systems are esterified as either triacylglycerols of phospholipids and their antioxidant behaviour may be significantly different as well.

Methods to follow oxidation

In this thesis, the effects of carotenoids were investigated at various steps of lipid oxidation. First, the detection of free radicals by ESR technique focused on the early stages of oxidation (I); secondly, the primary oxidation products, hydroperoxides were measured as PV (III-V) and their isomerization by HPLC (II) and thirdly, the decomposition of hydroperoxides to secondary oxidation products were measured by HPLC (V). Previous studies on the subject are difficult to interpret because of variations in experimental conditions and endpoints of lipid oxidation.

Paper I, which aimed to compare the free radical scavenging properties of eight carotenoids, describes a chemical model in which radicals were thermally generated from AMVN in solution (see *reactions 5,6*, p. 13). The ESR spin trapping technique (see p. 13) was applied since it detects the free radicals themselves. However, this method using PBN as a spin trap observed alkoxyl radical adducts of PBN since alkylperoxyl-PBN spin adducts are not very stable at room temperature (I).

The measurement of hydroperoxide formation focused on primary oxidation products. The ferric thiocyanate method for measurement of PV was chosen because of the small amount of sample needed when comparing with the iodometric titration and because of the avoidance of using chloroform in the analysis (III-V) and for suitability of measurement of primary oxidation products, hydroperoxides, at range of < PV 50. Therefore, the experiments (III-V) examined the low level of oxidation. The presence of carotenoids did not affect the determination of PV. This ferric thiocyanate method gave a PV value approximately twice as high as the results obtained by the iodometric titration. Furthermore, the results of PV strongly depend upon the method chosen and the results obtained by different methods

should be interpreted carefully (Mäkinen et al., 1995). Lampi et al. (1997b) concluded that the PV measurements were the most sensitive and repeatable when they compared four analytical methods including PV, AnV, [O_2] measurements and analyses of volatile aldehydes for characterization of the oxidation of RSO TAGS. In comparison, the other studies on the effects of carotenoids on autoxidation of lipids have mainly used PV and [O_2] to follow oxidation (see Table 2, p. 26). The present study (V) measured volatile aldehydes as DNPH derivatives by HPLC to achieve information on the specific secondary oxidation products. In comparison with the main carotenoid antioxidant literature, the most common method has been the measurement of the secondary oxidation products by TBA test (see above 2.5.3 and Krinsky, 1993). Recently, Stahl et al. (1998) noted that the presence of carotenoids did not affect the TBA assay itself.

In RSO TAGS, the detection of consumption of added carotenoids and γ-tocopherol during lipid oxidation experiments gave crucial information on the effects of carotenoid breakdown and carotenoid-tocopherol interaction on reaction mechanisms of carotenoids (III, IV). At certain proportions, a combination of carotenoid and γ-tocopherol inhibited the formation of hydroperoxides more efficiently than γ–tocopherol alone. In comparison, both the hydroperoxide formation of RSO TAGS and the consumption of carotenoid increased without added γ-tocopherol, whereas the breakdown of carotenoid was slower in the presence of γ-tocopherol (III). This evidence suggests the pro-oxidative role of carotenoid breakdown in lipid environment.

Other experimental factors in antioxidant/pro-oxidant studies of carotenoids

A variety of experimental factors e.g. concentration of carotenoids, temperature, pO_2, light, the presence of free radical initiators may all contribute to the antioxidant/pro-oxidant results obtained in this thesis. It was important to evaluate the pro-oxidant/antioxidant effect of carotenoids at variety of concentrations: the levels of carotenoids were 0.17 mM and 0.90 mM in solution (I) and from 0.45 μg/g in emulsified RSO TAGS to 360 μg/g in methyl linoleate (II-V). This thesis describes oxidation experiments at low temperatures 25-40 °C, which are relevant for food lipid models and lipid models in general. Furthermore, the effects of carotenoids on autoxidized lipids were examined both under dark and light (I-V). Under light, carotenoids itself are susceptible to oxidation due to the conjugated double bond structure. The oxidation of carotenoids is less severe in visible light than in UV light depending on the wavelength of irradiation (Christophersen et al.,1991; Jørgensen and Skibsted,1990).

As described above (2.4.4), pO_2 has a critical effect on carotenoid antioxidant/pro-oxidant action. Studies were mainly (I-V) performed under atmospheric oxygen except experiments at 2% O_2 (I). The results obtained are in agreement with the work by Burton and Ingold (1984) suggesting that β-carotene looses its antioxidant activity and shows an autocatalytic pro-oxidant effect especially at concentrations higher than 5×10^{-4} M and at pO_2 >150 torr. In other words, Kennedy and Liebler (1992) suggested that β-carotene undergoes both autoxidation and scavenging of radicals, and that the autoxidation is more significant at high pO_2. Antioxidant properties observed in *in vitro* studies done at atmospheric or higher pO_2 may not reflect antioxidant performance *in vivo* (Liebler et al.,1997). In most intact tissues, pO_2 is much less than this, e.g. on mammalian tissues pO_2 is usually

< 3% O_2 (Krinsky, 1993). At relatively low pO_2, addition mechanism (*reactions 15 and 16,* p. 21) would consume $ROO^{\bullet}$ and the carotenoid would act as a chain-breaking antioxidant (Britton, 1995).

The results in three different autoxidized lipid systems, i.e. methyl linoleate, RSO TAGS and emulsified RSO TAGS (II-V), are in disagreement with most earlier published studies. The oxidation experiments on lipids with radical initiators have been performed in organic solutions (I), whereas autoxidation experiments in this study used spontaneously autoxidized lipids. It is obvious that the experimental conditions in the presence of radical initiator differ markedly from experiments with autoxidized lipids, which may to some extent explain the controversy. The presence of initiators may affect the mechanism and kinetics of lipid oxidation.

Limitations of the experimental models and methods in the present thesis

The antioxidant/pro-oxidant role of carotenoids in the following lipid environments and chemical solutions *in vitro* were examined in this work: natural triacylglycerols, i.e. RSO TAGS (III-IV), emulsified RSO TAGS (V), methyl linoleate (I,II) and acetone and toluene solutions (I), respectively (for evaluation of these model systems see 6.1.1). Therefore, the results of this thesis should be restricted to *in vitro* lipid oxidation.

In lipid environment, carotenoids promoted the formation of hydroperoxides in light and dark (II-V). In light, both experiments with or without added photosensitizer resulted in pro-oxidant effect of β-carotene (IV). However, as mentioned above (2.5.2), using the PV method it was not possible to detect whether experiments with light, without an added photosensitizer, were $^1O_2$ initiated reactions. According to Chan (1987), a number of radicals, e.g. transition-metal ion, a radical generated by photolysis or irradiation, a radical obtained by decomposition of a hydroperoxide or a radical formed from an added initiator, can abstract hydrogen from RH to form $R^{\bullet}$. Thus, the measurement of hydroperoxides by PV gave unprecise information on the reaction mechanisms. The anti/pro-oxidant activity of carotenoids was based on the effect compared to a control sample without an added carotenoid (I-V). The ferric thiocyanate method was suitable for the determination of the PVs in the tested area of 0.2-100 meq/kg (Mäkinen et al., 1995), but not < 0.2 meq/kg. The limitation concerning the statistical analysis for papers III and V was that the statistical analysis takes account of the variability arising from sample preparation, storage and analysis only of two replicate samples.

A disadvantage of the present work was that the specific oxidation products of carotenoids were not measured. On the other hand, the level of specific oxidation products of carotenoids was too low to be detected by the methods currently available. To further understand the reaction of pro-oxidant mechanisms it would be important to analyze chemical composition of carotenoid and lipid breakdown products.

6.2 Free radical scavenger properties of carotenoids

The investigation of free radical scavenging properties of eight carotenoids including β-carotene, astaxanthin, canthaxanthin, zeaxanthin, lutein, cryptoxanthin, lycopene and bixin focused on the early stages of oxidation (see *reactions 5, 6,* p. 13 and reactions in original paper I). Based on the effect on the amount of spin adducts formed in acetone and toluene solutions, carotenoids acted as free radical scavenging antioxidants in chemical environment. The differencies between carotenoids in scavenging of free radicals were, however, small. In a single experiment, β-carotene was a pro-oxidant at a higher concentration. The structural differencies in carotenoids indicated structural differencies of the spin adducts. The present results of carotenoids in chemical solution are in agreement with the increasing data which have reported that carotenoids may scavenge a variety of free radicals studied by fast reaction techniques. These techniques directly detect the radicals themselves (Böhm et al., 1996; Conn et al., 1992; Edge et al., 1998; Everett et al., 1995; 1996; Hill et al., 1995; Miller et al., 1996; Mortensen et al., 1997; 1998; Mortensen and Skibsted, 1996a; 1996b; 1997a; 1997b; 1997c; 1997d; Simic, 1992). In addition, Ozhogina et al. (1995) reported β–carotene as a free radical scavenger based on a kinetic model. Furthermore, rather small differencies between activities of carotenoids support the recent findings that free radical scavenging properties of carotenoids may depend more on the type of the radical than the structure of the carotenoid (Mortensen et al., 1997).

In this thesis, different carotenoid mixtures in a chemical solution did not show synergism (I), whereas Stahl et al. (1998) demonstrated in a very different model system that mixtures of carotenoids were more effective than one single compound. In egg yolk PC multilamellar liposomes, lutein and lycopene were responsible for synergistic antioxidant properties. This was suggested to be related to different physico-chemical properties and specific orientation of carotenoids in the membranes. The comparison of antioxidant activities of carotenoids have often been performed among carotenoids only. However, all carotenoids differed remarkably more from α-tocopherol, which almost totally suppressed the formation of PBN adducts in acetone under these experimental conditions (unpublished data from study I). Thus, it is important to compare carotenoids with other antioxidants, e.g. tocopherols, which may give a totally different scale for the observed antioxidant activities.

6.3 Antioxidant effect of carotenoid-tocopherol interaction on autoxidized lipids

6.3.1 Carotenoid-tocopherol interaction

In autoxidized RSO TAGS and emulsified RSO TAGS, the combinations of carotenoid and tocopherol resulted in an antioxidant effect. Interestingly, at certain proportions, the combinations were even more effective antioxidants than tocopherol

alone thus showing synergism (III-V). The consumption of carotenoids was slower in the presence of tocopherols, whereas the consumption of tocopherols was not seen to be affected by carotenoids (III). This suggests that the beneficial effect of a combination of carotenoid and tocopherol as an antioxidant is due to the ability of tocopherol to inhibit the breakdown of carotenoids. As discussed above (2.6), recent evidence suggests that the mechanism of interaction between β-carotene and tocopherols may involve adverse *reactions 23 and 24* (see p. 30). In this thesis, the observations concerning carotenoid-tocopherol interaction suggest the mechanism of *reaction 23* (p. 30). The carotenoid radicals formed probably are reduced back to carotenoids by tocopherols. These carotenoid radicals are proposed to be formed by hydrogen transfer as well as adduct formation mechanisms in reactions between lipids and carotenoids (see detailed discussion 2.4.2, *reactions 15,16, 17*, p. 21*)*. This mechanism would inhibit the pro-oxidant action of carotenoids by inhibiting the further reactions of carotenoid radicals formed from hydrogen transfer and adduct formation mechanisms: *reactions* 19,20 and *reactions* 21,22, respectively (p. 23). In addition, it is possible that the carotenoid-tocopherol interaction may involve the recycling of tocopherol by carotenoid according to *reaction* 24 (p. 30) when the combination of carotenoid and tocopherol was more efficient antioxidant than tocopherol alone (III-V).

The present findings of carotenoid-tocopherol interaction support the previous data suggesting carotenoid-tocopherol interaction in other food lipids (Terao et al., 1980; Suzuki et al., 1989), biological systems (Palozza and Krinsky, 1992b; Woodall et al., 1996) and chemical solutions (Edge et al., 1998; Mortensen et al., 1998; Mortensen and Skibsted, 1997a; 1997b). The finding of the present thesis was that even a very small amount of tocopherol had an effect on the action of carotenoids (III-V). Thus, it is obvious that tocopherols protect carotenoids in food oils and living systems. In natural lipid systems, the presence of higher concentration of tocopherols than carotenoids may inhibit both breakdown and pro-oxidant actions of carotenoids. Furthermore, in membranes, the natural environment of carotenoids, proteins and other compounds may stabilize carotenoids as well (see e.g. above 2.5.3; Britton, 1995; Stahl et al., 1998). On the other hand, the present results suggest that a single carotenoid should be combined with tocopherols to achieve antioxidant, but not pro-oxidant effect (see also below 6.3.2).

6.3.2 Carotenoid colour, natural carotenoid products and lipid oxidation

The present thesis examined the antioxidant/pro-oxidant effects of the carotenoids, which are accepted as food additives for colouring (European Parliament and Council Directive, 1994). These were β-carotene, annatto, bixin, lycopene and lutein (I-V). In lipid environment, the observations that even a small amount of tocopherol protected carotenoid both from the breakdown (III-IV) and pro-oxidant effect (III-V), in autoxidized lipids, are of practical relevance. The light stability of carotenoid colours, which may be of concern in colouring of foods (Jørgensen and Skibsted, 1990; Johnson, 1995; Kearsley and Rodriquez, 1981; Marcus, 1994; Wissgott and Bortlik, 1996), could be improved by a combination of carotenoid and tocopherol. In practise, the present results suggest that a carotenoid should be protected from bleaching and pro-oxidant effect by phenolic compounds whether in food or pharmaceutical use.

Natural food additives including colorants (Arad and Yaron, 1992; Francis, 1996; Henry, 1996; Miller, 1991; Wissgott and Bortlik, 1996) and antioxidants (Frankel, 1989; 1993; Löliger, 1991; Pokorný, 1991), have become increasingly popular due to the strong consumer demand for more natural foods. For example, annatto is a natural pigment obtained from the seeds of the plant *Bixa orellana* and widely added to foods (Collins, 1992; Francis, 1996; Lauro, 1991). In contrast to a single carotenoid, the natural annatto colorant, containing bixin as a carotenoid, showed antioxidant effect in autoxidized lipids (III). However, it should be stressed that the antioxidant effect may be due to some unidentified components present in a natural carotenoid product. Natural β-carotene products derived from plants, fruits, vegetables and algae contain different amounts of 9-*cis* stereoisomer, whereas synthetic β-carotene is in all-*trans* form. In comparison of natural *versus* synthetic β-carotene, the results have suggested that 9-*cis* β-carotene derived from *Dunaliella* algal is a more effective lipid antioxidant *in vitro* and *in vivo* than all-*trans* β-carotene due to the insertion of *cis* position along the conjugated double bond chain (Ben-Amotz, 1998; Ben-Amotz and Levy, 1996; Levin and Mokady, 1994; Levin et al., 1997).

6.4 Pro-oxidant effects of carotenoids on autoxidized lipids

6.4.1 Pro-oxidant effects of β-carotene, lycopene and lutein on autoxidized lipids

In three different lipid models *in vitro*, β-carotene promoted both formation and decomposition of lipid hydroperoxides (II-V). In addition, other tested carotenoids, lycopene and lutein, promoted formation of lipid hydroperoxides (III). Thus, carotenoids as lipid pro-oxidants did not stabilize the spontaneously autoxidized lipids, whereas more radicals were formed, but not removed from the oxidizable lipid substrate. The present pro-oxidant results are in disagreement with the majority of earlier reported studies on carotenoids as lipid antioxidants in autoxidized food oils (see Table 2, p. 26), photosensitized oxidation of lipids (see Table 3, p. 27) and in studies using different lipid models in organic solution in the presence of radical initiators (see 2.5.3). The previous pro-oxidant evidence of β-carotene is rather weak. In addition to pro-oxidant results in autoxidized food lipids (see Table 2, p. 26), β-carotene has been reported to produce a pro-oxidant or lack of antioxidant effect only in few studies using a lipid model in solution in the presence of radical initiator (Kigoshi and Niki, 1992; Liebler et al., 1997; Palozza et al., 1995a). As already discussed (see 2.5, and 6.1), many factors may explain this discrepancy: the differencies in the lipid model and solution and methods involved, the possible presence of other antioxidants, the concentration of carotenoids and other experimental conditions such as pO_2.

The possible reaction pathways of carotenoid pro-oxidant action on lipid oxidation are summarized in Scheme 2, p. 50. The present observations in autoxidized lipids suggest that the pro-oxidant mechanisms of carotenoid at atmospheric pO_2

may involve *reactions 19-22* (see p. 23). The formation of carotenoid-peroxyl radicals, either autoxidation product of carotenoid CAR-OO$^{\bullet}$ (*reaction 19*, p. 23) or adduct formed with lipid ROO, ROO-CAR$^{\bullet}$ (*reaction 15*, p. 21) may have continued the free radical chain reactions and promoted the oxidation of lipids. The further reactions of CAR-OO$^{\bullet}$ and ROO-CAR$^{\bullet}$ may lead to a pro-oxidant effect: *reaction 20* and *reactions 21-22* (p. 23), respectively (see 2.4.2). The results suggest that both β-carotene and its breakdown product, retinal, do not react with 1) lipid ROO$^{\bullet}$ to inhibit the formation of ROOH and 2) lipid RO$^{\bullet}$ to inhibit the decomposition of hydroperoxides into aldehydes and other products. The increased decomposition of hydroperoxides indicates further that increased formation of RO$^{\bullet}$ may promote the oxidation of lipids by reacting with RH and ROOH (Chan, 1987).

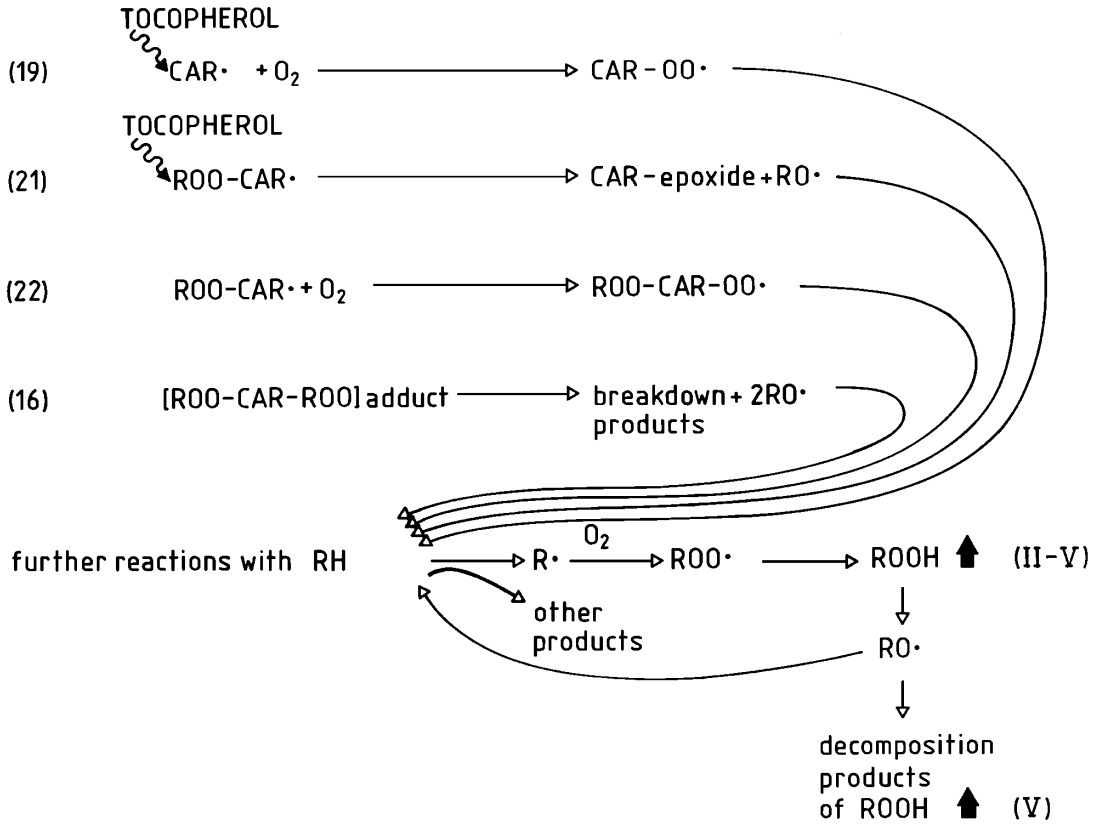
The present pro-oxidant results of β-carotene in autoxidized lipids (II-V) support the recent evidence that β-carotene is not a scavenger of lipid ROO$^{\bullet}$ (Mortensen and Skibsted,1998) (see 2.6). Furthermore, these findings also suggest that the autoxidation of β-carotene may lead further to lipid pro-oxidant action at atmospheric pO_2 (see above *reactions 19-20*, p. 23 and 2.4.2, 6.4.1). Burton and Ingold (1984) originally proposed this pro-oxidant action of β-carotene at higher pO_2 (see also 2.4.4). The autoxidation of β-carotene promoted the lipid oxidation, whereas an antioxidant is supposed to be itself oxidized yielding an antioxidant derived radical not promoting oxidation. The autoxidation of β-carotene leads to colourless products. Alternatively, Tsuchihashi et al. (1995) proposed that the following reactions of ROO-CAR$^{\bullet}$ with O_2 to yield ROO-CAR-OO$^{\bullet}$ and the reactions between ROO-CAR-OO$^{\bullet}$ and RH could be the pro-oxidant pathway.

6.4.2 Effect of β-carotene breakdown on autoxidized lipids

An important observation was that the carotenoid was consumed, i.e. colour disappeared, faster alone than in the presence of γ-tocopherol. Simultaneously, a single carotenoid was a pro-oxidant, whereas the combination of carotenoid and γ-tocopherol was an antioxidant (III-IV). On the other hand, the consumption of γ-tocopherol was not affected by the presence of carotenoids in the combined sample, which was a better antioxidant than tocopherol alone at certain proportions (III). The measurement of consumption of added carotenoids gave additional information on the pro-oxidant mechanisms of carotenoids and antioxidant mechanisms of the combination of carotenoid and γ-tocopherol during the lipid oxidation. Furthermore, the higher the concentration of lutein was, the higher the pro-oxidant activity was (III). This observation may indicate that the increased pro-oxidant activity may be due to the effect of increased destruction of carotenoid. These results raised the question of the effect of carotenoid breakdown products on lipid oxidation.

The basic electron rich conjugated double bond structure of carotenoids (Figure 1, p. 17) is susceptible to oxidation and the breakdown of β-carotene yields a complex mixture of shorter-chain derivatives including retinal, β-*apo*-15-carotenal (Figure 2, p. 24). Both β-carotene and retinal promoted the formation of methyl linoleate hydroperoxides (II). Further investigations could be carried out on the effects of oxidation products of different carotenoids on lipid oxidation.

Scheme 2. Summary of possible reaction pathways of carotenoid pro-oxidant action on lipid oxidation (see also reactions *16, 19, 21, 22 in text). Roman numerals II-V refer to original papers.*



Jørgensen and Skibsted (1993) observed more significant differencies in antioxidative properties of carotenoids after the carotenoids were completely bleached. There may be differencies in pro-oxidative/antioxidative effects of the breakdown products between different carotenoids.

It should be emphasized that the breakdown of β-carotene and other provitamin A carotenoids in the presence of oxygen is a natural phenomen in humans and in most animal species leading to vitamin A. β-Carotene is known to yield two molecules of retinal, which in turn are oxidized to retinoic acid or reduced to retinol. In addition, β–carotene is metabolized to a series of *apo*-carotenals, which undergo oxidation to apocarotenoic acids, and form retinoic acid (Krinsky et al., 1993; Omaye et al., 1997; Wang and Krinsky, 1997). Further knowledge on the biological activity and antioxidant/pro-oxidant activities of shorter-chain β-carotene breakdown products would be of interest.

6.5 General perspectives on the antioxidant/pro-oxidant role of carotenoids

In this thesis, carotenoids showed both antioxidant and pro-oxidant action *in vitro*. Antioxidants are not universal. An antioxidant may protect in one environment, but lack protection in another environment. Furthermore, the results of this thesis demonstrate that the choice of experimental conditions in test tubes may affect greatly the antioxidant/pro-oxidant activity of carotenoids (see also 2.4, 2.5, 2.6). Although the antioxidant/pro-oxidant roles of carotenoids remain controversial, the physical and chemical reactions between carotenoids and oxygen are the fundamental elements of carotenoid functions: antenna pigments for photosynthesis, quenchers of $^1O_2$ (2.3) and dietary sources as precursors of vitamin A via oxidative cleavage (6.4.2).

Recently, the results of observational studies on supplemental β-carotene raised an active discussion regarding the role of carotenoids and other antioxidants in humans (see 1). Based on current scientific knowledge, the situation of antioxidant/pro-oxidant action of carotenoids *in vivo* is not clear. Although carotenoids have been proposed to prevent diseases through antioxidant action there is a need for further understanding in terms of the basic chemical and biochemical principles (Britton, 1995; Edge et al., 1997), rather than hypothesized from indirect associations from diet. In all, a number of areas of research need more investigation to have a more complete picture of the role of β-carotene and other carotenoids in humans. It should be stressed that antioxidant mechanism is only one proposed mechanism for protective effects of carotenoids against cancer; others include regulation of gap junctional communication, modulation of immune response and retinoid-like effects on cellular differentiation (Omaye et al., 1997; Rousseau et al., 1992; Stahl and Sies, 1996). Although carotenoids are well-known as efficient $^1O_2$ quenchers (2.3), the possible antioxidant role of carotenoids as $^1O_2$ quenchers in humans needs clearly more investigation in the future. Stahl et al. (1997) indicated that $^1O_2$ quenching and induction of gap junctional communication, which have been discussed as biochemical mechanisms underlying cancer-preventive potential, may operate independently of each other.

So far, the evidence that carotenoids are lipid antioxidants or pro-oxidants have been mainly based on studies using different lipid models *in vitro.* In general, it is difficult to estimate the experiments performed in test tubes to activity in living systems. Halliwell (1997) proposed that carotenoids would not be effective antioxidants *in vivo* due to the weak antioxidant evidence *in vitro*. According to Britton (1995) the concentrations of carotenoids in mammalian tissues are much lower than those used to demonstrate antioxidant action in *in vitro* lipid models. To act as an antioxidant *in vivo*, a carotenoid would need to be incorporated into tissues in the correct location and at a suitable concentration relative to the oxidizable substrate.

At present, there is growing research interest in the interactions between carotenoids and a variety of free radicals (see above 2.6) involved in oxidative stress. For example, Scheidegger et al. (1998) have proposed that zeaxanthin may protect macular tissue from oxidative damage. Also, knowledge on the antioxidant/pro-oxidant properties of carotenoids at the gene level would be of interest. On the other hand, the current *in vitro* and *in vivo* evidence of pro-oxidant activity of carotenoids have awakened a new area of interest in the field of carotenoid research. The significance of pro-oxidant actions of carotenoids in biological systems re-

mains to be investigated (Palozza, 1998). To achieve a better understanding of carotenoids and dietary antioxidants, further investigations should emphasize on the interactions between carotenoids and tocopherols as well as other complex interactions between naturally occurring components of foods (Omaye and Zhang, 1998).

7. Conclusions of the Thesis

The results of this thesis demonstrate the crucial role of experimental conditions on the *in vitro* antioxidant/pro-oxidant action of carotenoids. There was a difference of carotenoid actions between the two experimental systems based on 1) the scavenging of initiator derived free radicals in chemical solution and 2) the effect on formation and decomposition of hydroperoxides of autoxidized triacylglycerols, emulsified triacylglycerols and methyl linoleate. In these two models, carotenoids were weak antioxidants and pro-oxidants, respectively. In slower reactions such as autoxidation of lipids, carotenoids may act as a pro-oxidant, whereas in faster reactions it may act as a free radical scavenging antioxidant. The following conclusions refer to *in vitro* lipid oxidation experiments in test tubes

1 Carotenoids are weak free radical scavenging antioxidants in chemical solution. In acetone, carotenoids except β-carotene were antioxidants. In toluene, carotenoids except bixin, lutein and a combination of lutein and β-carotene were antioxidants. On the other hand, the results showed that the differencies between β-carotene, astaxanthin, canthaxanthin, zeaxanthin, lutein, cryptoxanthin, lycopene and bixin in free radical scavenging were small.

2 Carotenoids are *in vitro* pro-oxidants in lipid environment based on the effect on both the formation and decomposition of hydroperoxides. The loss of yellow colour indicates the pro-oxidant action of carotenoids in lipids. This thesis raised the question of the effect of carotenoid breakdown products on pro-oxidant role of carotenoids as demonstrated with retinal.

3 In lipid environment, a combination of carotenoid and tocopherol at certain proportions may be a better antioxidant than tocopherol alone. Even a minor amount of tocopherol protects carotenoid from destruction and thus inhibits the pro-oxidant action of carotenoid.

4 As a summary it can be concluded that the naturally occurring combination of a carotenoid and a stabilizing compound such as tocopherol acts as a lipid antioxidant, whereas when tested as an isolated carotenoid it acted as a lipid pro-oxidant. Finally, the present findings help us to understand the balance of interactions between components in the natural environment of carotenoids.

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